used and is superior for the removal of inorganic phosphate.

The determination of fatty acid in lipid extracts before and after treatment on Sephadex columns is a reliable criterion for the recovery of all lipids except hydrocarbons, steroids, nonesterified ethers, and various other lipid components that occur in tissues in relatively small amounts. The recoveries of the order of 99% obtained probably indicate that the loss of lipid on Sephadex is negligible. It is particularly noteworthy that brain extracts containing sulfolipids and gangliosides and the fraction containing highly polar phosphatidic acid showed no significant loss of fatty acid. That sterols are not lost in the procedure is indicated by the good recovery of cholesterol obtained.

The data presented in Tables I, II, and III indicate that the limiting factor on the capacity of the columns is in the total volume of lipid extract and eluting solvent passed through the column and is not greatly affected by the concentration of the lipid. Because of the convenience of using small columns the use of as concentrated solutions as possible is desirable. However, the relatively dilute extracts obtained from most tissues can be treated without concentration by simply scaling up the size of the columns. This is particularly desirable with extracts of labile lipids which might be degraded during concentration. Regardless, optimum performance is obtained when up to 15 ml of a chloroform-methanol-water, 60:30:4.5, solution of lipid is eluted through the column with 5 ml of chloroform-methanol, 2:1, for every gram of Sephadex. At 4°, an additional 5 ml of chloroform-methanol, 2:1, is required to elute the lipid quantitatively. It should be emphasized that the actual concentration of lipid or contaminant is not critical but the combined volume of lipid solution and eluting solvent should not exceed 20 ml/g of Sephadex. Elution with excessive volumes will cause the elution of nonlipid material. Also, prior partitioning of extracts with water or salt solution results in poor retention of nonlipid contaminants, although this can be corrected by adding HCl or $MgCl_2$ to the lipid solution.

Finally, it may be pointed out that the use of Sephadex for this application has the advantage of being rapid and easily adapted to either a micro or macro scale. With lipid preparations subject to oxidation, it can be used in the cold or, with minor modifications, in an inert atmosphere.

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Studies of Chemically Reacting Systems on Sephadex. I. Chromatographic Demonstration of the Gilbert Theory*

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The presence of rapid reversible association in proteins has previously been demonstrated by either electrophoretic or sedimentation velocity experiments. In the present study, a chromatographic procedure employing Sephadex G-100 is described whereby similar qualitative infomation may be obtained. Patterns comparable with the schlieren peaks observed in the ultracentrifuge have been derived from the elution profiles of α-chymotrypsin in 0.01 M sodium phosphate, *p*H 7.9. All the features of the transport behavior of such systems, theoretically predicted by Gilbert, have been observed experimentally in a chromatographic procedure for the first time.

The possible effects of chemical interconversion of solute species during transport experiments has been the subject of several theoretical studies. For a substance existing in two isomeric forms, or in association equilibrium, as many as three peaks or spots can result when the rate of reaction is slow relative to the time of migration (Keller and Giddings, 1960; Cann and Bailey, 1961; Scholten, 1961; Mysels and Scholten, 1962). This type of behavior has been observed, e.g., in the chromatography of glucuronic acid and its lactone

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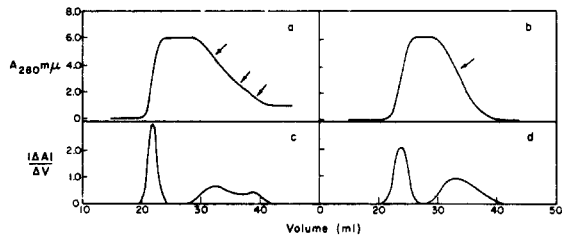

FIG. 1.—Elution profiles obtained in the chromatography of (a) α-chymotrypsin (3.8 mg/ml) and (b) DIP-chymotrypsin (3.2 mg/ml) on 1.25 × 32-cm columns of Sephadex G-100 previously equilibrated with 0.01 M phosphate, pH 7.9. Patterns (c) and (d) are the respective first-derivative curves, 0.50 ml having been used as the increment in volume. The arrows indicate inflection points.

(Partridge, 1948). Furthermore, for substances undergoing instantaneous chemical re-equilibration it is theoretically also possible to obtain apparent partial resolution (Gilbert, 1955, 1959; Bethune and Kegeles, 1961a,c). Experimental verification of this effect is found, e.g., in ultracentrifuge studies of α-chymotrypsin (Massey *et al.*, 1955) and β-lactoglobulin (Townend *et al.*, 1960).

Although the theory for the sedimentation and electrophoretic behavior of systems undergoing rapid reversible association (Gilbert, 1955, 1959) was developed from chromatographic principles, these effects have not heretofore been detected in chromatography. The present work was accordingly undertaken in order to apply a column procedure to the study of chemically reacting systems in rapid equilibrium. The procedure of gel filtration chromatography on columns of Sephadex (Porath and Flodin, 1959) seemed most suitable for the present purpose, since a second solvent, often detrimental to proteins, is not required. In addition, it has been established for a homologous series of dextrans that the elution rate is dependent upon molecular weight (Granath and Flodin, 1961), a necessary criterion for Gilbert effects to be observed in the chromatography of rapidly associating systems.

This paper reports the results of initial studies on the possible use of gel filtration for obtaining information about systems in rapid association equilibrium. For this purpose α-chymotrypsin in 0.01 M phosphate, pH 7.9, a system studied extensively in the ultracentrifuge by Massey *et al.* (1955), has been investigated.

EXPERIMENTAL

Materials.—Three-times crystallized, salt-free α-chymotrypsin (lot CDI 678-84B) and five-times crystallized soybean trypsin inhibitor (lot SI 5433) were obtained from Worthington Biochemical Corporation, Freehold, N.J. The ovalbumin was also a five-times crystallized product (lot 5709, Nutritional Biochemicals Corporation, Cleveland, Ohio). Phenol reagent (Folin and Ciocalteu, 1927) was obtained from Fisher Scientific Co., New York, N.Y. Sephadex G-100 (lot To 1554), purchased from Pharmacia Fine Chemicals, Inc., Rochester, Minn., was used without further refinement.

Diisopropylphosphoryl- (DIP-)[1] chymotrypsin was prepared from α-chymotrypsin according to the procedure of Jansen *et al.* (1949), and the sample was then exhaustively dialyzed at 4° against 7×10^{-4} M HCl prior to freeze-drying.

Estimation of Protein Concentrations.—All protein concentrations were determined spectrophotometrically at 280 mμ in a Beckman DU spectrophotometer; the values 20.1 (Egan *et al.*, 1957), 6.6 (Crammer and Neuberger, 1943), and 9.4 (Wu and Scheraga, 1962) were used for the extinction coefficients ($E_{1\,cm}^{1\%}$) of α-chymotrypsin, ovalbumin, and soybean trypsin inhibitor, respectively.

Column Procedure.—The proteins were dissolved directly in buffer (0.01 M Na_2HPO_4, pH adjusted to 7.9 with dilute HCl), and then applied to a 32 × 1.25-cm column of Sephadex G-100, previously equilibrated with the same buffer. A disk of filter paper was placed on top of the column to minimize disturbance of the upper section during the application of samples. Sufficient protein solution was applied to the column to ensure a plateau region in the resulting effluent profile (approximately 9 ml of ovalbumin, soybean trypsin inhibitor, and DIP-chymotrypsin, 14 ml of α-chymotrypsin). During this process and the subsequent elution with more of the same phosphate buffer, the flow rate was maintained at approximately 6 ml/hour. The effluent from the column was collected in fractions of about 0.3 ml each, using a Technicon fraction collector equipped with a drop-counting attachment. In order to obtain a more precise measurement of the volume of each fraction, the previously tared tubes were reweighed, a value of 1 being assumed for the density of the solution. A 0.25-ml portion of each fraction was then either diluted with phosphate buffer prior to spectrophotometric analysis at 280 mμ, or analyzed by the colorimetric procedure of Lowry *et al.* (1951). Experiments were performed at room temperature (22–24°).

Differentiation of Elution Profiles.—The chromatographic data were first represented graphically in the form of absorbancy at 280 mμ (A) versus volume (V) of effluent which had flowed from the column since the initial application of protein sample. First-derivative curves were then obtained from these elution profiles by computing the change in absorbancy (ΔA) for a 0.50-ml increment in volume (ΔV) and plotting the quantity $\Delta A/\Delta V$ versus $\bar{V}$, the mean value of the effluent volume in the interval ΔV.

RESULTS

A typical elution profile for α-chymotrypsin in 0.01 M phosphate, pH 7.9, is shown in Figure 1a, in which it is evident that, at the end of the experiment, the effluent still contained material absorbing at 280 mμ. Since these experiments were performed under conditions where considerable autolysis must be expected to occur, it is thought that this elevated baseline results from the elution of peptide fragments. The fact that the original baseline value of absorbancy is regained when the inactive DIP-derivative of chymotrypsin is chromatographed under the same conditions (Fig. 1b) lends support to this conclusion. However, the possibility of this anomaly arising from adsorption effects (Glazer and Wellner, 1962) cannot be eliminated.

In both of these elution profiles the advancing edge is much sharper than its trailing counterpart, indicating nonlinear isotherms (DeVault, 1943; Bethune and Kegeles, 1961a; Houghton, 1963; Oldenkamp and Houghton, 1963). A further point of the theory of Bethune and Kegeles (1961a) is that, for a rapidly polymerizing system involving aggregates greater than dimers, there should be additional points of inflection in the trailing edge; this is apparent in Figure 1a but not in Figure 1b.

To transform these profiles into patterns comparable with those observed in sedimentation and electropho-

[1] Abbreviation used in this paper: DIP, diisopropylphosphoryl.

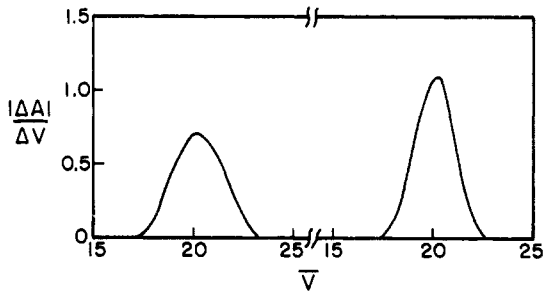

FIG. 2.—First-derivative curves of the advancing (left) and trailing (right) edges of the protein zone in the chromatography of ovalbumin (4.0 mg/ml) on Sephadex G-100 previously equilibrated with 0.01 M phosphate, *p*H 7.9. For the trailing edge effluent volumes were calculated by using as origin the volume at which elution with 0.01 M phosphate was commenced.

retic experiments, the first-derivative curves are required; these are shown in Figure 1c,d, where the difference between the two experiments, mentioned above, becomes much more apparent. Both patterns (Fig. 1c,d) are typical of reversibly associated systems, the former representing the case where trimers or larger polymers are present, the latter representative of a monomer ⇌ dimer system (Gilbert, 1955, 1959). Such a difference between the association behavior of native and DIP-chymotrypsin was not anticipated. In fact, Massey *et al.* (1955) report the result of one ultracentrifuge experiment at a much higher concentration in which apparent partial resolution was observed with DIP-chymotrypsin. The pattern obtained for the modified protein in the present study, which we confirmed in the ultracentrifuge, is typical of those reported for the sedimentation of α-chymotrypsin and its derivative at all other conditions of *p*H and ionic strength studied (Schwert, 1949; Smith and Brown, 1952; Steiner, 1954; Massey *et al.*, 1955; Dreyer *et al.*, 1955; Rao and Kegeles, 1958; Tinoco, 1957).

A feature of the derivative curves (Fig. 1c,d) is the sharp advancing and diffuse trailing boundaries, which is the reverse of the behavior of a nonassociating protein, e.g., ovalbumin, on Sephadex (Fig. 2). Similar hypersharp peaks are observed with rapidly associating systems in electrophoresis, where the ascending boundary is hypersharp and the descending boundary is diffuse (Egan *et al.*, 1957; Ogston and Tombs, 1957; Tombs, 1957, 1958; Timasheff and Townend, 1960, 1961).

The derivative curves of the trailing edges obtained at a series of concentrations of α-chymotrypsin[2] are shown in Figure 3. Comparison with schlieren patterns obtained in the ultracentrifuge (Massey *et al.*, 1955) is possible only at the two highest concentrations,[3] where fairly good agreement is observed between the two methods. Figure 3e,f correspond very closely to Figure 2a,b of Massey *et al.* (1955); in the present study the faster-moving peak is on the left. The slightly smaller size of the slower-moving boundary than that observed by Massey *et al.* (1955) may be a consequence of the failure to regain the original baseline value of absorbancy. In Figure 3c–f, apparent partial resolution into two peaks is observed, the size of the slower-moving (right-hand) peak remaining essentially constant. This feature of the Gilbert (1955, 1959) theory has, of course, been previously observed with chymotrypsin under these conditions in the ultracentrifuge studies of Massey *et al.* (1955). However, the Gilbert theory also predicts that at concentrations below a certain critical value, corresponding to the area under the slow-moving peak, only a single peak will be observed; Figure 3a,b illustrate this feature. The results depicted in Figure 3 thus provide perhaps the best demonstration so far of the Gilbert (1955, 1959) theory for the transport properties of a rapidly, reversibly associating system involving polymers greater than dimers in that it has also been possible to study the concentration range where only a single peak is observed.

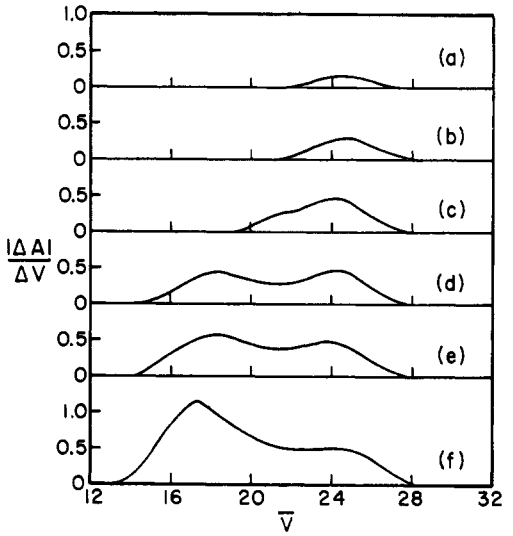

FIG. 3.—Derivative patterns of the trailing edge of the protein zone in the chromatography of α-chymotrypsin at a series of concentrations on Sephadex G-100. Experimental conditions as in Fig. 1. (a) 0.6; (b) 1.2; (c) 1.4; (d) 2.8; (e) 3.4; (f) 5.0 mg/ml.

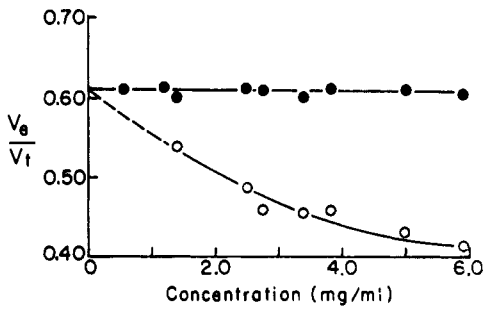

FIG. 4.—The concentration dependence of the rate of elution of the trailing edge in the chromatography of α-chymotrypsin on Sephadex G-100. ●—●, the slow-moving peak; O—O, the fast-moving peak.

It should be emphasized that the partial separation of peaks observed in Figure 3 is only apparent, no actual resolution having been achieved, and that it is quite incorrect to identify either peak as the monomeric or polymeric species. Thus, failure to observe the faster-moving peak in Figure 3a,b does *not* imply the absence of polymeric species at these concentrations.

It was also of interest to determine qualitatively whether the analogy between gel filtration and ultracentrifugation could be extended to include quantities corresponding to sedimentation coefficients. The concentration dependence of the rates of elution of the trailing boundaries is shown in Figure 4, where the manner

[2] Since the features of the Gilbert theory for monomer-dimer equilibrium are not as striking as those for systems involving higher polymers, native chymotrypsin, although less ideal experimentally than DIP-chymotrypsin, has been studied in greater detail.

[3] The diffuse nature of the peak makes ultracentrifuge studies of α-chymotrypsin under these conditions impractical below a concentration of 3.5 mg/ml, whereas solutions containing as little as 0.1 mg/ml can be studied by the chromatographic procedure.

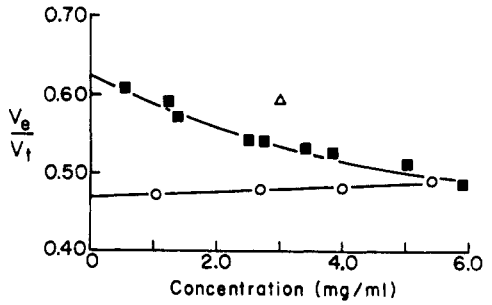

FIG. 5.—The concentration dependence of the rate of elution of the advancing edge in the chromatography of proteins on Sephadex G-100 previously equilibrated with 0.01 M phosphate, *p*H 7.9. ■—■, α-chymotrypsin; O—O, ovalbumin; △, soybean trypsin inhibitor.

of presentation is that used by Pedersen (1962). In this method the rate of elution is recorded as the ratio of the volume of solvent required for elution of the peak (V_e) to the total volume of the column (V_t). Since this quantity thus represents the fraction of the column volume which is accessible to a particular type of molecule, faster elution rates are indicated by smaller values. As is seen in Figure 4, the migration rate of the slow-moving peak is essentially constant, while that of the faster peak increases with increasing concentration in the range studied. This is in agreement with the ultracentrifugal results of Massey *et al.* (1955) and the theoretical predictions of Gilbert (1955, 1959).

In Figure 5 is shown the corresponding relationship for the advancing boundary, together with results for ovalbumin and soybean trypsin inhibitor; these values of V_e/V_t define the systems more precisely than the trailing counterparts in that they are weight-average quantities (Gilbert, 1959). As was anticipated, very little variation of V_e/V_t with concentration was observed for ovalbumin. However, the slight positive concentration dependence is significant [$V_e/V_t = (V_e/V_t)_0 (1 + 0.08\ C)$], and parallels very closely the corresponding sedimentation coefficient relationship [$s = s_0\ (1 - 0.076\ C)$] found by Creeth and Winzor (1962).

Since the concentration dependence of the sedimentation coefficients of nonassociating proteins is usually fairly small, and, for a polymeric system the sedimentation coefficient reflects the state of aggregation, the results shown in Figure 5 give, to a first approximation, a measure of the weight-average molecular weight as a function of concentration. As expected on this basis, the value of V_e/V_t for chymotrypsin increases with decreasing concentration. However, from this study it would appear that the use of other proteins of known molecular weights as markers does not appear feasible. The value of V_e/V_t for soybean trypsin inhibitor, mw 21,500 (Wu and Scheraga, 1962), at one particular concentration is less than the extrapolated value for chymotrypsin, the monomeric molecular weight of which is 25,000 by chemical (Jansen *et al.*, 1949; Hartley and Kilby, 1952) and physicochemical (Steiner, 1954; Tinoco, 1957; Rao and Kegeles, 1958) techniques. No serious error should be introduced by using a nonextrapolated value for soybean trysin inhibitor, since the concentration dependence of the sedimentation coefficient is negligible (Sheppard and McLaren, 1953). An even greater discrepancy is observed by comparing the results obtained for ovalbumin, mw 45,000 (Fevold, 1951; Halwer *et al.*, 1951), with those for chymotrypsin at higher concentrations. From the calculations of Gilbert (1955) it has been estimated that the weight-average molecular weight of chymotrypsin should be in the vicinity of 80–90,000; however, a value of less than 45,000 would be indicated by the chromatographic procedure, if ovalbumin were used as a reference molecule.

DISCUSSION

The results of this study of α-chymotrypsin on columns of Sephadex G-100 serve to illustrate the general applicability of the Gilbert (1955, 1959) theory for the behavior of systems in rapid association equilibrium to *any* transport experiment in which there is a difference between the migration rate of monomeric and polymeric species. On the basis of the partition chromatographic theory for the mode of action of Sephadex (Flodin, 1962), this work also lends qualitative support to the theory of Bethune and Kegeles (1961a,c) for the behavior of rapidly, reversibly associating systems in countercurrent-distribution experiments. In this connection Pedersen (1962) has questioned the partition chromatographic theory of gel filtration, and has preferred to rename the process *exclusion chromatography*. Although this is the first chromatographic study in which the Gilbert (1955, 1959) theory has been used to interpret elution profiles, Hirs (1955) has reported that broad and decidedly asymmetric peaks are obtained in the chromatography of α-chymotrypsin.

As a qualitative method of detecting and studying rapidly associating systems the present procedure compares quite favorably with the other methods available. Patterns essentially analogous to those obtained in ultracentrifugation may be derived from the elution profiles, and the method has the advantage, also exhibited by moving-boundary electrophoresis (Edsall *et al.* 1955; Egan *et al.*, 1957; Ogston and Tombs, 1957; Timasheff and Townend, 1960), that the hypersharp leading edge of the protein zone is observed in addition to the diffuse trailing edge, thus facilitating qualitative detection of rapid, reversible association. From the quantitative point of view the procedure suffers from the disadvantage that molecular size is not the only factor affecting the rate of migration (Gelotte, 1960; Porath, 1960; see, however, Andrews and Folley, 1963). Thus, as is seen in this study, gel filtration cannot be used alone, with the aid of proteins of known molecular weight as markers, to yield the molecular weight of the monomeric species involved in the association process.

However, the method does yield a weight-average quantity (Gilbert, 1959), which for a homologous series of polymers is a function of molecular weight (Granath and Flodin, 1961). Furthermore, because of the very low concentrations that can be used in this procedure (less than 0.1 mg/ml, compared with 1.0 mg/ml in the ultracentrifuge), extrapolation of V_e/V_t to zero concentration is greatly facilitated. From this point of view gel filtration has a decided advantage over the direct measurement of molecular weights, where difficulty with the extrapolation, of necessity from a higher concentration, has been encountered (Rao and Kegeles, 1958; Cohly and Scheraga, 1961; Millar, 1962). Thus use of chromatography on Sephadex, in conjunction with molecular weight estimations by the Klainer and Kegeles (1955, 1956) modification of the Archibald (1947) procedure at higher concentrations to determine the relationship between elution rate and molecular weight for the particular polymeric system, may well provide a better estimate of the size of the monomeric species. Such a study is currently in progress (Winzor and Scheraga, 1963). A quantitative study of this kind with α-chymotrypsin at *p*H 7.9 has not been attempted because of the autolysis which occurs under these conditions. This system, although nonideal in

this respect, was chosen in order to exemplify the most striking features of the Gilbert (1955, 1959) theory in this qualitative chromatographic study.

It would appear that chromatography on Sephadex is likely to provide a convenient general procedure for investigating reversible interactions, an application of gel filtration to the study of reversible interactions between macromolecules and small molecules having already been reported (Hummel and Dreyer, 1962). The present investigation of reversible polymerization is a special case of the more general reaction A + B $\rightleftharpoons$ C, and the method can obviously be extended to rapid protein-protein interactions of the latter type, for which the theoretical transport behavior has also been predicted (Gilbert and Jenkins, 1956, 1960; Bethune and Kegeles, 1961b).

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