body, treated to eliminate complement-fixing capacity, inhibits complement binding when mixed with untreated complement-fixing antibody (*14*). Mixtures in vivo of specific antibody of variable IgG subclass could affect the extent of tissue damage by determining the probability of the occurrence of two adjacent complement reactive molecules bound to antigen. Our observations would appear consistent with such a concept as applied to the occurrence of glomerulonephritis in patients with antibodies to nuclear antigens. Genetic or other factors might determine the subclass of IgG molecules which comprise the population of a given antibody present at any particular time.

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Proteins in Denaturing Solvents: Gel Exclusion Studies

Abstract. *Many proteins are fully denatured and separated into component polypeptide chains in 6M guanidine hydrochloride containing mercaptoethanol and a chelating agent. In this and similar denaturing solvents, polypeptide chains of molecular weights from 2,000 to 100,000 can be eluted from 6 percent agarose columns and separated according to molecular weight. By this procedure molecular weights may be assigned to proteins (including many normally insoluble in nondenaturing solvents) without the uncertainties that arise when native molecules of unknown shape are studied.*

The volumes accessible to macromolecules in columns of molecular-sieving media such as Sephadex (Pharmacia), polyacrylamide gels (Bio-Rad), or agarose depend upon the size and shape of the molecule; hence the elution volumes for a series of homologous macromolecules reflect their molecular weights (*1*). For estimation of protein molecular weights, the shapes and the hydrations of the sample and of calibrating proteins must be similar. This source of ambiguity in instances in which the shape is unknown can often be obviated if the proteins are denatured; it has been shown that many polypeptide chains show homologous physical behavior in 5*M* or 6*M* solutions of guanidine hydrochloride containing reducing agents (*2*, *3*); under these conditions the chains adopt a conformation close to a random coil (*2*). Therefore the relative weights of peptide chains (and, if the numbers of constituent chains are known, the molecular weights of the parent proteins) can be deduced from gel filtration in denaturing solvents.

I have studied the behavior of insulin (Sigma), bovine serum albumin, ovalbumin, β-lactoglobulin (all Pentex), aldolase, chymotrypsinogen, trypsin, lysozyme, and ribonuclease (all Worthington) on a variety of gel media. Each sample was checked for purity by ultracentrifugal and molecular-sieve analysis. Each protein was dissolved in 6*M* solution of guanidine hydrochloride containing 0.05*M* lithium chloride, 0.1*M* mercaptoethanol, and 0.01*M* ethylenediaminetetraacetic acid (EDTA), adjusted to *p*H 6.5 to 7.3, and chromatographed with 5*M* or 6*M* guanidine hydrochloride or 8*M* urea; each mixture contained these proportions of mercaptoethanol, EDTA, and lithium chloride. The *p*H of the eluting solution was adjusted to between 6.5 and 8. Lithium chloride was used for an irrelevant purpose and can be replaced by other electrolytes.

These experiments were performed at 23°C in Pharmacia 2 by 100 cm columns of siliconized glass or, more commonly, in 0.9 by 60 cm acrylic columns. Usually 0.2 to 3 mg of protein was applied in not more than 0.4 ml—that is, 4 percent of the exclusion volume of the smaller columns. The eluting solutions were pumped through the columns at a constant rate (not exceeding 2.5 ml/hour per square centimeter of column section) and collected in 40 to 60 fractions. On the shorter columns an analysis could be completed within 24 hours. The protein in the eluate samples was estimated turbidimetrically with trichloroacetic acid or sulfosalicylic acid. The procedure can be made more sensitive if the protein can be coupled to a dye (such as rhodamine isothiocyanate) or radioactively labeled, in vivo, or with a suitable reagent (reaction with $H^{14}CHO$, for example).

A large increase in intrinsic viscosity accompanies protein denaturation (*2*), and, in correlation with their greater physical dimensions, the denatured molecules are excluded from gel media that normally admit the native. Sephadex G-100 or beads of 10 percent agarose are suitable only for the characterization of denatured polypeptide chains up to 20,000 daltons in weight. The gel encompassing resolution of proteins over the most useful range of molecular weights proved to be Bio-Gel A-5M with a nominal 6 percent content of agarose. Figure 1 shows a series of elution curves of different polypeptide chains run on this material. The exclusion and internal volumes of the columns were marked by cochromatography of dextran blue (Pharmacia) and dinitrophenyl-alanine with the protein. The exclusion limit of the 6 percent agarose is about 100,000 molecular weight; for longer chains (for example, the α- and β-chains of collagen) 4 percent agarose columns may be employed; however, the peaks—at least on Sepharose 4B—were broader than those in Fig. 1.

The eluted peaks showed a considerable spread that precluded facile discrimination of chains of similar size; nevertheless, the maxima of the peaks (by interpolation between successive tubes) showed good consistency in all experiments. The distribution coefficient (*1*) for a solute is $K = (V_e - V_o)/(V_i - V_o)$, where V_e is the elution volume of

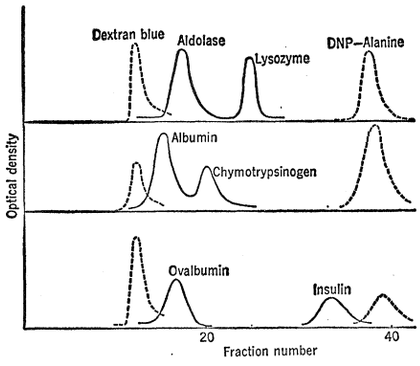

Fig. 1. Elution diagrams of proteins chromatographed on 6 percent agarose in 5*M* guanidine hydrochloride (top two curves) or 8*M* urea. Each solvent also contained mercaptoethanol and EDTA (see text). The profile of dextran blue was measured at 6300 Å; that of dinitrophenyl- (DNP) alanine, at 3500 Å. The turbidity of the protein solutions was measured at 4500 Å on a Gilford spectrophotometer.

the protein studied, V_o is the exclusion volume indicated by dextran blue, and V_i is the internal volume indicated by dinitrophenyl-alanine. For lysozyme in eight experiments K equaled 0.489 ± 0.016; for bovine serum albumin in five experiments, 0.100 ± 0.012.

Figure 2 shows the relation of the distribution coefficients to the molecular weights of the polypeptide chains studied by me. These results may be summarized by the expression $\log M = 4.01 - 2.105\,K$, where M is the molecular weight of the peptide chain. The

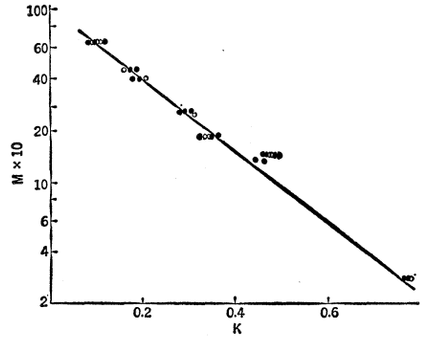

Fig. 2. Semilogarithmic plot relating the distribution coefficient K to the molecular weights of the polypeptide chains. The A and B chains of insulin were not resolved, and the logarithmic mean of their weights is used in the graph. Values for K were determined in guanidine hydrochloride (●) and in urea (○). The molecular weights of the chains were assigned as follows (in ascending order): insulin, 2,800; ribonuclease, 13,700; lysozyme, 14,500; β-lactoglobulin, 18,500; trypsin, 24,000; chymotrypsinogen, 25,000; aldolase, 40,000; ovalbumin, 45,000; and bovine serum albumin, 65,000. Lysozyme was consistently retarded from its expected position.

precision of these measurements suggests that the molecular weights may be estimated probably within 10 percent. The relation between K and M can certainly be expected to vary with different batches of gel, but calibration can be readily effected by chromatography of albumin, chymotrypsinogen, and insulin.

Several previous molecular-sieve studies of denatured proteins have been made without reduction of disulfide bonds (*4*). The intrinsic viscosities of peptide chains, denatured with and without breakage of disulfide bonds, are very different (*2*), and the importance of adequate reduction therefore merits emphasis if full denaturation is to be attained; that is, the concentration of thiol reagents must be sufficient to overcome any aerial oxidation that may occur, and a *p*H higher than 6 must be maintained to permit rapid reduction of disulfide (*2*). Another recommended addition to the denaturing solution is a chelating reagent to scavenge the metal from any metalloprotein so as to permit full unfolding of the polypeptide chains.

These experiments have demonstrated the utility of solutions of urea and guanidine hydrochloride, and concentrated solutions of lithium chloride probably could be used similarly. However, in view of the varying degrees to which detergent molecules are bound to proteins, the homologous behavior of detergent-denatured proteins cannot be assumed. The urea-treated proteins could suffer carbamylation during study; thus electrophoretic heterogeneity may be produced by the chromatography (*5*).

The validity of the molecular weights, determined by molecular sieving, must depend on the complete denaturation of the polypeptide chains and the absence of adsorptive interaction between protein and gel; for either of these reasons the molecular weight could be underestimated. The consistent retardation of lysozyme, in both urea and guanidine hydrochloride, presumably must be explained by one of these factors. Thus it is clearly desirable to confirm by some independent procedure, such as viscometry (*2*) or ultracentrifugation (*3*), any molecular weights determined by sieve chromatography. Conversely the molecular weights determined by these other procedures may well merit vindication by analysis by sieve chromatography, especially if the homogeneity of the polypeptide is in doubt.

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Uroporphyrinogen III Cosynthetase in Bovine Erythropoietic Porphyria

Abstract. *The activity of uroporphyrinogen III cosynthetase is much lower in hemolyzates from mature cattle with congenital erythropoietic porphyria than in hemolyzates from mature normal cattle. The porphyric hemolyzates do not inhibit the cosynthetase activity present in normal hemolyzates or in extracts of mouse spleen.*

Uroporphyrinogen III cosynthetase determines the structure of the tetrapyrrole formed by the enzyme uroporphyrinogen I synthetase (*1, 2*). In the presence of cosynthetase, synthetase catalyzes the conversion of the monopyrrole porphobilinogen to uroporphyrinogen III, a physiological intermediate in the biosynthesis of protoporphyrin IX. In the absence of cosynthetase, synthetase catalyzes the formation of uroporphyrinogen I, a product which is not converted to heme or chlorophyll by biological systems. The mode of action of cosynthetase is unknown; it does not catalyze interconversion of the uroporphyrinogen isomers. Bogorad proposed that synthetase catalyzes the formation of a linear polypyrrole which cyclizes spontaneously to form uroporphyrinogen I, and that cosynthetase is an enzyme which catalyzes cyclization of the polypyrrole to form uroporphyrinogen III (*3*).

Animals and humans with the hereditary disorder congenital erythropoietic porphyria excrete in the urine large amounts of uroporphyrin I, an oxidation product of uroporphyrinogen I. Increased excretion of uroporphyrin I could result from a hereditary deficiency of the cosynthetase (*3, 4*), but direct measurements of cosynthetase activities have not been made. Cosynthetase activity in crude extracts may now be measured with the use of partially purified synthetase from hematopoietic mouse spleen (*2, 5*). With this method,