Gel Chromatography in Denaturing Solvents: A Method for the Study of Protein Subunit Composition

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Gel chromatography in two denaturing solvents, dilute aqueous sodium dodecyl sulfate (SDS) or aqueous 6 *M* guanidine hydrochloride (GuHCl), was used to measure protein subunit molecular weights. Gel chromatography on 6% agarose in 6 *M* GuHCl permitted separation of polypeptide chains from 1000 to 50,000 daltons. Accurate molecular weight estimates on P-200 in 0.1% SDS were obtained between 15,000 and 70,000 daltons. The utility of the method was demonstrated by an examination of the subunit nature of the transferrins and apoferritin. It was shown that human serum transferrin, ovotransferrin, and lactoferrin were each composed of a single polypeptide chain of 77,000 mol wt. The subunit molecular weight of apoferritin was found to be 18,200 by gel chromatography of ^{125}I-labeled apoferritin in 6 *M* GuHCl. Together with a native mol wt of 435,000 this suggested a 24-subunit model for apoferritin.

Proteins which are composed of more than one polypeptide chain accomplish their biological role, in part, as a result of their subunit–subunit interactions. Thus, a knowledge of the subunit composition of a protein is basic to understanding the mechanism by which it functions. Polypeptide chains are held together by the same types of forces which hold different parts of each chain in a unique conformation. For the most part, this involves noncovalent interactions which are nonpolar in nature (Perutz *et al.*, 1968), but in some cases covalent linkages in the form of interchain disulfide bonds also occur (Noelken *et al.*, 1965).

To be confident one has dissociated a protein to its constituent polypeptide chains, it is best to employ a dissociating agent whose effectiveness as a denaturant is well documented. Two solvents which have been well characterized as protein denaturants are concentrated aqueous guanidine hydrochloride (GuHCl) (Tanford, 1968) and aqueous sodium dodecyl sulfate (SDS) (Shapiro *et al.*, 1967; Reynolds and Tanford, 1970a,b). Tanford and coworkers demonstrated that reduced proteins in concentrated GuHCl possessed no detectable residual noncovalent structure and were conformationally altered to random coils (Tanford, 1968). Recently, Reynolds and Tanford (1970b) have shown that polypeptide chains from reduced proteins in SDS solution bind identical amounts of SDS on a g/g basis: 1.4 g SDS/g of protein. Their hydrodynamic measurements suggested that the peptide chain–SDS complexes exist as slightly flexible rods of near-uniform diameter.

It is important to note that the products of denaturation by either denaturant assume a characteristic conformation, the dimensions of which vary predictably with molecular weight. If a native protein is subjected to reducing conditions in the presence of either of these denaturants, it is dissociated to its constituent polypeptide chains, all of which possess the same conformation.

Molecular sieving of the type that occurs in gel filtration and disc gel electrophoresis functions by the distribution of solute molecules between the solvent within the pores of the gel and the solvent outside of these pores. This distribution depends upon the size and shape of the hydrodynamic particle formed by the solute. Thus, in either of the two denaturing solvents mentioned above, the distribution of a reduced polypeptide chain among the pores of crosslinked gel should be a unique function of polypeptide chain molecular weight. This has been shown to be true for gel chromatography in denaturing solvents (Davison, 1968; Fish *et al.*, 1969, 1970) and for SDS–polyacrylamide gel electrophoresis (Shapiro *et al.*, 1967; Weber and Osborn, 1969).

It is the purpose of this report to discuss the separation and molecular weight estimation of protein subunits by gel chromatography in denaturing solvents.

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REAGENTS

Proteins. All proteins used for column standardization were from the sources listed previously (Fish *et al.*, 1969, 1970).

Lactoferrin, purified from a commercial preparation of α-lactalbumin or from whole milk, was a gift of F. J. Castillino. Ovotransferrin (conalbumin) was a 6× crystallized preparation purchased from Mann Research Laboratories, New York, N.Y.

Apoferritin was prepared according to the method of Bjork and Fish (1971) from 6× crystallized horse ferritin (Miles Laboratories, Inc., Kankakee, Ill.).

CHEMICALS

Ultrapure GuHCl was purchased from Heico, Inc. (Delaware Water Gap, Pa.) and was used without further purification.

SDS was obtained from Mann Research Labs and was used without additional purification.

^{125}I was purchased from Amersham-Searle Corp., Arlington Heights, Ill.

All other chemicals were the best commercially available.

PROCEDURE

Ultracentrifuge Studies. Sedimentation equilibrium studies were performed according to the short-column, high-speed technique (Yphantis, 1964) in a Beckman model E analytical ultracentrifuge. Equilibrium runs were made at constant temperature between 20 and 25° C in double-sector cells equipped with sapphire windows. Samples for measurement were dissolved in the appropriate solvent and dialyzed against solvent for at least 36 hr prior to ultracentrifugal analysis.

The partial specific volume used for apoferritin was 0.733 and was calculated from its amino acid composition.

Preparation of Proteins for Gel Chromatography. Reduced, carboxymethylated protein polypeptide chains for gel chromatography in 6 *M* GuHCl were prepared as outlined previously (Fish *et al.*, 1969).

Proteins for gel chromatography in SDS were prepared by one of the two following procedures.

Proteins were reduced and carboxymethylated in the presence of 6 *M* GuHCl, the GuHCl was dialyzed away with water, and the precipitated polypeptide chains were redissolved in the SDS solvent by dialysis. SDS could not be added directly to the GuHCl solution, as the two form an insoluble precipitate.

Proteins were reduced by incubation for 5 min in a boiling water bath in the presence of 1% β-mercaptoethanol and 2 mg SDS/mg protein in 0.1 *M* sodium phosphate, pH 7.0.

Blocking of the sulfhydryl groups to prevent oxidation was not necessary in the SDS solvent system. The sulfhydryl groups on the polypeptide chains in the SDS complex are protected both from the surrounding medium and from each other (Fish *et al.*, 1970).

Blue Dextran 2000 (Pharmacia, Piscataway, N.J.), 3 mg/ml, DNP–alanine or DNP–glycine, 0.2 mg/ml, and 20 mg/ml of sucrose were added to solutions before their application to the column.

Preparation of ^{125}I-Labeled Apoferritin. One milligram of apoferritin was labeled with ^{125}I according to method A outlined by Talmage and Claman (1967); 1 mCi of ^{125}I was used, and the volumes of all reagents were reduced by one-tenth. The ^{125}I-apoferritin was separated from the unreacted ^{125}I and other reagents by passing the solution through a column of Sephadex G-50.

Preparation and Operation of Gel Chromatography Columns. The preparation of gel filtration columns in the appropriate solvent system was identical to that previously described (Fish *et al.*, 1969). Flow rates for the columns were 1.5–2 ml/hr/cm^2, and 1-g fractions were collected.

Gel chromatography in the presence of 6 *M* GuHCl was carried out on 6% agarose (Bio-Rad Laboratories, Richmond, Calif.) or 4% agarose (Pharmacia).

Gel chromatography in the presence of SDS was carried out on P-200 acrylamide (Bio-Rad Laboratories). The SDS solvent contained 0.1 *M* sodium phosphate, pH 7.0, and 0.1% SDS. Sodium azide, 0.02%, was also added to prevent microbial degradation of the gel.

Polypeptide chains eluting from the columns were monitored by their absorbance at 280 nm. In some instances, where the extinction coefficient of the polypeptide chain at 280 nm was quite low or where very low protein concentrations were used, samples were monitored at 230 nm. Blue Dextran was monitored at 630 nm and DNP–amino acid was monitored at 360 nm.

The elution position of ^{125}I-labeled apoferritin was monitored by counting the weak β-emission of selected fractions in a Packard Tri-Carb liquid scintillation spectrophotometer (Packard Instruments, La Grange, Ill.).

Treatment of Data. The elution position of each solute was expressed in terms of a distribution coefficient, K_d. K_d is equal to $Ve - Vo/Vi - Vo$ where Ve is the elution position of solute expressed as g of eluant, Vo is the g of solvent in the column external to the gel matrix, and $Vi - Vo$ is the g of solvent within the gel matrix. Experimentally, Vo is the elution position of Blue Dextran, and Vi is the elution position of DNP–amino acid. These markers were included in each run. Greater accuracy can be achieved by using eluate weight rather than volume (Fish *et al.*, 1969).

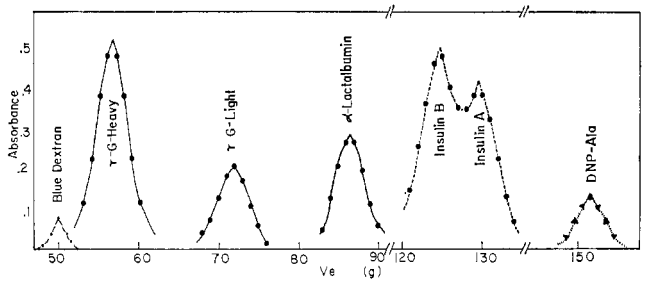

Figure 1. Example of an elution profile of a mixture of reduced, carboxymethylated protein polypeptide chains from 6% agarose in 6 *M* GuHCl. —●— ●—, absorbance at 230 nm; —●—●—, absorbance at 630 nm; - - -●-●- - -, absorbance at 230 nm; ▲- - -▲- -, absorbance at 360 nm

Standard curves for molecular weight estimation were prepared by plotting log mol wt *vs.* K_d (Andrews, 1965). Data may also be expressed in terms of the radius of gyration, R_G, or the radius of an equivalent sphere (Stokes radius), R_s, as a function of K_d (Fish *et al.*, 1969, 1970).

RESULTS AND DISCUSSION

Gel Chromatography in 6 *M* GuHCl. Gel chromatography columns are routinely calibrated with several protein polypeptide chains of known molecular weight. A typical elution pattern from a 6% agarose column in 6 *M* GuHCl is shown in Figure 1. This figure illustrates an important aspect of the method: the separation of polypeptide chains of different sizes is accomplished concomitantly with the estimation of their molecular weight. This is demonstrated by the complete separation of the heavy and light chains of immunoglobulin G. The semipreparative nature of columns of this type proves to be a definite asset in subunit studies. Quantities of protein can be applied to the column to provide enough material in the elution peak for additional characterization. It is possible to distinguish between identical polypeptide chains or nonidentical chains of equal size by quantitative end group analysis or peptide mapping of material from the column. Protein preparations which are not entirely homogeneous may be purified by this method, and more accurate amino acid analyses and sedimentation equilibrium measurements may be performed from the column-purified material.

It was possible to separate the two chains of insulin on this particular 6% agarose column. No additional chemical experiments were performed to prove the identity of each chain, but this elution pattern for insulin was repeated upon numerous occasions. This suggests that gel chromatography in denaturing solvents may find widespread use for the separation of the difficultly-soluble peptides derived from enzymatic or chemical cleavage of protein polypeptide chains.

Figure 2 shows the data from a number of calibration runs plotted in a manner similar to that of Andrews (1965). The elution positions are normalized in terms of a type of distribution coefficient, K_d. A sigmoid-shaped curve is obtained which will provide accurate molecular weight estimates between 1000 and 50,000 daltons. The precision and accuracy of this method was estimated by repeated column runs on protein polypeptide chains of known molecular weight. The precision of K_d determinations was about 2%, while the accuracy of molecular weight determination was within 10%. These compare favorably with other methods of molecular weight determinations in denaturing solvents (Mann *et al.*, 1970; Weber and Osborn, 1969).

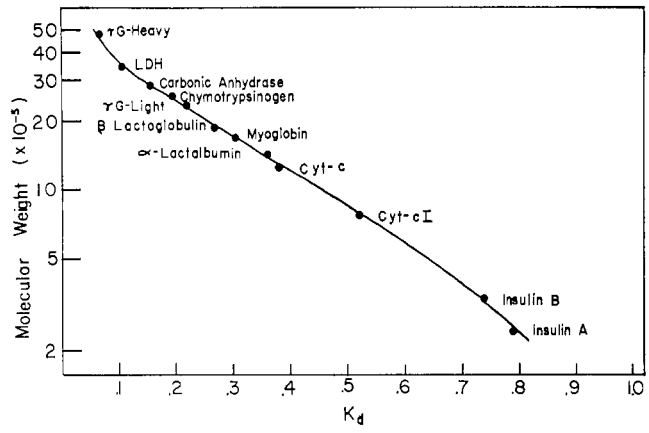

Figure 2. Graphical interpretation of the elution data from 6% agarose in 6 *M* GuHCl. A semilogarithmic plot of the mol wt *vs.* K_d. LDH, lactate dehydrogenase; cyt c-I, cyanogen bromide peptide of cytochrome c, residues 1-65

The working ranges of these gels vary between lots and manufacturers for a given degree of crosslinkage. The agarose preparation represented by Figure 2 has a pore size distribution optimal for polypeptide chains in the 3000–25,000 mol wt range. Other 6% gels have had optimum pore size distributions ranging from the above to a mol wt range of 10,000–70,000 daltons. Obviously, a better resolution at one end of the molecular weight range means a poorer resolution at the other extreme.

The disulfide bonds of proteins must be reduced for the molecular weight estimate by gel chromatography in denaturing solvents to be valid. Thus, it is not possible to tell whether the polypeptide chains are held together in the native protein through noncovalent interactions, as in the case of lactate dehydrogenase, or whether they also possess interchain disulfide bonds, as does immunoglobulin G. It is possible, however, to resolve this question by sedimentation equilibrium measurements in 6 *M* GuHCl in the absence of reducing agent.

Gel Chromatography in SDS Solution. As mentioned above, polypeptide chains in SDS solution are not random coils, but when complexed with SDS, they take on a conformation which possesses a high degree of order (Reynolds and Tanford, 1970a). This polypeptide–SDS complex is best described hydrodynamically as an ellipsoid of constant minor axis whose major axis, or length, varies uniquely with the molecular weight of the protein polypeptide chain moiety of the complex. This means it is also possible to estimate polypeptide chain molecular weights in SDS solution by gel chromatography. This is illustrated in Figure 3. The accuracy of molecular weight measurements by this technique is as good as in 6 *M* GuHCl for polypeptide chains down to about 10–15,000 mol wt. Polypeptide chains below this molecular weight range exhibit erratic behavior in their elution positions. This is probably explained by the fact that a rodlike particle begins to approximate a sphere when the magnitude of the length approaches that of the diameter. This sets a lower limit of about 10–15,000 for a reliable estimation of molecular weight by gel chromatography and gel electrophoresis in SDS solution.

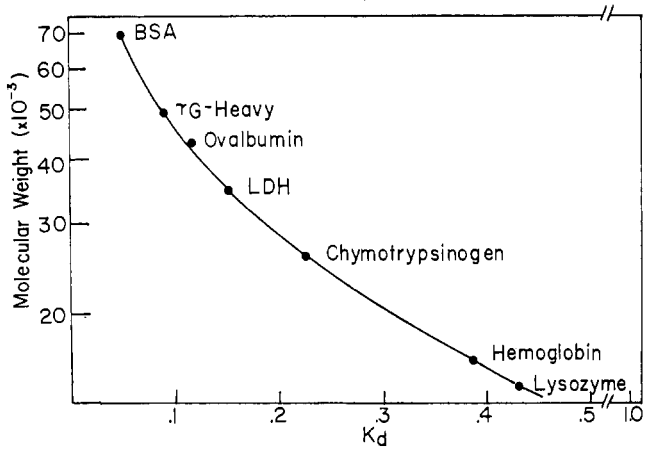

Figure 3. Graphical interpretation of the elution data from P-200 in 0.1% SDS. A semilogarithmic plot of the mol wt *vs.* K_d. BSA, bovine serum albumin; LDH, lactate dehydrogenase

Applications of Gel Chromatography in Denaturing Solvents. THE TRANSFERRINS. In subunit studies, one fact is quite often taken for granted: the fact that all covalent crosslinks between chains have been broken by the dissociating solvent employed.

The dimensions of randomly coiled linear polymers, as measured by intrinsic viscosity, vary predictably with molecular weight, and the introduction of crosslinks reduces these dimensions (Tanford, 1968). Therefore, if the intrinsic viscosity of a reduced polypeptide chain were less than that expected from its mass measured by sedimentation equilibrium it would be a good indication that not all crosslinks had been broken. This would mean that one might not be dealing with the smallest constituent polypeptide chains.

This approach was used by Kawahara and Tanford (1966) in the determination of the number of subunits in aldolase, and by Mann and coworkers (1970) in the study of human serum transferrin.

The transferrins provide an excellent example to illustrate this point. They are proteins involved in the binding and transport of iron and are found in serum, milk, and egg white. Until recently, their subunit nature had been the subject of considerable controversy. Human serum transferrin possesses two equivalent iron binding sites and two identical carbohydrate moieties per mole of protein (Aisen *et al.*, 1966). This suggests two identical subunits in the native protein. Experimental evidence had been offered to support both the single-chain and the two-chain models.

Table I contains our data from gel chromatography of the reduced transferrins in 6 *M* GuHCl and data from the literature on molecular weights of the transferrins by sedimentation equilibrium. It can be seen that the native molecular weights of the three transferrins agree quite well, and that the molecular weights of human serum transferrin and lactoferrin remained unchanged under dissociating conditions.

Mann *et al.* (1970) also found that human serum transferrin was highly crosslinked by disulfide bonds. However, when they reduced it and determined its intrinsic viscosity in 6 *M* GuHCl, they found the intrinsic viscosity to be consistent with that expected for a randomly coiled polypeptide chain of 77,000 mol wt. This provided evidence that indeed all covalent crosslinks had been broken under the reducing denaturing conditions employed.

In denaturing solvents, gel chromatography and intrinsic viscosity measure the same hydrodynamic parameter. Thus, if all covalent crosslinks in a protein have been broken, the polypeptide chain molecular weight estimated by gel chromatography in 6 *M* GuHCl should equal that determined by sedimentation equilibrium under the same conditions. If the molecular weight by gel chromatography is significantly less than that by sedimentation equilibrium,

Table I. Elution of Various Transferrins from 4% Agarose in 6 *M* GuHCl

Protein	Source	K_d	Native mol wt by sedimentation equilibrium
Human serum transferrin	Purified from serum	0.315[a]	76,600[b] (Mann *et al.*, 1970)
	Commercial	0.317	
Hen egg ovotransferrin	Commercial	0.316	76,000 (Greene and Feeney, 1968)
Lactoferrin	Purified from milk	0.316	77,100[c] (Castillino *et al.*, 1970)
	Commercial	0.315	Associating system[d] (Castillino *et al.*, 1970)

[a] Mol wt estimate from this K_d was 77,000. [b] Mol wt of reduced protein in 6 *M* GuHCl by sedimentation equilibrium was 71,600–76,300 (Mann *et al.*, 1970). [c] Mol wt of reduced protein in 6 *M* GuHCl by sedimentation equilibrium was 72,500–77,200 (Castillino *et al.*, 1970). [d] Mol wt of material near the meniscus was 76,000 and $\bar{M}_z$ was 200,000.

crosslinks still remain, and the minimal polypeptide chains may not have been realized.

A comparison of the K_d values of the three transferrins after reduction, carboxymethylation, and gel chromatography on 4% agarose in 6 *M* GuHCl clearly shows that the transferrin chains from various sources are the same size. From a K_d of 0.315, a mol wt of 77,000 was estimated from a standard curve prepared in the same manner as in Figure 2. This molecular weight agrees with sedimentation equilibrium measurements under the same conditions and thus indicates that all covalent crosslinks in the transferrins have been broken. This comparative study establishes that the two equivalent iron binding sites of the transferrins occur on a single polypeptide chain.

APOFERRITIN. Apoferritin is the protein shell of the iron storage protein, ferritin. It is commonly quoted in the literature that apoferritin consists of 20 subunits of 24,000 mol wt each (Klotz and Darnall, 1969). This model is based upon a determination of the subunit molecular weight by ultracentrifugal measurements in SDS. After correction for SDS binding, a value of 24,000 was obtained (Hofmann and Harrison, 1963). Due to the large amount of SDS which binds to proteins (Reynolds and Tanford, 1970b), this approach may be susceptible to considerable error.

It was felt that the subunit composition of apoferritin was still open for investigation, and that gel chromatography in denaturing solvents was an ideal method to employ.

The molecular weight of native apoferritin was measured by sedimentation equilibrium. Figure 4 shows a plot of log fringe displacement *vs.* the square of the distance from the center of rotation for a sedimentation equilibrium run on native apoferritin. The points on this run were linear from a displacement of less than 50 μ to the bottom of the cell. For ten runs at various speeds and at various protein concentrations and pH, an average mol wt of 435,000 ± 15,000 was obtained. This is the first time the molecular weight by sedimentation equilibrium has been reported for apoferritin, and it agrees well with the value reported by Rothen (1944) from sedimentation and diffusion measurements.

Sedimentation equilibrium measurements were made on apoferritin in 6 *M* GuHCl in the presence of reducing agent. A mol wt of about 18,000 was measured under this set of conditions. The value of 18,000 is considerably less than the 24,000 value which others had reported (Hofmann and Harrison, 1963).

To obtain as accurate an estimate of molecular weight by gel chromatography in 6 *M* GuHCl as possible, ^{125}I-labeled apoferritin was chromatographed simultaneously with two standards of known amino acid sequence: α-chymotrypsinogen A, mol wt 25,700; and myoglobin, mol wt, 17,200. The column employed was that of Figures 1 and 2.

The elution profile of the three proteins is shown in Figure 5. Qualitatively it can be seen that the molecular weight of the apoferritin subunits is closer to that of myoglobin than of chymotrypsinogen. The ^{125}I-apoferritin peak represents about 25 μg of protein and illustrates one means of increasing the sensitivity of detection at least 50-fold for this method.

If one refers to Figure 2, it can be seen that the elution positions of these proteins correspond to an area of the standard curve which is essentially linear.

Therefore, the molecular weight of the apoferritin subunits was estimated assuming a linear relation between log mol wt *vs.* K_d for the two standards as shown in Figure 6. A mol wt of 18,200 was obtained for the apoferritin subunits from

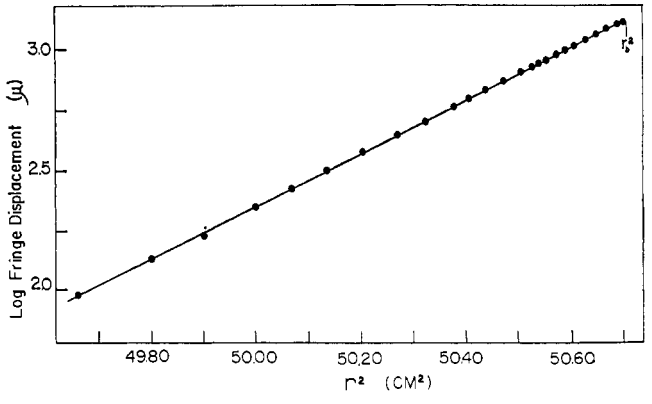

Figure 4. Sedimentation equilibrium data for a solution of native apoferritin (0.02%) in 0.1 *M* NaCl, pH 5.5. Plot of log fringe displacement *vs.* square of distance from the center of rotation, r^2. Points represent the average of five fringes. The line is drawn from a least-squares analysis of all fringe displacements greater than 100 μ. r_b^2 is the bottom of the cell

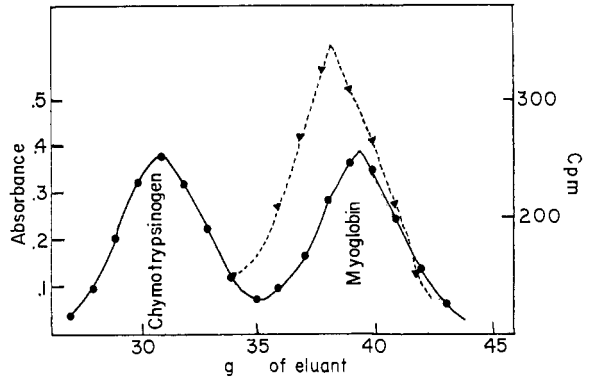

Figure 5. Elution profile of ^{125}I-labeled apoferritin chromatographed simultaneously with α-chymotrypsinogen and myoglobin on 6% agarose in 6 *M* GuHCl. —●—●—, absorbance at 280 nm; ▼- - -▼- -, counts per minute

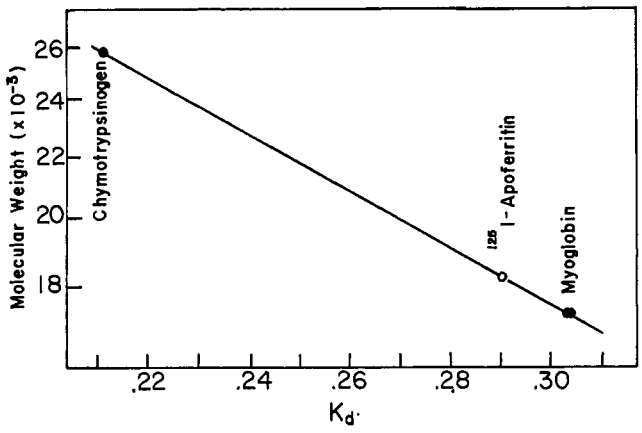

Figure 6. Estimation of the subunit molecular weight of ^{125}I-apoferritin from the elution data from 6% agarose in 6 *M* GuHCl. All three proteins were chromatographed simultaneously

two experiments of this type. Gel chromatography in SDS yielded a mol wt of 18,400.

Thus, all of our data point to a subunit structure for apoferritin of 24 units, each of mol wt 18,200.

Criteria for the Choice of Denaturant Solvent System. Reduced proteins in aqueous solutions of 6 *M* GuHCl or dilute SDS assume conformations such that their hydrodynamic shape varies predictably with molecular weight. Consequently, gel chromatography can be used to determine polypeptide chain molecular weight in either of these solvents.

Complete dissociation of protein subunits is accomplished by very low concentrations of SDS; this makes SDS considerably cheaper to use than GuHCl. When the SDS–gel electrophoresis technique of Maizel (Shapiro *et al.*, 1967) is employed, subunit molecular weights may be estimated on μg quantities in a relatively short period of time. It is important to note that due to the shape assumed by the protein–SDS complex, molecular weights of polypeptide chains less than 15,000 daltons cannot be estimated accurately in SDS. It is also relatively difficult to remove the protein-bound SDS, and this decreases the desirability of SDS as a denaturing solvent for preparative gel chromatography of protein subunits.

On the other hand, the nature of the conformation adopted by reduced polypeptides in 6 *M* GuHCl, that of random coils, allows molecular weight estimations on polypeptides as small as 1000 daltons. Due to physical limitations which arise in such a dense solvent as 6 *M* GuHCl during high-speed sedimentation equilibrium, gel chromatography in 6 *M* GuHCl is the method of choice for the molecular weight estimation of protein subunits less than about 15,000 daltons.

The semipreparative nature of gel chromatography in 6 *M* GuHCl and the relative ease of removal of the denaturant allow the recovery of adequate amounts of protein for additional physical and chemical characterization.

Gel chromatography in denaturing solvents is an excellent supplement to the analytical ultracentrifuge. It provides a measure of molecular weight based on hydrodynamic size (polypeptide chain length). When the polypeptide chain length, as measured by gel chromatography, is compared to the molecular mass measured by sedimentation equilibrium, it is possible to determine that all covalent crosslinks have been broken.

Although empirical in nature, gel chromatography in denaturing solvents is a very powerful tool for the study of the subunit composition of proteins.

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