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ADSORPTION STUDIES OF ENZYMES AND OTHER PROTEINS

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I. Introduction

Twenty-five years ago adsorption was the principal technic advocated for the characterization and purification of enzymes. With the demonstration that enzymes were proteins, precipitation and

other procedures based on the high molecular weight of proteins came to the fore as a means for the study of enzymes. Because of this new concept of enzymes and the limitations of the adsorption procedure the method for a time was almost completely abandoned. Within the last decade advances in adsorption technics exemplified in various types of chromatography have led to simple but precise means for the study of biological substances of low molecular weight. With the obvious success of chromatography in many biological problems the method has been applied in recent years to the study of enzymes and other proteins. This review will summarize the results of these studies and the study of related adsorption phenomena, and discuss the limitations of adsorption procedures particularly where imposed by the high molecular weight and complexity of proteins.

The early literature on the adsorption of enzymes has been reviewed by Kraut (98). This literature will be referred to only briefly here to illustrate the variables affecting adsorption, and certain aspects of the single (batchwise) adsorption technic. It will be seen from the recent examples cited that the single adsorption method, in conjunction with other procedures, again has a role as an important step in the purification of enzymes.

There will be only brief references to equipment and procedure in the present review. Chromatographic technics in general have been thoroughly dealt with in many books and reviews. Chromatography of proteins is still very much of an art and the best references for procedures are the papers of those who have been successful in applying chromatography to proteins.

II. Theory and Principles

The theory and principles of adsorption in relation to chromatography have been thoroughly discussed in a recent book (21) and several shorter articles of general interest (121,233,199,12). New developments are now reviewed annually (197,102,198,27,200,96,201). Only those aspects will be referred to here that appear to bear on the practical application of chromatography to solutions of proteins.

A. ADSORPTION ISOTHERMS AND THEIR RELATION TO CHROMATOGRAPHY

The quantitative aspects of adsorption are described in terms of adsorption isotherms which formulate the amount adsorbed, m, as a function of the equilibrium concentration, c, of the adsorbed substance. When $m = kc$ the isotherm is linear. Usually the isotherm is not linear but decreases with increase in c. For these cases the formulation has followed that of Freundlich or that of Langmuir.

The Freundlich isotherm has the following form: $m = k_1c^{k_2}$. This equation becomes linear when k_2 is one; there is a continuous increase in m with increase in c. By taking the log of each side of the equation it becomes $\log m = k_1 + k_2 \log c$. When $\log m$ is plotted against $\log c$, a straight line will be obtained and the intercept will equal $\log k_1$ and the slope of the straight line will equal k_2.

The Langmuir isotherm has the following form: $m = k_1k_2c/(1 + k_2c)$. In this case a linear equation is obtained when k_2 is very small. Further, this equation provides for linear results in very dilute solutions, and the attainment of a maximum at some higher concentration. This equation can be put in the form $c/m = 1/k_1k_2 + c/k_1$. By plotting c/m against c a straight line is obtained and the constants can be obtained from the slope and intercept. The derivation and significance of these equations has been treated by Cassidy (21); he and others (199,123) have covered the theories relating adsorption isotherms to the separation and shape of the bands obtained on passing a solution down a column of adsorbent (chromatography).

The data required for the adsorption isotherm can readily be determined by shaking a series of concentrations of the substance or preparation under investigation each with a unit amount of adsorbent. The equilibrium concentration, c, is determined in the solution and the amount adsorbed per unit of adsorbent, m, is obtained by difference. In some reports a solution of one concentration is used throughout but the amount of adsorbent is varied (99,187). The results by this procedure will differ somewhat from the previous except for ideal conditions; the first procedure provides data useful for theoretical treatment but the latter appears to have advantages for mixtures of proteins in solution (98,187). Kraut and Bauer (98,99) reported that the shape of the adsorption curve, m versus c, obtained by varying the amount of adsorbent revealed whether the enzyme was pure and whether the accompanying proteins were more or less strongly adsorbed at various dilutions. The three types of curves that can be expected were illustrated. However it was found in some cases that enzymes that were still impure gave the ideal type of curve. Utkin (229) has reported data for pepsin adsorbed on calcium phos-

phate, obtained by varying both the amount of adsorbent and the concentration of the enzyme.

In enzyme studies total protein and enzyme activities can be determined and the isotherm for each obtained. In general, substances are less well adsorbed from a mixture than from pure solutions. However, the relative adsorptions usually remain the same. Knowledge of the adsorption isotherm of a substance is of practical value in several respects. If it is linear the band obtained in chromatography will be sharp; the greater the departure from linearity the greater will be the tailing of the band. Further, the greater the adsorption isotherm the slower the band will move in chromatography and vice versa. Linear adsorption isotherms have rarely been reported for proteins. However, in many cases they have conformed to the Langmuir isotherm and accordingly would be expected to become linear in sufficiently dilute solution. Moore and Stein (140) have emphasized the importance of adsorption data for predicting behavior of a protein on a column. Prior to their successful chromatography of ribonuclease, test tube experiments were performed with a number of adsorbents such as alumina, silica, cellulose, and several acidic and basic ion exchange resins. Only in the case of the resin IRC-50 was there obtained finite, reversible distribution of the protein between buffer and adsorbent.

In a number of instances the adsorption isotherms of proteins have been determined. The following have conformed to the Langmuir equation: egg albumin and gelatin on collodion (83), gliadin on quartz (95), egg albumin and gelatin on quartz (115). The data obtained by Hitchcock (83) are shown in Figure 1 both in a direct plot and in a manner showing the conformance to the Langmuir equation. In other instances the data were in agreement with the Freundlich equation, as shown by the adsorption of gelatin on charcoal (90), hemoglobin on kaolin (35), and amylase on starch (184). In the last two examples the amount of adsorbent was varied. In one report, adsorption of hemoglobin on kaolin plotted logarithmically did not give straight lines (153); however over a limited range straight lines were obtained (154). Addition of salts and change in *p*H altered the shape of the adsorption curves (35). Crystalline trypsin has been studied (179a) with a number of adsorbents as varied as cholesterol, alumina, and $Al(OH)_3$. In all instances the adsorption isotherms conformed to the Freundlich equation. In other studies the amount of adsorbent has been varied and the results presented in graph form: adsorpti of egg albumin on aluminum hydroxide (141), trypsin on charco (151), various proteins on silica gel (187), and alkaline phosphata on Filter-Cel (253). The adsorption isotherm of serum albumin calcium phosphate became nearly linear on the addition of phospha (207).

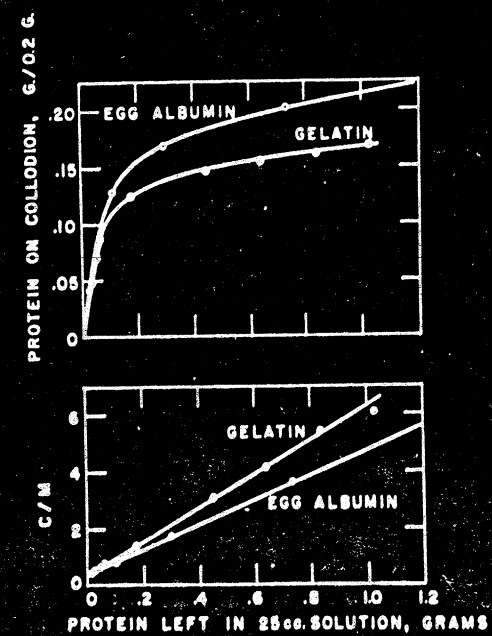

Fig. 1. Effect of protein concentration on adsorption of isoelectric gelatin and egg albumin on collodion membranes (83). Protein left in 25-cc. solution = C, equilibrium concentration; protein on collodion = M, protein adsorbed.

In two instances adsorption data have been obtained that bea the physical dimensions of the adsorbed layer of protein. Lindau Rhodius (115) in a study of the adsorption of egg albumin onto qu concluded that 3.6 mg. of albumin was adsorbed per square mete quartz surface at the isoelectric point. This figure was obtaine three different methods. In the original derivation of the Langm equation k_1 represented a volume constant of the adsorbed monol and k_2 an affinity constant; for the infinitely concentrated solu

the maximum amount adsorbed will be k_1k_2/k_3. Lindau and Rhodius (115) used this calculation to determine the maximum amount of albumin adsorbed, and determined the surface area of the adsorbent by a comparison of adsorption on quartz plates of measured area and on quartz powder. This procedure gave 3.5 mg. of albumin adsorbed per square meter of quartz.

The electrophoretic mobility of quartz particles in various concentrations of egg albumin shows a sharp break which is considered to represent the point at which maximum coverage of the quartz is obtained. The amount adsorbed by this method was 3.1 mg. per square meter. Williams and Choppin (234) using a similar procedure found that 3.5 mg. of egg albumin was adsorbed per square meter of synthetic rubber latex. The surface area was determined by microscopic inspection and estimation of the size of the latex spheres. Lindau and Rhodius with their third method obtained an average figure of 4.2 mg. of albumin per square meter by placing the quartz in a concentrated solution of albumin and subsequently washing with distilled water. The amount of egg albumin bound to unit area of adsorbent is considered to be in agreement (115,234) with Svedberg's calculations of the dimensions of the albumin molecule.

In view of the approximate agreement of the results of their last method with the other methods Lindau and Rhodius assumed that the retained protein represented an irreversibly bound monolayer of protein whereas any additional layers had been washed away. Williams and Choppin (234) pointed out that the S-shaped curves they obtained in a direct plot of their adsorption data are considered to represent multilayer adsorption. Zittle and Della Monica (253) reported that alkaline phosphatase appeared to be adsorbed on Filter-Cel in layers since there was a constant amount of enzyme per milligram of adsorbent that could not be eluted and a portion that could be eluted. The latter varied with the total enzyme adsorbed, which was determined by the concentration of the enzyme.

Both Lindau and Rhodius (115) and Williams and Choppin (234) found that one-third as much albumin is adsorbed at *p*H 3 as at the isoelectric point. This decrease in adsorption at lower *p*H values has been discussed in terms of hydration of the protein molecule (115), and active centers on the adsorbent (234). The albumin-on-quartz surface changed from hydrophilic to hydrophobic character at about 58% coverage of the surface (115); this phenomenon may explain the reversible type of adsorption curve obtained with proteins and the un expected irreversibility of the adsorbed protein monolayer (147).

More extensive studies of the type just quoted are desirable for a understanding of the adsorption of protein on solid adsorbents. It i important to know, for example, whether proteins are spread on suc surfaces as they are at liquid-gas interfaces.

B. VARIABLES AFFECTING ADSORPTION

1. *p*H

Changes in *p*H affect adsorption in two ways. The charge on th adsorbent is sometimes changed, as is the case with the ampholyte A (OH)$_3$ (47,188). Also ionization of the substance being adsorbed ma take place. This is an especially complex factor with proteins b cause of their amphoteric nature. In general, as shown in Figure 2 fo the data of Hitchcock (83) for the adsorption of albumin and gelati

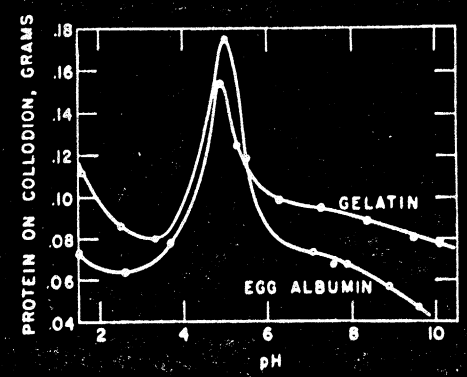

Fig. 2. Effect of *p*H on adsorption of gelatin and egg albumin from 2% solutions by collodion membranes (0.2 g.) (83).

on collodion, a maximum is obtained in the neighborhood of the is electric point with a relatively sharp decrease on either side. This h been observed for hemoglobin on kaolin (153), serum albumin kaolin (36), catalase on kaolin (78), sucrase on Al(OH)$_3$ (51), and e albumin on rubber latex (234). In the last example there is also an crease in adsorption at extremes of *p*H (3 and 7) giving a W-shap adsorption curve. The increase in adsorption at low *p*H values h

been observed in several instances (83,153). The adsorption maximum although probably related to the isoelectric point is also determined by the properties of the adsorbent; for example the adsorption maxima of egg albumin on seven adsorbent carbons varied from *p*H 3.0 to 6.0 (75a). The adsorption maxima of gelatin with different carbons showed some variation also (75a).

With many enzymes and other proteins it is not practical to study a wide range of *p*H values but a region will be found, presumably one limb of the total curve, where a change from maximal to minimal adsorption will be found for a range of *p*H values. Examples exist in the early enzyme literature (98) and recent studies of the following systems have given similar results: egg albumin on silica gel (153), alkaline phosphatase on Filter-Cel (223), prothrombin on aluminum hydroxide (143), lysine decarboxylase on aluminum hydroxide (60), xanthine oxidase on kaolin (37). However, the adsorption of immune globulin on silica gel was not altered by change of *p*H in the range 4 to 8 (153) and the adsorption of phycoerythrin on calcium phosphate in the presence of NaCl and phosphate was not affected by *p*H variation between 5.2 and 7.8 (207); change in *p*H had little effect on the adsorption of trypsin on charcoal (151).

Change in *p*H is a feature of most single adsorption-elution technics and it is bound to be an important factor in the operation of a column. For example fractional elution with several concentrations of acid has been successfully used in the purification of ACTH (62). Change in *p*H is employed in the purification of cytochrome c on the resin IRC-50 (155).

2. *Solvent*

The adsorption of proteins is determined to an important extent by the solvent from which they are adsorbed. Because of the lability and insolubility of most proteins in organic solvents, studies in such environments are usually impossible. However, important observations have been made with aqueous solutions of organic solvents. In general, adsorption of proteins is increased by organic solvents (188,239, 241). The magnitude of this effect is shown by the effect of the ethanol concentration on the movement of enzymes on filter paper chromatography (17a) (see Fig. 8). The interesting observation has been made that ethanol decreases the adsorption of amino acids on charcoal but at the same time the adsorption isotherm becomes more linear (223). An important study has been made of an enzyme in a two-phase system where one of the phases was predominantly organic (122); this is discussed in greater detail in section IV,B. The study of insulin in a two-phase system has been reported (74).

The influence of salts on adsorption is complex. In some cases the presence of salt prevents adsorption (84), and salts have been used to elute enzymes adsorbed from low-salt solutions (213,94,191,119,165, 117,116). In other cases however salts enhance adsorption and in some instances adsorption occurs on adsorbents that are otherwise inert to proteins. The ability of salts to increase adsorption has received considerable quantitative study (222,187,83,156,39,171,207). Additional studies are badly needed, however, to determine the type of enzyme or other protein, or the adsorbent, or conditions of adsorption by which salts increase adsorption in one instance and decrease it in another. Tiselius (222, 222a) has studied the ability of salts to increase the adsorption of certain proteins and termed it "salting out" adsorption. This procedure appeared to be promising, if readily reversible, for the protein could be adsorbed in a high-salt solution and eluted with a salt-poor solution. Anson (8) has pointed out that salting out adsorption of cathepsin onto tissue solids is involved in the purification of this enzyme. Other applications of this phenomena to the fractionation of proteins probably can be found.

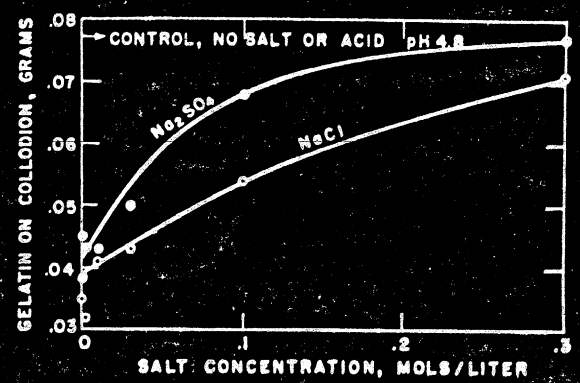

Fig. 3. Effect of salts on adsorption by collodion membranes of gelatin from 2% solutions in 0.02 *M* HCl (*p*H 2.5) (83).

Certain salts have specific effects with particular adsorbents. Phos-

phate, for example, will prevent adsorption on tricalcium phosphate and conversely can readily elute adsorbed proteins from this adsorbent (207,51). It is believed in part that the effect is due to displacement by adsorption of the phosphate on the calcium phosphate. The adsorption of the red protein phycoerythrin on filter paper, on the other hand, is increased by potassium phosphate (222).

Hitchcock (83) studied in detail the effect of salt concentration and *p*H on the adsorption of gelatin on collodion. It was found (83,156) (see Fig. 3, p. 327) that in acid solutions (*p*H 2.5) salt increased the adsorption up to the maximum obtained in the isoelectric region (*p*H 4.8) without salt. This was also observed for egg albumin on collodion, and in addition it was observed that at *p*H values alkaline to the isoelectric point adsorption with salt present was much above that at the isoelectric point (39). It is evident from the results in Figure 3 that NaCl and Na_2SO_4 are about equally effective at the same ionic strength. This was also found for the effect of NaCl and Na_2SO_4 on the adsorption of several proteins on silica gel (187).

The adsorption of egg albumin on silica gel was greatly increased by ammonium sulfate (19-fold at 2 *M* concentration) (222). Phycoerythrin, insulin, and pepsin were similarly affected by salts (222). Adsorption of phycoerythrin on calcium phosphate was greatly increased by NaCl; as little as 0.05% will bring about almost complete adsorption of the protein. Sodium phosphate opposed the effect of NaCl (207). The adsorption of one form of alkaline phosphatase on diatomaceous silica (Filter-Cel) is enhanced by salt (253). Shepard and Tiselius (187) studied the effect of salt on the adsorption of a number of proteins, especially serum proteins, on silica gel. Only the adsorption of immune globulin, which was high without salt, was unaffected by salt. Another type of behavior was shown by egg albumin, which was not adsorbed without salt but which showed increased adsorption in proportion to the salt added. The adsorption of hemoglobin on kaolin was slightly reduced by salts (35,154); the closeness of the *p*H (7.4 to 7.6) to the isoelectric point might explain the negligible effect. Hemoglobin at *p*H 7 was not adsorbed on silica gel or filter paper by the addition of salts (222). The adsorption of horse serum albumin on kaolin was little affected by salts at *p*H 5.4 to 5.6 (36). The interest in salting out adsorption is indicated by the number of studies reported recently. Further study is desirable, however, to determine the general applicability of the method. It should be noted that irreversibility of adsorption has been observed in this type of system also (253).

3. *Concentration of Adsorbate (Solute). Amount of Adsorbent*

These variables are paramount in the determination of the adsorption isotherm and have been discussed in detail earlier (II,A). In general adsorption increases with increase in concentration of protein until the adsorbent is saturated. With increase in the amount of adsorbent the total adsorption increases until the solution is exhausted. In the operation of a column it is desirable to use low concentrations of protein, where the adsorption isotherm approaches linearity.

4. *Temperature*

Only brief observations have been made of the influence of temperature on the adsorption of proteins. Adsorption of low molecular substances in general decreases with increase in temperature. In the case of proteins however in most cases adsorption is increased by a rise in temperature. Only in the case of the adsorption of amylase on starch, which may be a special type of adsorption (see III,B,3) was the opposite found; with this enzyme adsorption was considerably greater at 0° than at 20° (85a). The adsorption of ptyalin on filter paper (42), diastase on charcoal (228), egg albumin on collodion (39), alkaline phosphatase on Filter-Cel (253), and proteinase on bacterial cells (141a) show increased adsorption with increase in temperature. In the case of egg albumin on collodion adsorption was twice as great at 25° as at 1°. This effect of temperature suggests that adsorption of proteins involves a certain degree of unfolding of the protein on the surface of the adsorbent since this type of reaction is endothermic. The unfolding of proteins at liquid-liquid and liquid-gas interfaces is well known (147,148). The adsorption of a protein on a solid adsorbent need not lead to denaturation for, since the protein is held rigidly, it would have a better opportunity to return to its native state than when adsorbed at more mobile interfaces (217).

Whether this effect of temperature on the adsorption of proteins can be utilized in the single adsorption technic or in the operation of a column remains to be seen. It has been reported (209) that elution of invertase from $Al(OH)_3$ is favored by a temperature below 15°. In the case of amino acids chromatography at higher temperatures per-

mits faster flow rates due to increase in the rate of diffusion and exchange, and specific adsorptions are changed (139,140).

5. *Rate of Adsorption*

The time required for the adsorption of proteins is considerably greater than that required for low molecular substances. A number of studies have been reported with dilute solutions of protein (124,95) where change in the electrical properties of the adsorbent were used as a measure of the protein adsorption thereon. The rate of adsorption of gliadin on glass (124) and on quartz (95) is unimolecular with respect to concentration of protein, and the rate of adsorption is slower when the gliadin is negatively charged (*p*H 6.41, 5.66) than when positively charged (*p*H 3.2, 3.7) (glass and quartz are negatively

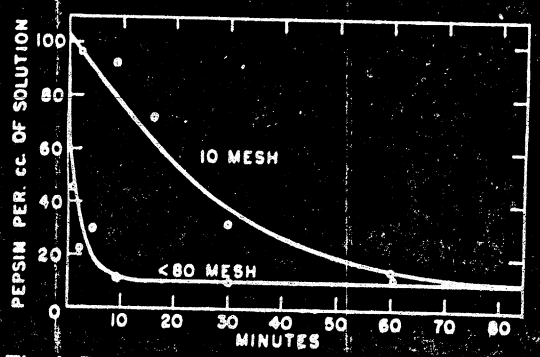

Fig. 4. Rate of adsorption of pepsin on dried egg albumin of different particle size (150).

charged) (95,124). The rate of adsorption is faster at higher temperatures (37° *vs.* 1°) (228). When the adsorbent has an internal structure accessible to proteins particle size is also a rate-determining factor, as shown in Figure 4 for the adsorption of pepsin on egg albumin at *p*H 2.5 (150). In the case of the adsorption of amylase in a concentration of about 0.2% on various starches most of the adsorption takes place within 2 minutes; particle size here also is a determining factor (184). Claesson and Claesson (26) in a study of the adsorption of polyvinyl acetates on activated carbon found that 40 to 60 minutes was required for adsorption. It was pointed out (25) that the low rate of diffusion of high molecular substances must be responsible. This requires that the rate of flow in adsorption columns must be extremely slow for adsorption equilibrium to be reached. This may be a source of trouble in the chromatography of many proteins. Shepard and Tiselius (187) found this a limiting factor in the study of various serum proteins and egg albumin. Thirty minutes was required for single adsorptions and for the development of satisfactory steps in frontal analysis 24 to 48 hours was required. However, not all proteins have such low rates of adsorption as shown by their successful chromatography; it should be noted that the greatest success has been with proteins of low molecular weight (ribonuclease, lysozyme, cytochrome c). Differences in the rate of adsorption have been taken advantage of in the operation of a column under nonequilibrium conditions (62).

6. *Special Features of Protein Adsorption*

It has already been pointed out that proteins because of their size have a low rate of diffusion which in turn means a low rate of adsorption. This in turn limits the speed at which continuous-flow operations (chromatography) can be performed.

Claesson (25), who has summarized chromatographic studies with high polymers, states that in homologous series there exists a maximum adsorbability for a molecular weight of about 10,000, with decreased adsorption at lower and higher weights. The studies on a series of dextrans were performed by Ingelman and Halling (86a) with the adsorbent carbon Carboraffin Supra. The adsorption isotherm of each dextran was determined. From these and other studies one can conclude that size alone will probably be a factor in the case of the adsorption of proteins as well.

There are other special features of protein adsorption that appear to be consequences of their large molecular weight. Lack of reversibility of adsorption is encountered with some low molecular substances (12) but it is almost regularly encountered with proteins; proteins, with few exceptions, cannot be eluted with the solvent from which they are adsorbed (13,130,194). Further, proteins adsorbed from high salt containing solutions cannot always be eluted at low salt concentrations (253). In most cases a large shift in *p*H is necessary to bring about elution (194). Also it appears to be difficult to obtain equilibrium at intermediate *p*H values. A few instances have been cited where enzymes adsorbed from alcohol-containing solutions have been eluted with water (237). In some cases the proteins can be only partially eluted (194,253). Such results may reflect a firmly held

layer and an outer, elutable layer held by secondary forces (194). Other puzzling features of this problem are that the equilibrium appears to be shifted by dilution (130) or by addition of adsorbent (95) whereas the proteins cannot be eluted from the adsorbent.

Irreversibility of adsorption might be expected when adsorption is almost stoichiometric as with the adsorption of pepsin on tricalcium phosphate (229), ACTH on cellulose (160), and albumin and hemoglobin on the resin Dowex 50 (193), but it is more difficult to understand, of the adsorptions so frequently reported, which fit the Freundlich or Langmuir equations, since the equations conform to conditions of equilibrium (147). This aspect of protein adsorption must be better understood before adsorption technics are generally applicable to the study of proteins.

Another important aspect of protein adsorption concerns the retention of the properties of the native protein. In a number of cases enzymes have been reported to retain their catalytic properties in the adsorbed state (38,60,69,78,101,128,131,145,166,239); in other cases negative results were reported (11,46), but in these cases apparently the adsorbed enyzme was not kept in suspension. Cobra venom and diphtheria toxin, however, were not active in the adsorbed state (14). There seems to be no instance in which an eluted enzyme was shown to be inactive. This retention of activity may be due to the rigidity with which proteins are held (see II,B,4). Langmuir and Schaefer have pointed out (107) that loss of chemical and biological activity of proteins may be due to the destruction rather than the formation of the monolayer. The activity of enzyme monolayers has been reviewed recently (177).

In cases where enzyme elution is resistant to quite high *p*H values it is found that, when elution is carried out with a displacing reagent such as phosphate from the adsorbent calcium phosphate, the enzyme is obtained in the active state. Serum globulins although firmly bound to $Al(OH)_3$ and mastic could be eluted with alkali and obtained in the undenatured state (194).

It should be kept in mind that coenzyme and apoenzyme may be separated by adsorption with resulting disappearance of enzyme activity. This has been reported for the old yellow enzyme when the riboflavin-containing coenzyme was adsorbed on the kaolin frankonite (232), and alkaline phosphatase when an essential cofactor was separated on a column of alumina (48); coenzyme I was separated from glyceraldehydephosphate dehydrogenase by treatment with Norite (214a).

C. USE OF ADSORPTION TECHNIQUES

1. *Purification*

Twenty-five years ago it was hoped that the use of adsorbents would provide a means for purifying enzymes. Since the technic employed was simply to mix the adsorbent and solution together, separate the adsorbent, and elute the enzyme, either the adsorbent or the elution had to be fairly specific for a particular enzyme, to provide much purification. All adsorbents studied were only relatively specific and accordingly the purification accomplished was not complete. The technic, however, is still widely and successfully used, particularly in the early stages of the purification of enzymes as noted in section IV,A. It appears that it is most successful when used to remove undesired proteins and other substances (104,151a,187), when elution problems need not be considered. Also in the early stage of purification when the enzyme is present in small concentration it is less likely to be adsorbed because of competitive effects.

2. *Determination of Homogeneity*

There are few examples in this category since most of the chromatographic systems employed were not sufficiently precise for such a purpose. However, there is one outstanding example; it is outstanding since the enzyme in question, ribonuclease, appeared to be homogeneous by all other methods used in its study. Hirs, Stein, and Moore using a cation exchange resin column showed that there was several per cent of a second component that was also a ribonuclease (82a). Independently Martin and Porter using a two-phase chromatographic system made the same observation (122).

3. *Resolution of Mixtures*

Adsorption technics in many cases have been adequate to resolve a mixture of enzymes to show that the several activities did not reside with the same enzyme molecule. This has been accomplished with the single adsorption technic but most successfully with chromatography. In a recent example of the first technic Kalckar (113) separated adenosine deaminase and alkaline phosphatase by the use of $Al(OH)_3$, and

Reddi and Giri (166a) have described a procedure, based on differential elution from C_γ alumina, for separating the thiaminases (active at *p*H 3.6 and 6.5) in the fresh water mussel (*Lamellidens marginalis*). Willstätter *et al.* (241) have described methods for separating pancreatic lipase, amylase, and trypsin. Young and Hartman (244) studied the effect of 21 adsorbents on pancreatic lipase, amylase, and trypsin. Several pairs of adsorbents were found that would completely free the pancreatic extracts of two of the enzymes leaving the third in full activity.

The use of paper and column chromatography for separating enzymes is described in section IV.

III. Methods Employed in the Study of Enzymes and Other Proteins

A. TYPES OF ADSORPTION TECHNIQUE

1. Single (Batchwise) Adsorption and Elution

This procedure, briefly described earlier, involves mixing adsorbent and solution, and removing the adsorbent after adsorption has taken place. If the enzyme is in the solution the used adsorbent can be discarded. If the enzyme is on the adsorbent a suitable eluant must be used for recovery of the enzyme. Some of the recent results obtained with this procedure are listed in section IV,A.

In addition to adsorptions on solids single adsorptions of proteins have also been described at charged oil-water interfaces and the method applied to the separations of proteins (44).

2. Continuous Flow Adsorption (Chromatography)

The essential feature of this procedure is the use of a column of adsorbent which in principle subjects the flowing solution to a large number of single adsorptions. Under ideal conditions this would bring about a separation of the components of the solution. Since ideal behavior is rarely found variations of the simple elution technic such as frontal and displacement analysis are sometimes employed. Full treatment of the theoretical considerations for these methods is given by Tiselius (221) and Cassidy (21). Shorter outlines of the different methods are available a number of places (12,121,199,223). The apparatus required for column chromatography, preparation of the column, etc., as well as paper chromatography, are fully described elsewhere (13a,21,71,245), and will not be treated here.

Elution Development. In this method the protein is placed on the top of the column and developed with the same solvent or some other solvent from which the proteins in question have different degrees of adsorption. Weiss (231) has given a theoretical treatment of elution chromatography for components with nonlinear adsorption curves but assuming practically instantaneous equilibrium between the solution and the adsorbent. Weil-Malherbe (230) showed that the elution curves obtained moved at the expected rate and the shapes conformed to Weiss's theory. When k_2 in the Freundlich adsorption isotherm is close to 1, that is, when the adsorption curve is linear, the elution curve is almost symmetrical, whereas a "tail" develops if k_2 is greater than 1.

The initial application of this technic to the separation of the enzymes lactic dehydrogenase and diaphorase (flavoprotein) was not very successful; the enzymes were spread out and incompletely separated (2). The general conclusion seems to be that many proteins are not amenable to the simple chromatographic elution technic (82a, 105). Excellent results have been reported, however, with certain enzymes of low molecular weight and high isoelectric point (82a,122,155, 211) (see section IV). Preliminary experiments (40) indicated that a stepwise elution of serum albumin and globulin had been accomplished with 0.035 *M* and 0.105 *M* phosphate at *p*H 6.7.

Frontal Analysis. In this procedure the protein solution is run into the column continuously. Only the first, the least adsorbed, component is obtained in the effluent in pure form. This method, however, is a means of determining the number and amount of each component at the advancing front from the retention volume and the height of the steps. The retention volume is defined as the volume of liquid which emerges from the column before the solute appears; it is equal to the ratio of the adsorption per gram to the concentration under study, multiplied by the weight of adsorbent (223). In this case the concentration put on the column is equivalent to the equilibrium concentration in the adsorption isotherm. From the above relationship adsorption data can be calculated from observed retention volumes.

The frontal analysis procedure has the advantage that the adsorbed substance does not have to be eluted again and it can be used therefore

even in the case of irreversible adsorptions (24). The theory of frontal analysis has been developed by Tiselius (219,220) and extended by Claesson (23,24) to calculation of the concentration of the components in the advancing fronts. This technic has been applied to the study of casein (219), egg albumin (141), oxidized insulin (224), and serum albumin (40). Other examples are given in greater detail in section IV,F.

Displacement Chromatography. After the protein solution is placed on the column a substance that is more strongly adsorbed is used to develop the column. By this means tailing is avoided. The theory of this technic has been discussed by Claesson (23,24). Concentration steps are obtained as in frontal analysis but in this case the height of the step is characteristic of the substance and determined by the adsorption isotherm and the length of the step is proportional to the amount of substance. The limitation of this procedure is the difficulty in finding a developer that will displace the unknown mixture of proteins quantitatively. The elution of adsorbed proteins with phosphate solutions under certain conditions represents a displacement of the protein by the phosphate (207). Examples of displacement by more rigorous reagents like pyridine are given later (see Table I).

Partition Chromatography. Paper Chromatography. Partition chromatography employing a two-phase liquid system, in its simplest form, is based not on adsorption but on partition between two solvents. Since a column or sheet (filter paper) of inert substance is frequently used to support the immobile phase the method has come to be classified with adsorption technics. Actually in the study of low molecular substances in such systems adsorption has been found to be taking place as well as partition; in many cases the use of a single solvent was found to be successful (21,223). Moore and Stein (140) in discussing paper chromatography state that because of solvent-paper interaction these systems are more properly termed liquid-gel systems.

Chromatography on paper, with which so many partition studies have been performed, is described in terms of R_F values. The R_F value is defined as the ratio of the movement of the solute band to the rate of movement of the solvent front. The R_F value will lie between 0 for a strongly adsorbed component showing no movement and 1 for the unadsorbed component accompanying the solvent front. The R_F value is related to the retention volume (see frontal analysis) by the ratio of the solution volume of a column to the sum of this volume and the retention volume (223). R_F values can also be calculated from partition coefficients, the volumes of the two phases, and the volume of the adsorbent (123).

Because of the lability of proteins in organic solvents partition chromatography would not be expected to be generally applicable to proteins. However, ribonuclease, a relatively stable enzyme, has been chromatographed in a two-phase system with striking success (122). Countercurrent distribution studies with insulin in a two-phase system (2-butanol–1% aqueous dichloroacetic acid was most suitable) have been described (74). The requirements to be met for the application of this technic to proteins are discussed. Filter paper chromatography of enzymes and other proteins is reviewed in section IV, F. A laboratory manual on paper chromatography (13a) and a guide to filter paper and cellulose powder chromatography (10a) are available. The latter contains a discussion of the properties of filter paper of interest in chromatography.

Other Types. An important recent development is the electrophoresis of proteins on paper (41,105). Tiselius (223) has pointed out the resemblance of this technic to chromatographic procedures. This method appears to offer all the advantages (separation of components, simplicity, etc.) of chromatography of low molecular substances on filter paper with none of the drawbacks that have hampered the application of the method to proteins. Summaries of the current literature in this rapidly advancing field have appeared recently (96, 201). Sections of recent books on paper chromatography are devoted to electrophoresis on paper (10a,13a).

A separation of proteins has been obtained by subjecting the solution of proteins to a salt concentration gradient in a column (182).

Considerable study has been devoted to the continuous separation of proteins by adsorption on foams (21). The variables affecting the formation of protein foams have been studied intensively (217). Cholinesterase has been purified by a continuous foam procedure (10); a fungal proteinase and peptidase have been separated by the foam technic (141a).

Diffusion of proteins in a column of agar or gelatin has been made the basis of a method for the recognition of specific antibody–antigen combinations. Antigen and antibody are placed at opposite ends of

the column and allowed to diffuse toward each other. At some point the concentration of each component will be optimal for specific precipitation. The number of components will be evident from the number of bands of precipitate. This technic is well exemplified in its application in investigations of diphtheria toxins (15,164).

B. ADSORBENTS, SOLVENTS, AND ELUANTS. SELECTIVE METHODS

1. Type and Selection of Adsorbents, Their Source or Preparation

In the consideration of adsorbents a distinction may be made between dry, solid adsorbents, many of them available commercially, and hydrated precipitates or gels. In general the latter type will be much more powerful adsorbents on an equal dry weight basis because of their larger surface area. This is illustrated by the comparative data that have been reported for the adsorption of gelatin on the hydroxides and oxides of aluminum, iron, and nickel (74), the adsorption of albumin on hydrated and anhydrous calcium phosphate (207), and the adsorption of crystalline trypsin on various oxides and hydroxides (179a). The gel type of adsorbent decreases in adsorbing power on aging since the particle size tends to increase with a decrease in total surface area (98). This type of adsorbent has had numerous applications in the single adsorption technic (see IV,A). The dry solids are used almost exclusively in columns because of the convenience in preparation of the column and their uniformity. Also, regulation of the degree of adsorption and the flow rate is possible through variation of the particle size.

Another distinction may be made between nonionic, with which the adsorptive forces are considered to be physical, and ionic adsorbents. With the latter type adsorptive forces are electrical. However, in aqueous solutions this distinction is not sharp for electrically neutral adsorbents will take up ions from the environment and become charged. Further, the *p*H of the solvent influences the charge on the adsorbent.

Cassidy (21) has compiled in tabular form data on the charge on various adsorbents; eleven pages are devoted to the description of other properties of various adsorbents. Dietz has also compiled data on numerous adsorbents (33). Other books and articles are devoted to carbons (32,75), diatomaceous silica (7,18), and aluminum oxides (100).

Michaelis and Ehrenreich (129) determined the charge on various adsorbents from their ability to adsorb acid and basic dyes. With these adsorbents they showed that some enzymes behaved like acids, others were amphoteric. Unfortunately the enzymes were not pure and later studies showed that the adsorption of the enzyme changed in the course of purification, but the method suggests an approach to the problem of selecting suitable adsorbents.

Synthetic ion exchange resins of the sulfonic acid type have been used for the chromatography of proteins, IR 100 for the separation of pectin-methylesterase from pectin-galacturonase (125), and Dowex 50 for the resolution of a mixture of proteins from egg white (192,193). This type of resin has been criticized for use with proteins (119,210) because of the difficulty of maintaining a suitable *p*H. This can be circumvented by freeing the protein solutions of salts, or by the use of the weakly acidic, carboxyl type of resin. The latter type of resin, IRC 50, has been used in the study of bacterial pectinase (210), and Moore and Stein (140) found this resin the most useful of several adsorbents tested for studies of ribonuclease and lysozyme. Cytochrome c also has been successfully chromatographed on IRC 50 (155). A list of the various types of synthetic resins is available (102).

The preparation of various precipitates and hydroxides suitable for adsorption of enzymes is reviewed by Kraut (98). The preparation of various types of aluminum hydroxides, so frequently used, is readily available (76). Special uses for these various hydroxides have been described (238). An easily prepared $Al(OH)_3$ has been described by Marshall and Welker who applied it to the adsorption of proteins and enzymes (120). The physical properties of various alumina gels have been described (71a). Calcium phosphate, another frequently used adsorbent, can be prepared by several methods (94,191,207,119a). Specific properties of hydrated precipitates are difficult to reproduce; they depend on mode of precipitation, amount and kind of reagents, temperature, duration of preparation, age, etc. (119a).

Cellulose will adsorb some enzymes (38,213) but in general substances like filter paper are poor adsorbers of proteins at neutrality (187); however adsorption on filter paper is quite strong in acid solutions (105,241) and it is increased by ethanol (17a). Crystalline trypsin, however, is not adsorbed on filter paper in acid solution (179a). Aminized and phosphorylated cotton has been used for ion exchange (85,167); production of papers of this type is under consideration.

Any substance that can exist as a separate phase with an aqueous

system can be used as an adsorbent. Schormüller (179a) has investigated 33 adsorbents for their ability to adsorb crystalline trypsin from solution. Trends within series of various types of adsorbents are illustrated in these studies. For example, adsorption of trypsin by straight chain, saturated fatty acids decreases with increase in chain length; whereas with the dicarboxylic acids the reverse is true. Charcoal can be used to remove contaminating proteins, but it is not recommended for the isolation of proteins since there is no satisfactory eluant (98). Iron hydroxide may have special use since it is less soluble in alkali than $Al(OH)_3$ (54). Benzoic acid has been used for the purification of insulin (136) and gonadotropic hormone (91).

In addition to the selection of type of adsorbent the particle size of the adsorbent is an important factor. It not only determines the total surface area per unit weight of adsorbent but it also influences the rate of adsorption (187) and the rate of elution (155). Further, the adsorbent should be easy to pack into a uniform column since irregularities distort the zones. New information on adsorbents is now summarized annually (27,96,102,197,198,200,201).

2. *Solvents. Eluants*

The choice of solvent from which adsorption is to take place is not a decisive factor for the success of an adsorption procedure. In general the choice is made so that adsorption is maximal for either the single adsorption-elution procedure or the operation of a column. In the latter, because of nonequilibrium conditions development of the column is frequently an elution also. In addition the choice of solvent is made so that it is possible to change to conditions for maximal elution. From the discussion in previous sections it appears that adsorption is maximal at some *p*H near the isoelectric point of the protein to be adsorbed. Further, adsorption is enhanced by the addition of salts or organic solvents to the solution.

For elution the choice of conditions is very much wider, not all of them applicable to the operation of a column however. Moloney and Findlay (137) have outlined the procedures available for eluting adsorbed proteins:

1. Changing the adsorbent chemically.
2. Changing the temperature.
3. Decreasing the chemical potential of the solute: (*a*) dilution: (*b*) change of solvent.
4. Displacement.

Elution of insulin adsorbed on charcoal was approached from the standpoint of this classification (137). Changing the adsorbent chemically could not be done with charcoal; the temperature was altered together with other factors; dilution and change of solvent were ineffective even though for the latter the *p*H was varied from 1 to 9.8 with various buffers and insulin solvents such as phenol and glacial acetic acid were used. Displacement was successful. Long chain fatty acids in alcohol (lauric acid was the best) and benzoic and salicylic acids displaced the insulin from charcoal. The eluant deemed the best was 12% benzoic acid in 60% ethanol. Some additional examples of elution results are shown in Table I under the same classification.

In addition to the examples given in the table it should be noted that the effective elution by phosphate solutions of proteins adsorbed on $Al(OH)_3$ is considered to involve a transformation of the adsorbent to a phosphate, which is considerably less adsorbent than the hydroxide. When considerable time is required for elution a change of the adsorbent probably occurs. The elution of proteins adsorbed on $Ca_3(PO_4)_2$ by phosphate probably represents a displacement since phosphate is adsorbed on $Ca_3(PO_4)_2$ (207). With acid eluants a transformation to $CaHPO_4$ (239) occurs.

Elution, like adsorption, as one might expect, is a function of *p*H. This has been reported in many instances in the older literature (98). A striking example of the effect of *p*H is shown by the elution of lysozyme from bentonite with pyridine; there is a sharp elution maximum at *p*H 5.0 decreasing to negligible elution at *p*H 6.5 and 3.0 (4).

The tailing shown by many protein bands in chromatography can be reduced by a regular alteration in the properties of the eluant, such as the salt concentration or the *p*H, as the development proceeds (134,207,223).

3. *Specific Adsorbents and Eluants*

Adsorbents differ in their ability to adsorb certain proteins and to a degree are relatively specific. However, it would be desirable to have an adsorbent of absolute specificity such that a single adsorption would suffice to remove the wanted protein from a mixture.

The binding of substrate and enzyme which is highly specific might provide the desired specific forces. It would be necessary to have a solid resembling the substrate sufficiently so that it could be the site

TABLE I: ELUTION OF ENZYMES AND OTHER PROTEINS BY VARIOUS PROCEDURES

Procedure	Enzyme or protein	Adsorbent	Eluant	Remarks	Ref.
1. Changing adsorbent chemically	Insulin	Benzoic acid	Ether-water	Insulin recovered in water phase	136
	Gonadotropic hormone	Benzoic Acid	Acetone	Hormone recovered as flocculent precipitate	91
	Pepsin	$Al(OH)_3$	0.5% HCl	$Al(OH)_3$ dissolved at this *p*H, removed by dialysis	101
	Catalase	$Ca_3(PO_4)_2$	Potassium oxalate	Cryst. calcium oxalate does not adsorb catalase	81
	Prothrombin	$Mg(OH)_2$	CO_2 under slight pressure	Adsorbent dissolves	185
2. Changing temperature	Invertase	$Al(OH)_3$	Solvent	Elution favored by temp. below 15°	209
3. Changing the chemical potential of solute	ACTH	Cellulose	0.1 *N* HCl	Couldn't be eluted with 0.1 *N* acetic acid	160
	α-Amylase	Starch	Water	Adsorbed from 40% ethanol	183
	Chicken tumor virus	Celite No. 503	Water	Adsorbed from 0.15 *M* NaCl	171
	Pectin esterase	Celite Analytical Filter Aid	1 *M* NaCl	Adsorbed from water	116
4. Displacement	Insulin	Charcoal	12% benzoic acid in 60% ethanol		137
	Lysozyme	Bentonite	5% aq. pyridine	Numerous other eluants were ineffective	4
	Trypsin inhibitor	Bentonite	Pyridine		103

of enzyme-substrate bonding and yet usable under conditions that it would not be altered by the enzyme. Certain adsorptions appear to be of this type.

Certain lipases could be adsorbed on tristearin and cholesterol presumably because they are potential substrates (98,240). α-Amylase from malt can be adsorbed on starch (85a). The reaction is highly specific, for the adsorption of β-amylase could be prevented by use of maltose. This adsorption differs from the usual protein adsorption in that it is favored by low temperature. It was found that there is no pronounced *p*H dependence. Ethanol is required for the adsorption. The amylase from *Clostridium acetobutylicum* was purified 33-fold by one adsorption from an alcoholic solution at low salt concentration; a high salt concentration prevents the adsorption (84). This method has been frequently used (59,149). The adsorption of malt α-amylase in 40% ethanol at 0° has been studied in other details (183,184); adsorption isotherm data fit the Freundlich equation. By studying the effect of starch hydrolytic products (maltose, limit dextran, α-maltose) it was concluded that the site for the adsorption of the enzyme on the starch was not the same as the site of union of enzyme with substrate.

The adsorption of polygalacturonase, which hydrolyzes pectic acid, on alginic acid (poly-β-D-mannuronic acid) (117) may be a specific adsorption of the type discussed. The adsorption takes place in an aqueous system at *p*H 3. The adsorption of proteolytic enzymes on protein precipitates (150,202) and on bacteria (141a) may be specific in nature. The attempt to adsorb alkaline phosphatase on cellulose phosphate (167), a potential substrate, was unsuccessful (255).

Highly specific adsorbents for antibodies have been obtained by coupling the protein antigen to cellulose and using the complex to adsorb the specific antibody. The elution was done at *p*H 3.2. The procedure has been applied to the purification of antibodies to serum albumin (19).

Selectivity of a different nature is obtained by using a cation-exchange sulfonated polystyrene resin of different degrees of cross bonding which determines the size of the pores in the resin (159,215). By varying the cross bonding it is possible to obtain an adsorbent which will adsorb amino acids but exclude proteins. The technic may provide a means of fractionating intermediate polypeptides as well. A selective adsorbent for amino acids has proved useful in the hydrolysis

of proteins (227). Charcoal adsorbs most amino acids but it is a poor adsorbent for proteins (219,220). Ferric hydroxide, on the other hand, adsorbs proteins but not amino acids (54). Calcium phosphate has been useful in freeing the saccharase in yeast extracts from the yeast gum (203a). The enzyme is retained by this adsorbent whereas much of the gum can be washed away with water. Filter-Cel and other Celites (diatomaceous silica) (151a,179a) adsorb the mucinlike substances present in tissue extracts.

von Euler and Hahn (49) have used adsorption to distinguish between protein nucleates (saltlike bond) and nucleoproteins. Nucleic acids of average molecular weight of 14,000, and of less than 10,000 were used to prepare model substances of the first type; the nucleoprotein was prepared from thymus gland. Frontal analysis was used with the adsorbents Carboraffin C, Al_2O_3, and Floridin XS. With this procedure the nucleic acid separated as distinct steps from complexes with piperidine, arginine, and scombrine; the nucleic acid was most strongly adsorbed to Al_2O_3 and least adsorbed to the acidic Floridin XS. On the contrary the components of the nucleoprotein moved together. However, it should be noted that the nucleoprotein was very poorly adsorbed on all these adsorbents, which might be expected of the high molecular weight probably represented by such a complex. Pentose nucleic acid can be separated from desoxypentose nucleic acid by adsorption on Norite in 0.14 *M* NaCl (244a). This selectivity appears to be due to a difference in molecular weight rather than in the chemical nature of these two types of nucleic acid.

The elution of adsorbed enzymes by specific substrates has been reported from time to time (101) but others have reported negative results (78,131,144,228). Some of the early positive reports appear to be due to the fact that the enzyme is quite often active in the adsorbed state; however, the effect of substrates on adsorbed enzymes appears to deserve reinvestigation. The adsorption of prothrombin on barium sulfate (204a) shows an unusual specificity; it can be eluted only with citrate. Other adsorbed proteins are eluted with carboxyl compounds in proportion to the affinity constants of the acid for the barium ion.

C. DETERMINATION OF ENZYMES AND OTHER PROTEINS IN THE ELUANT OR LOCATION ON THE COLUMN OR SHEET AFTER CHROMATOGRAPHY

A fraction collector is a useful device for collecting flow samples from the chromatographic column. Stein and Moore have described (196) a collector which is now available commercially for collecting a constant volume or at constant time intervals. Schroeder and Corey (180) have described an automatic weight-driven, time-controlled collector and give references to five other types of collector that can be built in the laboratory. Hehre and Carlson (77) have described a similar device controlled by a motor-driven solenoid. Once the samples are collected the protein can be determined by any of a number of procedures (106,118,138), or in the case of enzymes specific assay methods can be used.

Automatic devices have also been described for continuously recording the concentration of protein in the solution flowing from a chromatographic column. Drake (40) has described the use of polarography for this purpose and has applied the method in the chromatography of serum globulin and albumin. The schlieren lens system, so widely used in electrophoresis, has been adapted to chromatographic studies (218). The method has been used in the study of a mixture of proteins from egg white (192,193). Other methods based on refractive index have been described (221). An automatic instrument has recently been described using an interferometric arrangement or the refractive index directly (93); the most advantageous application of either procedure is discussed.

For the location of proteins on paper, the use of bromophenol blue (41,105) or other dyes (53,88,157) provides the simplest and most sensitive technic. However, it should be noted that some amino acids and peptides bind bromophenol blue also (61). Pepsin and other proteolytic enzymes are reported not to bind certain dyes (20). Many reports have appeared describing specific methods for locating enzymes on columns or paper; the application of the chromogenic substrates or reagents have been made by brush (175,246,248), or by juxtaposition as in the agar plate technic (64,168,169), the use of movie film for proteinase (64,229a), and in the use of a substrate-saturated paper (131a). Substrates and reagents have also been applied by dipping the paper into them (189,190) or by spraying them on the paper (229a). Zechmeister and Rohdewald (247) have listed methods for identifying fourteen enzymes by the brush technic on columns of alumina. Enzymes on paper can be determined by eluting successive sections with a suitable solvent and locating the enzyme by means of assay, as has been done in studies of a number of enzymes (17a,55, 254). Devices have been described for measuring the color (16,68, 105,111,142) or the radioactivity (236) in strips of paper. A paper holder for a Beckman spectrophotometer has been described (154a). The paper is made more transparent by treatment with paraffin oil. New developments in methodology have been reviewed annually since 1949 (201).

IV. Application of Adsorption, Principally Chromatography, to Particular Enzymes and Proteins or Systems

A. GENERAL APPLICATION

The use of the single adsorption-elution technic during the period of Willstätter's work has been summarized by Kraut (98). A table covering four pages lists examples of the application of the method. That this is still a useful technic is exemplified by numerous recent uses: fractionation of blood clotting factors (Hyflo Super-Cel, $BaSO_4$) (132), crystalline condensing enzyme (calcium phosphate gel) (152), horse-radish peroxidase (calcium phosphate) (94), penicillinase (Hyflo Super-Cel) (127), invertase (bentonite) (1), L-lysine decarboxylase (C_γ $Al(OH)_3$) (60), separation of adenosine deaminase and phosphatase ($Al(OH)_3$) (89), thymonucleodepolymerase (bentonite) (109), 5-nucleotidase (C_γ $Al(OH)_3$) (79), deaminase for threonine and serine (calcium phosphate) (242), uricase ($Zn(OH)_2$, C_γ $Al(OH)_3$) (5), sweet potato phenolase (lead acetate–probably lead phosphate formed, $Al(OH)_3$) (43), crystalline pyrophosphatase (calcium phosphate gel) (104), xanthine oxidase (charcoal, kaolin) (37), ACTH (oxycellulose) (9). This last adsorption is so selective that it appears to be specific; a 40-fold purification was obtained by a single adsorption. Other interesting examples of the use of the single adsorption technic follow: crystalline trypsin inhibitor from soybean (bentonite) (103), cytochrome oxidase (magnesium carbonate) (50), hormones (Permutit) (165), catalase ($Al(OH)_3$) (110), phosphorylase ($Al(OH)_3$) (30,133, 186), lysozyme (bentonite) (4), L-amino acid oxidase (calcium phosphate gel) (191), prothrombin ($Al(OH)_3$) (143). The last three papers cited report extensive studies of the variables involved for the most effective application of the single adsorption technic. The references listed above provide only a few examples of the many that appear in the literature. Aluminum hydroxide and calcium phosphate are the adsorbents most frequently used. It may be relevant that phosphate solutions are powerful eluting agents for both these adsorbents.

The chromatography of enzymes and other proteins is a comparatively recent development. Grundman reviewed the method in 1941 and cited a few examples of its application to enzymes (71). Zechmeister devoted a portion of his recent book to enzymes and proteins (245) and recently Zechmeister and Rohdewald reviewed some aspects of enzyme chromatography (247). Sections of recent books have been devoted to enzymes and other proteins (10a,13a). Turba (226) has reviewed the application of chromatography to the study of proteins. Moore and Stein (140) in their review on chromatography have presented material on enzymes and other proteins. In Table II are listed the many recent studies of enzymes and other proteins where the column technic has been employed. Certain individual enzymes and viruses are discussed in detail later, as well as the many examples of filter paper chromatography of enzymes and other proteins. A number of interesting studies on polypeptides have appeared recently—gramicidin (208), peptides (97,158), tissue peptides (52), ACTH peptides (113,114), licheniformins (mol. wts. 3800 to 4800) (17b). However these studies have been performed in organic solvents and would not be generally applicable to proteins.

Many of the examples listed in Table II of the use of adsorbent in a column probably are not operating under the condition of reversibility required for true chromatography. In some cases it will be found that a separation has been made of components strongly adsorbed and components not adsorbed. Such an operation might be termed adsorption filtration. The technic is useful and more efficient than the single adsorption technic (140). Continued studies of this type, together with adsorption data, might reveal other enzymes and proteins that could be chromatographed under conditions of true reversibility.

B. RIBONUCLEASE

The successful chromatography of ribonuclease has recently been reported from two laboratories. In one instance the procedure was developed as a specific method for purifying small amounts of ribonuclease (122); in the other instance the method was found to be suitable for ribonuclease after the examination of several different proteins and numerous adsorbents (82a,140). Both studies revealed crystalline ribonuclease, homogeneous on the basis of electrophoretic and ultracentrifuge studies (176), to be composed of two enzymically active components, and indeed it appears to occur in the pancreas as the two forms (122).

Martin and Porter (122) chromatographed ribonuclease on a two-phase system composed of ammonium sulfate-water-ethylene glycol monoethyl ether (Cellosolve). The partition coefficient of ribonuclease in several mixtures was in favor of the Cellosolve phase; this phase preferentially wetted Hyflo Super-Cel and served as the stationary phase. For most of the work columns with an inside diameter of 1.2 cm. containing 6 g. of Hyflo were used; such a column could handle 1 to 5 mg. of ribonuclease dissolved in 2 ml. of the aqueous phase, which was used to develop the column. The results obtained with a two-phase system composed of 20 g. ammonium sulfate,

TABLE II: SEPARATION OF PROTEINS BY CHROMATOGRAPHY

Proteins or enzymes separated	Adsorbent used	Developing or elution solution	Remarks	Ref.
Taka-Diastase	Paper (chromatopile)	Ammonium sulfate	—	134
Z-enzyme, phosphatase, β-amylase	Paper (chromatopile)	Ammonium sulfate	Enzymes eluted by concentration gradient method (134) starting with half-saturated ammonium sulfate.	161
Phosphatases (snake venoms)	Cellulose (Whatman No. 5 paper, shredded) (column)	0.1% NaCl	Phosphodiesterase eluted, 5-nucleotidase remained on column. This technic gave no separation of intestinal phosphatases.	86
ACTH	Oxycellulose	0.3 and 0.7 *N* monochloracetic acid, 0.1 *N* HCl		62
Cellulase	Calcium phosphate	0.05 *M* KH_2PO_4, Na_2HPO_4	Method of Swingle and Tiselius (207). 2-Fold purification but total recovery low; marked tailing on elution.	233
Cellulase	Calcium phosphate	Phosphate		207
ACTH	Cellulose (Solka-Floc BW-200)	0.1 *N* acetic acid, 0.1 *N* HCl		160
Lactoperoxidase	Calcium phosphate, Silica-Celite	Phosphate	Separation of enzyme from red protein.	162
Serum albumin and globulin	Calcium phosphate and Super-Cel	0.035 *M* phosphate, followed by 0.105 *M* phosphate, both at *p*H 6.7		40

Proteins or enzymes separated	Adsorbent used	Developing or elution solution	Remarks	Ref.
D-Amino acid oxidase	Hydrated calcium phosphate, Super-Cel	Phosphate (0.005 to 0.025 *M*)		207
Phycoerythrin (red), phycocyan (blue)	Hydrated calcium phosphate, Super-Cel	Phosphate	Red and blue bands readily separated with red in lead.	207
α-Amylase	Wheat starch and Celite	Adsorbed from 40% ethanol, eluted with water		183
Amylase, proteinase (*Aspergillus tamarii*)	Potato starch	20 to 30% ammonium sulfate	Enzymes were separated.	190
Catalase (liver)	Calcium phosphate	Phosphate buffer, *p*H 8	See ref. 204 for illustration of catalase on column.	3.203, 204
Emulsin (β-D-glucosidase, α-galactosidase, chitinase)	Bauxite	0.1 *N* NH_4OH		250
Emulsin	Bauxite			146
Emulsin (chitinase, chitobiase)	Bauxite			249
β-Glucosidases of emulsin and fungous extract	Bauxite B, Brockmann's alumina	Acetate, *p*H 4.6	Cellobiase and salicinase were separated.	251
Tannase, esterase	Brockmann's alumina	*p*H 7.6	Esterase retained on column.	225
Snail (*Helix pomatia* extract)	Bauxite			252
Rennin	Floridin XS, Al_2O_3	Phosphate, *p*H 6, 0.15 *M* phosphate, *p*H 5.6	Purification less than 2-fold.	179
Tuberculin	Fibrous alumina	Aqueous, *p*H 7.4	No purification.	67

Table continued

TABLE II (*continued*): SEPARATION OF PROTEINS BY CHROMATOGRAPHY

Proteins or enzymes separated	Absorbent used	Developing or elution solution	Remarks	Ref.
Hemoglobin	Al_2O_3	Water	Two bands.	6
Gonadotropic hormone	Permutit (Folin)	10% ammonium acetate in 38% ethanol		92
Phenolases	Al_2O_3	Water		45
Thyroglobulin	$Al(OH)_3$ on kieselguhr			174
Bacterial growth factor (polypeptide)	Alumina (Alcoa F-20)	NH_4OH-NH_4Cl buffer, *p*H 11	Basic active protein moved away from inactive protein.	191a
Dipeptidase, aminopolypeptidase	Zinc carbonate	Water, followed by disodium phosphate	First enzyme eluted with water; second with phosphate.	226
Succinic dehydrogenase, diaphorase	Calcium carbonate	Saturated disodium phosphate	Ratio of activity of first to second enzyme increased 10-fold.	50
Alkaline phosphatase	Alumina		Separation of enzyme and cofactor.	48
Cottonseed allergen picrate	Alumina	50% ethanol	Two bands obtained.	195
Lactic dehydrogenase, flavoprotein	Alumina			2
Egg white proteins; serum albumin and hemoglobin	Dowex 50 resin		Frontal analysis used.	192, 193
Immune globulin, egg albumin, fetuin, pea seed globulin	Silica gel and Super-Cel	Sodium phosphate, *p*H 7; sodium phosphate, *p*H 7.7, plus 0.5 *M* Na_2SO_4	Frontal analysis used.	187
Egg albumin	Carbon (Carbo Activ); $Al(OH)_3$ (Mataki gel)	0.005 *N* NaCl	Frontal analysis used.	141
Bacterial pectinase	Amberlite IRC 50	0.1 *N* HCl		210
Pectin methylesterase, polygalacturonase	Resin IR 100		Methylesterase removed.	125
Lysozyme	Carboxyl type resin IRC-50	0.2 *M* sodium phosphate, *p*H 7.18		211 212*
Ribonuclease	Resin IRC-50	0.2 *M* sodium phosphate, *p*H 6.45		82a*
Cytochrome c	Resin IRC-50	0.1 *M* NH_4OH-NH_4OAc, *p*H 9.0 to 10.8	Four fractions obtained.	155*
ACTH	Resin IRC-50	0.2 *M* phosphate, *p*H 6.8, 6.65	ACTH slowly moving band with 50 to 100% of activity, less than 5% of total protein.	36a
Ribonuclease	Organic phase of 2-phase system supported on Hyflo Super-Cel	2-phase system of water–Cellosolve–$(NH_4)_2SO_4$. Aqueous phase used as developer.		122*

* Discussed in greater detail in text.

24 g. of Cellosolve, and 56 g. of water are shown in Figure 5A. A number of control experiments indicated that the two components did not arise in the course of the purification of ribonuclease. A partially purified ribonuclease, quickly prepared by the use of trichloroacetic acid to minimize proteolysis, showed the same active components in about the same ratio, from which it was concluded that the enzyme existed in the pancreas in the two forms.

Martin and Porter (122) calculated that the movement of the enzyme bands was less than expected from the partition coefficient. They concluded that the adsorption must be taking place at the interface between the two phases. This seemed likely too because of the

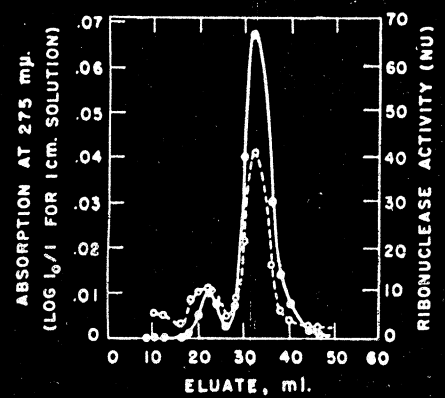

Fig. 5A. Chromatogram of ribonuclease with two-phase system (122): (—) enzyme activity; (—) adsorption at 275 mμ (protein).

high efficiency of the system with a rate of flow about the same as used with simpler molecules. With adsorption at the interface the low diffusion rate of proteins was not a limiting factor. The Hyflo Super-Cel alone was inert since it did not adsorb the enzyme unless the Cellosolve phase was present.

Martin and Porter (122) point out that a bacterial catalase was purified by partition in a two-phase system (80). Mammalian catalases (3,203,204) and lactoperoxidase (162) have been purified by chromatography. The evidence suggests that the heme-containing proteins to which the above belong might be another group of proteins amenable to chromatography.

Hirs, Stein, and Moore found (82a) that ribonuclease could be chromatographed on the carboxylic acid resin IRC-50. A finely ground preparation (XE-64, 250 to 500 mesh) was employed with 0.2 *M* sodium phosphate, *p*H 6.45, as the developing solution. With 3 mg. of crystalline ribonuclease in a column 0.9 by 30 cm. with a flow rate of 1 to 1.5 cc. per hour the results shown in Figure 5B were obtained. The position of the main peak can be changed by varying the *p*H of the buffer; at a lower *p*H it travels more slowly, *i.e.*, it is more strongly adsorbed; at a higher *p*H it travels more rapidly. This is contrary to expectation since the isoelectric point is at *p*H 7.8 (176), usually the *p*H of maximum adsorption. The authors point out that the activity of the ribonuclease is undiminished by chromatog-

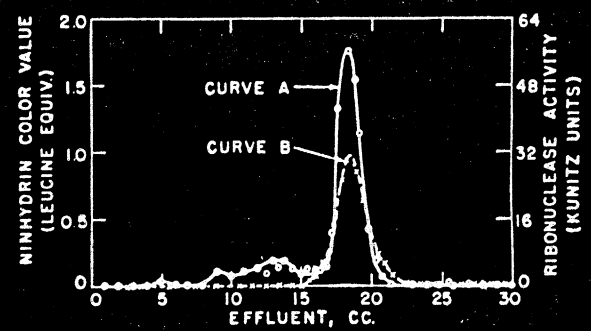

Fig. 5B. Chromatography of ribonuclease on resin IRC-50 (82a). *Curve A*, crystalline ribonuclease; (●) ninhydrin color (protein); (O) ribonuclease activity. *Curve B*, rechromatography of material from peak of curve A: (×) ninhydrin color.

raphy. The protein and enzyme activity are in parallel. Rechromatography of material from the peak of curve A gave the single symmetrical peak curve B.

C. LYSOZYME

Lysozyme, another enzyme with a low molecular weight (14,000) and an alkaline isoelectric point (*p*H 10.5), was chromatographed on resin IRC-50 by Tallan and Stein (212). Chromatography of 5 mg. of a recrystallized preparation of lysozyme carbonate, with 0.2 *M* sodium phosphate, *p*H 7.18, as the eluant, gave the results shown in Figure 6; 60% of the activity put on the column appeared in peak A, 40% in peak B. The protein, measured by the ninhydrin reaction,

paralleled the activity. Electrophoretic studies were not reported for this preparation. This preparation of lysozyme was about 1 year old; chromatography of two freshly prepared, twice crystallized samples, showed that over 95% of the enzyme appeared in peak A with only a small amount in peak B. Both these preparations appeared to be homogeneous on electrophoresis at *p*H 4.6, 7.8, and 9.2. Chromatography of egg white showed that the native lysozyme corresponded to peak A. Most of the egg white proteins was not adsorbed and emerged in the first 10 ml. of effluent; a low peak at 37 ml. corresponding to peak A contained all the lytic activity.

Transformation of lysozyme to the carbonate alone did not alter the chromatographic behavior; however it was found (211) that a preparation of the carbonate that contained 7% of peak B initially,

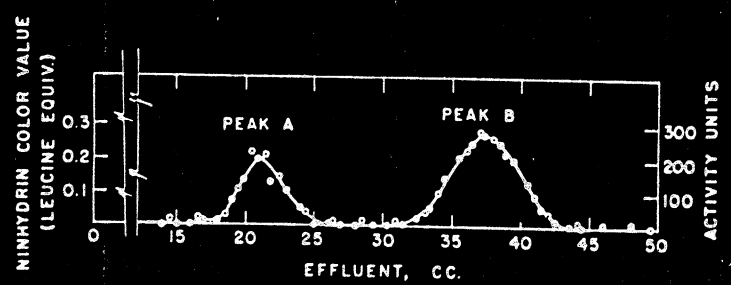

Fig. 6. Chromatography of lysozyme on the resin IRC-50 (212): (●) ninhydrin color value (protein); (○) lysozyme activity.

on storage at room temperature, steadily changed so that in 170 days 45% of B was present. Under the same conditions of storage the isoelectric lysozyme and the chloride remained unchanged.

No preparation of lysozyme by the usual crystallization procedures was found to be completely free of peak B. A preparation that showed only peak A was obtained by the use of large columns of IRC-50 (211, 212).

D. CYTOCHROME c

Cytochrome c has veen purified by chromatography on a column of the resin IRC-50 by Paléus and Neilands (155). The cytochrome c was partially purified with trichloroacetic acid (method of Keilin and Hartee) before chromatography. The resin, purchased in bead form, was ground to a powder in a mortar. Since the elution rate appeared to be influenced by particle size (higher for smaller particles) the powder was made more uniform by passing through a 0.075 mm. mesh screen; dust was removed by mixing with water and decanting the supernatant wash. The resin, in a glass tube 0.7 by 20 cm., was conditioned with 5% H_2SO_4, water, 5% ammonia, and finally washed to a constant *p*H with 0.1 *M* ammonium hydroxide-ammonium acetate, *p*H 9.0. Cytochrome c (15 mg. in a volume of 0.15 ml.) from salmon, dog, and chicken hearts was studied on this column; the initial developing solvent was the *p*H 9.0 buffer used for the final prewash. The cytochrome c was not eluted with this solvent but a golden-colored eluant was obtained containing considerable protein. When the *p*H was increased in steps of 0.1 *p*H unit a deep red cytochrome band began to move at *p*H 9.9 (fraction I); another band (fraction II) appeared on continuous development. A small amount of cytochrome c remaining on top of the column was finally eluted with 0.5% ammonium hydroxide (fraction III). The iron content of the cytochrome eluate was raised to 0.42% by this procedure; spectrophotometry showed that fraction I was reduced cytochrome c, fractions II and III oxidized cytochrome c.

This method was applied on a larger scale to beef heart cytochrome c; 4.5 ml. (507 mg.; iron content 0.34%) was placed on a washed column 2 × 15 cm. The elution was carried out as before. The golden-yellow, noncytochrome impurity, appeared in the 22 to 37 ml. of eluate (dry weight 101 mg.). The cytochrome fraction I appeared at 182 to 482 ml. and fraction II in a volume of 500 ml. These fractions had the same spectral properties as before. On a dry weight basis, 66, 155, and 22 mg. of fractions I, II, and III were obtained. Fraction II with an iron content of 0.466% represented the highest purity; fractions I and III contained 0.401 and 0.353% iron, respectively. Thus this method is applicable to the large scale preparation of cytochrome c in high purity. In commenting on the difference in the adsorption of oxidized and reduced cytochrome c the authors refer to a paper by Zeile and Reuter (252a). The latter found that at *p*H 3.8 in acetic acid reduced (hydrosulfite) cytochrome c was not adsorbed on kaolin whereas the oxidized form was.

E. VIRUSES

A number of adsorption studies of viruses have appeared, many of them employing the adsorbent in columns. The salting-out adsorp-

tion was very effective; the virus was strongly adsorbed from a salt solution and eluted with water or dilute buffer. Since the virus was either adsorbed or not adsorbed, very much the same purification could be accomplished with the single adsorption technic, although the column could be expected to be somewhat more efficient (140).

Delbrück in 1940 (34) used a technic of the salting-out type for the purification of *Escherichia coli* phage. The phage was adsorbed on Jena sintered glass filters (grade 5 on 3) from 0.06 *M* phosphate, 1% $MgCl_2$ or NaCl; the phage was eluted with water. With 0.03 *M* phosphate no adsorption was obtained. Ground glass, silica, and Fuller's earth were also good adsorbents but elution was not as satisfactory.

Riley (170) adsorbed the Rous sarcoma virus from 0.9% NaCl and eluted with water. A 5-fold purification was obtained but it appears there was only a 40% recovery. Riley later (171) made an extensive study of the various factors involved in this method. Adsorption occurred until the NaCl concentration was less than 0.01 *M;* this adsorption was relatively independent of *p*H change. Since only about 25% of the virus was recovered a large portion was not eluted or was inactivated. In spite of this loss a 6-fold purification of the virus was obtained. Riley has summarized the literature on the use of the single adsorption technic in the purification of viruses. Also he points out the implication of adsorption studies for the determination of the filtrability of viruses through Berkefeld filter candles, which are composed of compressed diatomaceous earth. Riley applied the same technic to the purification of subcellular, enzymically active (succinoxidase, dopa oxidase) granules (172). The adsorption was done at 2 to 10° and the elution at room temperature. In view of the effect of temperature on protein adsorption noted earlier the reverse might have been more effective. This type of adsorption technic is conveniently done on a Buchner (173). Similar studies have been made of pneumonia virus of mice and influenza virus (31) with a number of Celites. Adsorption was strong on Super-Cel, Celite 505, Filter-Cel, and Analytical Filter-Aid and poor on Celite 503 and Hyflo Super-Cel. In addition to low salt a high *p*H was required for elution. It has been pointed out (253) that there was a correlation between the adsorption and the processing that the Celite had been subjected to, heat-treated samples being least adsorbing. The clay in the Celites, which is dehydrated in the heat treatment, was suggested as the site of the adsorption. However surface area, which is reduced by fusion of the very porous diatom particles (7), may be an important factor as well. Since the alkaline phosphatase in a lipide complex is adsorbed on the Celite Filter-Cel but in the free state is not adsorbed, it has been suggested that the lipide component of the complex viruses and cellular particles is responsible for adsorption on Celites (253). In general diatomaceous silica is a poor adsorbent for protein.

Leyon (112) has applied salting-out adsorption to Theiler's virus both on sheets and columns of paper; the purification obtained was not determined. Paper chromatography has been described (28) for the detection of tobacco mosaic virus in extracts of tobacco plants. At *p*H 4 in a dilute buffer the virus was adsorbed to the paper; at *p*H 4.5 and higher the virus moved with the solvent, whereas most of the normal proteins and chlorophyll remained at the point of origin. Two like papers were employed; on one the virus protein was located with the Sakaguchi test; on the other the virus was eluted with buffer and its activity determined. Chromatography of tobacco mosaic virus on paper has been successful in 40% ethanol (68a). In this solvent the normal plant proteins remain immobile while the virus protein migrates. Electrophoresis on paper was used also to distinguish the virus protein. A cation exchange resin has been applied successfully to the purification of certain viruses (142a, 142b).

F. FRONTAL ANALYSIS

The application of frontal analysis to serum albumin and immune globulin on silica gel (187) has given the results shown in Figure 7. The experiments for A to C were performed in sodium phosphate, *p*H 7.0, $\mu = 0.1$; for D the *p*H was 7.7, $\mu = 0.1$, with 0.5 *M* sodium sulfate. Since the experiments lasted 24 to 48 hours 0.1% formalin was added as a preservative. Figure 7A shows the results with 0.05% immune globulin with a column volume of 780 cmm.; the effluent was collected in 3.3 ml. portions. The globulin was strongly adsorbed and first appeared in the seventh portion of effluent. Under these conditions albumin appeared in the first portion of effluent. Figure 7B shows the results obtained with a mixture of albumin and globulin; Figure 7C shows the same mixture with the cylinder volume twice as large. With 0.5 *M* sodium sulfate (Fig. 7D) albumin showed an enhanced adsorption (retention volume of about 10 ml.); however

with this salt concentration the resolution of a mixture of albumin and globulin was not as satisfactory as those illustrated. The same procedure was also applied to fetuin and pea seed globulin (187).

Frontal analysis has also been used in a study of egg white proteins (ovalbumin, ovomucoid, and conalbumin) (192,193), bovine plasma albumin, and human carbon monoxide hemoglobin (193). A cation exchange resin, Dowex 50 (200 to 500 mesh), was used for these studies at a temperature of 0.76°. Concentration changes in the ini-

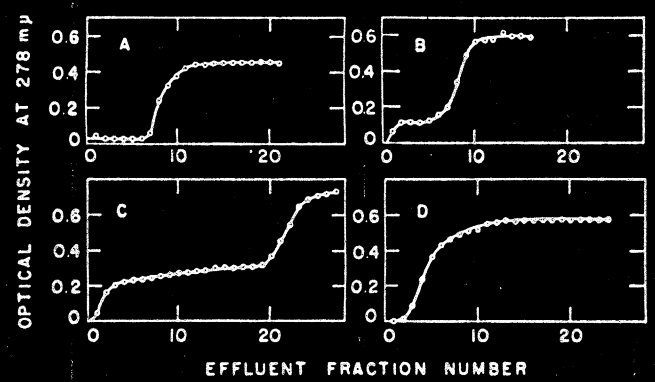

Fig. 7. Frontal analysis chromatograms of serum albumin and immune globulin and of mixtures thereof (187): (A) immune globulin; (B and C) immune globulin and serum albumin; (D) serum albumin.

tial study (192) were observed with a schlieren scanning system but later studies (193) were made with an instrument that automatically and continuously recorded changes in refractive index (93).

Studies of the egg white proteins indicated that resolution could be obtained in the *p*H range 6.5 to 8.5, ionic strength 0.01 to 0.1. However the degree of resolution and even the order in which the proteins appeared in the effluent (ascertained by subsequent electrophoresis) changed with the age of the proteins. It was concluded that the concentration dependence of the adsorption of the individual proteins must be responsible for the variable results. For further studies a mixture of plasma albumin and hemoglobin was employed. This mixture was resolved in the *p*H range 4.1 to 8.5, the albumin being the least adsorbed, but the concentration ratio of albumin to hemoglobin had to be 1 or greater for resolution. Variation of the ionic strength from 0.01 to 0.1 had no influence on the resolution. However the cation on the resin and the protein cation had to be dissimilar (ammonium and sodium for example) for resolution to be obtained. Retention volumes of albumin were found to be influenced by the cation but cation exchange was not essential for adsorption (*i.e.*, adsorption did occur in a system containing only ammonium cations). The authors point out that the resolution detected with their optical system could not be duplicated with an ordinary fraction collector.

A comparison of the known concentrations of the proteins in the mixture with that found in the frontal analysis steps showed that there was no mutual displacement. Measurements of retention volumes at *p*H 7.1, ionic strength 0.01, provided data for calculation of the adsorption isotherms. Adsorption was slight (3 mg. albumin/1 g. resin; 4 mg. hemoglobin/1 g. resin), and linear with respect to concentration. Few proteins have shown such stoichiometry over a wide range of concentration (up to 4% in the case of albumin). Preliminary elution experiments (eluant not stated) indicated that plasma albumin could be recovered from the resin in the native state.

G. FILTER PAPER CHROMATOGRAPHY

The simplicity of chromatography on paper has led many workers to use the technic with solutions of proteins. In no instance do there appear to have been experiments to determine the extent of adsorption on paper or whether it was reversible. Examination of the results shows that in most cases the enzyme or other protein was either immobile, or moved freely with the solvent ($R_F = 0$, or $R_F = 1$. For definition of R_F see section III, A, 2); there were few instances of intermediate adsorption. Accordingly the method does not represent a true chromatographic column and its application will be limited to separating a mixture of proteins into two fractions, that adsorbed and that not adsorbed in the particular solvent used.

Quastel and colleagues in a series of papers (55–57, 70, 205, 206) have described the chromatography of enzymes and other proteins on paper. The capillary ascent technic (235) was used, in which the developing solution is placed in the bottom of a closed jar and the paper, most conveniently stapled in the form of a cylinder, is dipped into the solution which rises by capillarity. Various aqueous buffers and salt solutions on Whatman No. 1 filter paper were employed. Few details of handling the paper are described. Some of the pro-

teins were spotted by adding hemin and subsequently using the benzidine test to reveal the hemin-protein complex. In instances where the hemin did not associate with the protein the protein was usually visible as a lighter area after the spotting reagent was used. In a later publication (57) the bromophenol blue technic (41) was used. Bovine serum albumin, β-lactoglobulin, γ-globulin, casein, egg albumin, and commercial enzyme preparations were studied in the first report (55). In most cases the advance of the bulk of the protein followed closely the solvent front, except at *p*H 4 to 5, the isoelectric region of most of the proteins studied. None of the chromatograms illustrated (55) suggested that the method would be useful for separating proteins. It would have been desirable to have a more intensive study of a few well-defined mixtures of pure proteins. The strong protein spot remaining at the origin suggests that the paper should have been dampened with solvent (*cf.* 73,105) before the protein was applied. Experiments with commercial urease demonstrated (55) that the bulk of the activity moved as a unit and that all the activity could be accounted for by assay of the paper on which it was adsorbed. The urease (0.04 ml.) was developed with a 2% cysteine HCl-1% glycine solution at *p*H 6. After development for 21 cm. the paper was partially dried and 3-cm. sections of the paper assayed for urease. The bulk of the urease was found 6 to 12 cm. from the origin.

In a subsequent publication (56) surface-active substances (Tween 81 and 85 were used most frequently) were added to the protein solution and reported to give good separation of the protein fractions in two-dimensional chromatography. In one chromatogram of serum as many as 21 "fingers" can be counted in the second dimension. Although the method was reported to give excellent duplication of results the chromatograms shown were not reassuring. In this report (56) the chromatograms show the advancing edge of the solvent visible as an irregular line, held back at the point the protein is present. Pretreatment of the paper with solvent might have avoided this condition. Hall and Wewalka (72) have criticized the technic of Franklin and Quastel and believe the patterns are artifacts due to the reluctance of the proteins to be moved by the developing solvent. Electrophoresis on paper was used to show that the "fingers" of the pattern did not represent distinct fractions but were heterogeneous throughout. This report brings out some of the pitfalls of chromatography of proteins on paper. These criticisms were not answered d rectly (58) but it was stated that the patterns obtained were chara teristic. For example serums could be classified (57) by this pr cedure. This is not unexpected for serums differ principally in the albumin-globulin ratio; the former are little adsorbed and the latt are strongly adsorbed, particularly the γ-globulin fraction. This reflected in the gross over-all distribution of protein in the patter Cabib (17a) has observed that chromatography of a long, narro band of protein on paper will give a number of "fingers" very simil to the results obtained in the second dimension by Franklin and Qua tel. These apparent components are believed due to a nonunifor diffusion of the solvent across the protein band. The influence electrolytes on the diffusion of proteins in paper has recently bee studied by Lederer (111a).

Paper chromatography was also applied (70) to plasma of anima given radioactive thyroxine. The radioactive material of the plasm is arranged in a pattern similar to that of the proteins. Thyroxi alone did not move with the aqueous developer used but when di solved in plasma it moved with the protein. Electrophoresis o paper was applied to this problem with elegant results (66). Th radioactive thyroxine in plasma was found to move with the sam mobility as the α_1-globulin and presumably was bound to it. Othe using the same technic have found (108) the radioactivity in the seru of a patient given I^{131} more closely coincided with the α_2-globulin.

Further study was devoted to plasma and plasma fractions (57 In this report a separation of cytochrome c and serum albumin is i lustrated in two-dimensional chromatography; the albumin move with both 0.1 *M* sucrose and 0.1 *M* sodium potassium tartrate where cytochrome c moves only with the second solvent. Chromatogram of snake venoms are shown also. The method has been applied als to a study of the effect of fat meals and heparin on blood plasma (205 and to a study of multiple sclerosis (206).

McKerns (126) used the method of Franklin and Quastel (55,56 to prepare a chromatogram of purified rennin; two papers were used one for a determination of protein, the other for activity. All th protein spots had enzyme activity. Casein before and after the ac tion of rennin was subjected to chromatography; no free amin acids were detected. Electrophoresis on paper has been used to sho that the isoelectric point of rennin is 4.5 (181).

Tauber and Petit (214) have used a technic similar to that of Franklin and Quastel (57) but with a more satisfactory staining procedure (methyl orange and eosin Y). "Patterns" are shown for cytochrome c, catalase (all parts of the pattern had catalase activity), γ-globulins, and normal human blood serum. It was concluded that separation of proteins was not obtained by this procedure, but it was believed that the discovery of specific developing solutions for each type of protein

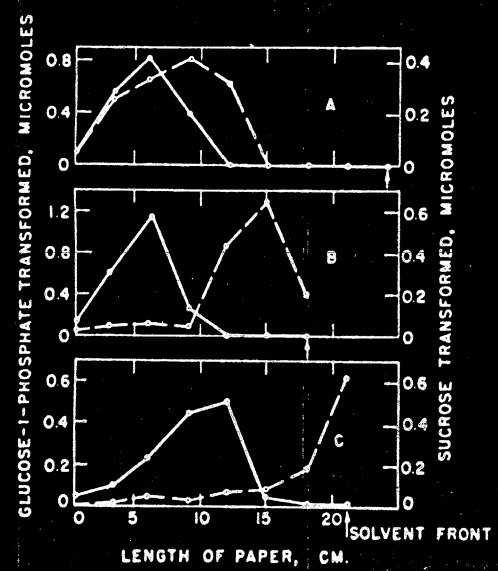

Fig. 8. Chromatography of a purified extract from *Saccharomyces fragilis* with ethanol-phosphate buffer mixtures (172). Unbroken line, phosphoglucomutase activity. Broken line, invertase activity. Concentration of ethanol in the solvent: (A) 30%; (B) 25%; (C) 20%.

might lead to success. Cabib (17,17a) has given striking evidence that with suitable developing solutions an excellent separation of certain enzymes can be obtained on paper. The separation of phosphoglucomutase and invertase in ethanol-phosphate buffer mixtures at *p*H 7 is shown in Figure 8. Cabib has reported additional studies with the enzymes in yeast extracts. The ammonium sulfate concentration-gradient technic (134) was unsuccessful; the enzymes spread over a large zone of the paper. Moreover a regular gradient could not be maintained. Ethanol-phosphate buffer, although successful with the enzymes in *Saccharomyces fragilis* extracts, when applied to brewer's yeast extracts gave two peaks of invertase activity. Others (65,87,168) have made similar observations with other enzyme preparations. It remains to be seen whether such results represent chromatographic artifacts or actual components. It should be noted that alkaline phosphatase could be obtained in forms that were, and were not, adsorbed, on Filter-Cel (253,256). Cabib (17a) found that the yeast enzymes hydrolyzing maltose and α-methyl glucoside could not be detected by assay after chromatography. Chromatography of plasma proteins in aqueous ethanol was not very satisfactory (17a). The adsorption of albumin was increased by ethanol, the R_F changing from 0.86 to 0.60 as the ethanol was increased from 10 to 40%. The serum globulins appeared as a streak on chromatography; variation of the ethanol concentration and the *p*H afforded little improvement. The globulins and the albumin appeared to interact since the R_F of the albumin was less than in pure solution. Simonart and Chow (190) have successfully separated the amylase and proteinase in extracts of *Aspergillus tamarii* by chromatography on Whatman No. 4 paper with 30 to 35% ammonium sulfate as the developing solution. The enzymes had intermediate R_F values with the amylase moving more slowly than the proteinase. Aqueous ethanol (35 to 70%) did not separate the enzymes. A column (1 × 20 cm.) of potato starch was also used successfully to separate these enzymes. The developing solvent was 20 to 30% ammonium sulfate. A recent report (13b) on the phenomenon of "double fronting" in chromatography of proteins on paper with organic solvent mixtures indicates that great care must be used in interpreting the results. Under certain conditions R_F values are meaningless and several spots can be obtained with a pure protein.

Mitchell *et al.* (134) have described the chromatograph of a mixture of enzymes (Taka-Diastase) on a filter paper chromatopile (135). In the chromatopile a stack of paper is clamped together and handled as a column. The arrangement provides ease of sampling by removal of the desired sheets of paper. The salting-out adsorption method was used; with a constant salt concentration broad bands of activity (adenosine deaminase, amylase, phosphatase) were obtained. By using a salt gradient (60% saturated ammonium sulfate decreasing to 14%) sharper bands were obtained and a satisfactory separation of

the enzymes resulted, the phosphatase least adsorbed, then amylase and deaminase. However in second runs with the last two enzymes both failed to move under the same conditions. It is evident a displacing substance was present in the original enzyme preparation. By using a *p*H gradient in 30% saturated ammonium sulfate movement of the enzymes was obtained above *p*H 7.3 and 7.7, respectively. In addition to the usual difficulties of protein chromatography, this method, although permitting ease of sampling, gives in cross section cone-shaped bands due to difficulties of clamping the paper with consequent irregularities of flow of solvent.

Paper chromatography can be useful in special applications. Castaneda (22) using a spot test found that a hematoxylin-stained suspension of *Brucella* was not moved on paper by 0.9% NaCl but it was moved if normal serum was added to it first. When specific antiserum was added the *Brucella* was fixed and did not move with NaCl. Hess and Roepke (82) used a variation of this test; the serum was developed with 0.05 *M* potassium acid phthalate, *p*H 6.2, and the hematoxylin-stained *Brucella* suspension was brushed on as a spotting reagent. It was found that the specific antibody moved with the developing solvent whereas a nonspecific reactant, present in certain serums, did not move. Mixtures of the two could be separated. Williams and Smith (236) describe a device for the continuous recording of protein radiochromatograms. Normal serum permitted the movement of labeled (I^{131}) bovine serum albumin whereas the buffers that were tried resulted in streaking of the albumin. The labeled antigen fixed specific antiserum and its amount could be determined from the radioactivity. The recorder gives the results in graph form and the amounts can be determined from the areas under the curves. Normal and fetal hemoglobin are reported (178,178a,178b) to be distinguishable on paper by means of 90% pyridine or 0.2 *N* NaOH as the developing solvent. With both solvents the hemoglobin color of the adult sample is transported while that of the fetal sample remains relatively fixed. Ponder (163) has studied the lysins in blood and tissues by paper chromatography and electrophoresis on paper.

The usefulness of indicators for spotting proteins on paper has been reported (157). Reid (168) has separated pectin esterase and polygalacturonase by chromatography on paper. The enzymes were located with the agar plate method. This spotting technic has been used by Giri *et al.* (63,64) in their studies of enzymes by paper chromatography. Amylase and trypsin in commercial trypsin were separated (64) by development with 50% acetone containing 1.5% NaCl. The trypsin was least adsorbed in this system. Giri *et al.* (65) have subsequently studied mixtures of amylases, phosphatases, and phosphorylases. Their methods are described in detail; a descending solvent technic is used similar to that of Consden, Gordon, and Martin (29). Aqueous acetone (20 to 50 v/v) and NaCl (2 to 20 w/v) were the best developing agents. By this means alkaline phosphatase of kidney was localized in two spots ($R_F = 0$, $R_F = 1$) with either 0.33 *M* NaCl or 30% acetone as the solvent. Movement of the enzymes is slower in aqueous acetone than in salt solutions. Salivary and mold α-amylases were separated as were the amylases in germinating rice. Increase in the concentration of enzyme decreases its rate of movement and the enzyme spreads over a longer area. Jermyn (87) used paper chromatography with aqueous acetone as the developing agent to demonstrate a multiplicity of enzymes in *Aspergillus oryzae* and horse-radish extracts, several spots with the same enzyme activity appearing. Electrophoresis on paper also showed the presence of numerous enzymes.

Thomson (216) has used paper chromatography qualitatively to show the presence of conarachin and arachin in the course of the fractionation of peanut (*Arachis hypogaea*) protein. The developing solvent was 10% NaCl. The results differed from the usual finding that protein is strongly adsorbed or not adsorbed; both proteins were poorly adsorbed and yet appeared as distinct bands in the illustration given. Wynn and Rogers (243) have used the method of Consden *et al.* (29) to investigate proteins. The report is of most interest to those investigating unknown fluids, such as urine, containing protein as well as substances of low molecular weight. Aqueous phenol and collidine were the solvents used; in the former the proteins had R_F values of 1, in the latter 0. If collidine is used first the protein subsequently will not move with phenol.

V. Summary and Conclusions

A consideration of the factors affecting the adsorption of proteins on solid adsorbents reveals several factors that impose limitations on the chromatography of many proteins. These factors are the frequently encountered irreversibility of adsorption, the slow rate of adsorption, and the nonlinearity of adsorption curves. The last two

factors can be overcome to a degree by working with a slow rate of flow of the developer through the column, and with dilute solutions Irreversibility is a serious limitation to the use of adsorption technics but it may be overcome by working under nonequilibrium conditions, or by the utilization of powerful displacing agents. In view of these limitations it would appear to be desirable to explore more fully the factors mentioned for individual proteins. In any case chromatography of proteins should not be expected to proceed as smoothly as that of the amino acids, for example.

A study of the adsorption of proteins on solid adsorbents should provide information of wider interest as well. A study of the protein molecule in the adsorbed state might provide information about its physical dimensions and its ability to undergo reversible changes. It seems to be well established that many enzymes in the adsorbed state can still function as enzymes. This must have to do with the orientation of the specific activity sites on the enzymes. By analogy to adsorption at liquid-liquid and liquid-gas interfaces it would be expected that proteins adsorbed at a liquid-solid interface would be in a spread condition. The increase in adsorption with increase in temperature supports such an interpretation. However when enzymes or other proteins can be eluted they appear to have their specific, native properties. It is suggested that the rigidity with which they are held makes it possible for the proteins to return to their original shape. A return to a haphazard arrangement would be more likely under conditions of heat spreading (denaturation) or spreading at more mobile interfaces.

Adsorption studies in general should provide information of interest in the purification of proteins. In many cases proteins in solution must adsorb to a degree on protein precipitates. An effective purification procedure may require recognition of such phenomena. The effect of salts on the adsorption of proteins needs additional study, to determine the systems or conditions in which in one case salts will reduce adsorption and in another case increase adsorption.

Consideration of the specific application of chromatographic methods to proteins, indicates that the use of filter paper has not been successful except in a few instances. With certain enzymes and proteins and suitable developing solutions satisfactory chromatography has been obtained. Where the simplicity and separation of components provided by the paper technic is desired for complex mixtures like plasma, however, it appears that electrophoresis on paper will be generally most applicable. However, further studies in this field are desirable and the results to date suggest that additional specific applications of paper chromatography will be found.

The striking success of column procedures applied to lysozyme, ribonuclease, and cytochrome c, proteins of relatively low molecular weight and high isoelectric point, suggest that other members of this group, perhaps other proteins of low molecular weight as well, will be amenable to chromatography. There are reasons for believing that the hemin-containing proteins will be amenable to chromatography also.

The numerous examples of the application of the single adsorption technic in protein purification are a measure of its usefulness. It appears that a search for specific adsorbents might lead to results that would further broaden the application of this method. A study of adsorbents simulating the specific substrates of particular enzymes is seriously needed to determine the nature of the phenomena and whether this type of adsorbent can be made to order. There is no good evidence that enzyme substrates can serve as specific eluants but the subject deserves investigation. Specific eluants based on other chemical or physical properties might be designed.

All in all the study of the adsorption of proteins on solid adsorbents can be expected to lead to much useful information as well as to a broadening of the application of adsorption technics to the purification and characterization of enzymes and other proteins.

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PRINCIPLES AND PROCEDURES IN THE ISOLATION OF ENZYMES

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I. Introduction

In this presentation we should like to outline the principles and techniques, to systematize the multitude of procedures which have led to successful enzyme purification, and to mention some of the more promising new methods which have recently appeared in the literature. We shall also try to draw from the more recent literature material illustrative of the more classical procedures—ones which have withstood the test of time.

The reader is referred to the following earlier discussions: the encyclopedic *Methoden der Fermentforschung* (1), *Handbuch der Enzymologie* (2), *The Enzymes* (3), and *Crystalline Enzymes* (4). These references present details for the preparation of individual enzymes and also some general principles. Furthermore, many manipulative details involved in separating proteins and assaying their purity have been treated by Fevold and by Li in the recent book *Chemistry of Amino Acids and Proteins* (5).