Separation of Proteins by Ammonium Sulfate Gradient Solubilization*

T. P. King

ABSTRACT: The classical method of protein separation by ammonium sulfate precipitation can be carried out in the reverse manner. Namely, the proteins are first precipitated in the presence of a carrier and then they are solubilized with a decreasing salt gradient. An advantage of the reverse procedure is that it makes it possible for one to choose the best recovery of the desired protein with the least amount of contaminants. The reverse procedure has been reported by other workers but the various parameters involved had not been studied. The present results show that the width of a solubilized protein peak covers a 5–7% saturation range of ammonium sulfate for 85% recovery of the sample, and that the salt saturation at the protein peak depends not only on the nature of the protein but also on its amount and the gradient used. With a given gradient there is an optimum as to the amount of proteins which may be separated; too large an amount will give a decreased resolution of the peaks and too small an amount will give poor recovery. The simplicity of the solubilization procedure facilitates investigations of the influences pH and temperature on the desired separation. The usefulness of this procedure is illustrated with the isolation of the two major allergens from ragweed pollen, antigens E and K.

One of the classical methods of protein fractionation is ammonium sulfate precipitation (Taylor, 1953; Dixon and Webb, 1961). The method is widely used for the initial separation of crude extracts because it is suitable for preparative scale work and because it can have selectivity different from those of ion-exchange or gel filtration chromatographies. The critical salt saturation required to precipitate a protein depends on the nature and the concentration of the protein as well as on the pH and the temperature of the solution. An increase of 5–10% salt saturation above the critical value is usually sufficient to precipitate 90% of the protein initially in solution. In actual practice the full selectivity of the method is seldom attained because of the need to have exact control of the conditions for taking the appropriate 5–10% saturation range cut.

The salt precipitation procedure can also be carried out in the reverse manner. Namely, the protein mixture is first completely precipitated in the presence of a carrier and then solubilized with a decreasing salt gradient. In a single operation, the proteins are separated into a series of fractions with small differences of salt saturation thus making it possible to choose the best recovery of the desired protein with the least amount of contaminants. This reverse procedure has been reported earlier by other workers (Zahn and Stahl, 1953; Zittle and Della Monica, 1955; Keil *et al.*, 1962). However, a systematic study of the influences of the sample load, the salt gradient and its pH on the separation has not been previously reported.

This paper reports a number of observations on the use of this reverse procedure as well as on its application to the separation of two ragweed pollen allergens (King *et al.*, 1964, 1967).

Materials

Crystallized bovine plasma albumin sample (lot B-70411) was from the Armour Co. Low ragweed pollen of the 1963 crop was obtained from the Division of Biologic Standards, National Institutes of Health. Celite 545 was from the Fisher Scientific Co. Rabbit antisera specific for antigens E and K were prepared as described previously (King *et al.*, 1964, 1967).

Methods

Gradient Solubilization of Bovine Plasma Albumin at 25 ± 1°. To a solution of albumin (26 or 260 mg) in 200 ml of buffer containing 22 or 10 g of Celite 545, solid ammonium sulfate was added, with stirring, in a 10-min period to reach 80% salt saturation. After stirring for 30 min the suspension was packed under 15-cm Hg pressure to form a column of 2.2 × 19 cm. The column was eluted at 25° with a 340- or 615-ml linear gradient decreasing from 80 to 0% saturation in ammonium sulfate, or with a 600-ml gradient from 80 to 40% saturation. Due to differences in the densities of the starting and the limiting solutions, unequal volumes of the two solutions were present initially in the two cylindrical vessels (5.6-cm i.d.) of the gradient forming device; for example, the volumes of the two solutions were 285 and 330 ml for the 615-ml gradient. The gradient contained one of the following buffers: (1) 0.05 M Tris–0.03 M HCl, pH 7.97, (2) 0.16 M sodium acetate–0.04 M acetic acid, pH 5.26, and (3) 0.04 M sodium acetate–0.16 M acetic acid, pH 4.01. The flow rate of the gradient was maintained at 30 ml/hr with a pump and fractions of 5- or 10-ml volume were collected. The salt saturation of the fractions was determined by conductance after a 40-fold dilution with water.

In these experiments, as well as those with ragweed pollen proteins, the fractions immediately preceding the peak were found to be saturated with the protein. On standing, slight evaporation caused a slight increase in salt saturation and some salting out of protein. Therefore, the tubes were thoroughly mixed prior to diluting a small sample for protein determination by absorbance at 280 nm. The supernatants of these fractions provided a convenient means for the determination of the solubility curve of proteins.

Antigens E and K from Ragweed Pollen. All the operations

* From the Rockefeller University, New York, New York 10021. *Received September 27, 1971.* This work was supported in part by a United States Public Health Service Grant AI-08445.

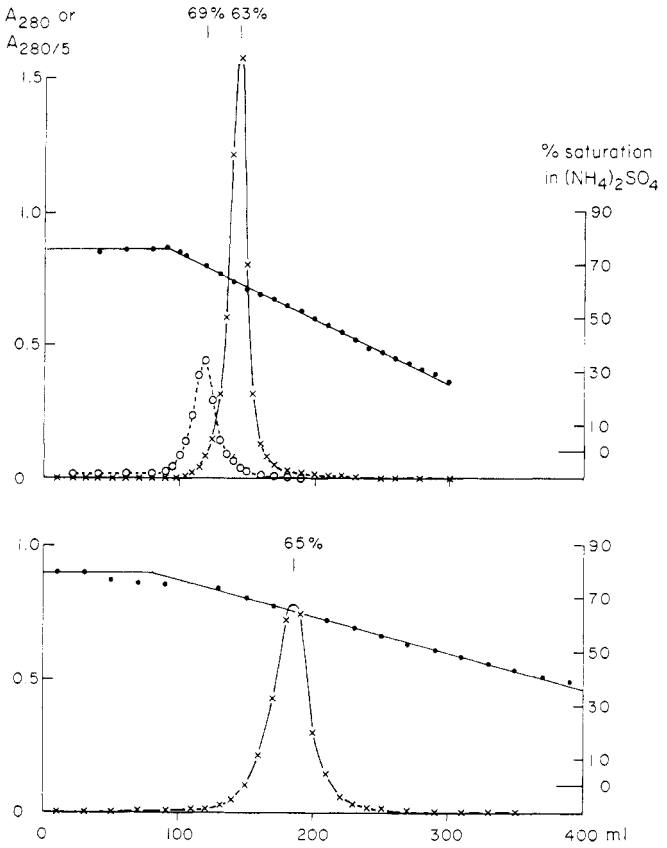

FIGURE 1: Ammonium sulfate gradient solubilization of bovine plasma albumin on a column of Celite 545. The top graph shows the results of expt 1 and 2 (denoted by open circles and crosses, respectively) and the bottom graph shows the result of expt 3. See Table I for experimental details. The salt saturation at the peak position is given above each curve.

were carried out at 25 ± 1°. Ragweed pollen (100 g) was defatted with ether, dried *in vacuo*, then extracted in 500 ml of water with stirring for 2 hr. After removal of the pollen, 25 ml of 1 M Tris and 0.6 HCl was added to the extract to bring the pH to 7 followed with 22 g of Celite 545. Ammonium sulfate was added with stirring to 80% saturation in a 10-min period and stirring was continued for 45 min. The Celite mixture was filtered dry by suction, resuspended in 150 ml of 80% saturated ammonium sulfate solution containing 0.05 M Tris-HCl (pH 7.97) and packed to form a column of 2.2 × 19 cm. The column was eluted at 30 ml/hr with the 615-ml gradient buffered with Tris-HCl as described above for albumin. All the fractions were strongly pigmented. Therefore for the protein determination, trichloroacetic acid precipitation was carried out using 50-μl aliquots of the fractions and 1 ml of 10% trichloroacetic acid, and the precipitate was analyzed by absorbance at 290 nm after dissolution in 1 ml of 0.2 N NaOH. The antigen concentration was determined quantitatively by immunodiffusion with specific rabbit antisera (King *et al.*, 1964).

An alternate procedure for handling 2.7 l. of extract from 500 g of pollen is as follows. To the extract containing 60 g of Celite 545 was added solid ammonium sulfate to 40% saturation. The mixture was filtered to give the antigen K enriched cut. After adding 60 g of Celite to the filtrate, solid ammonium sulfate was added to 65% saturation. The mixture was filtered to give the antigen E enriched cut. The antigen K and E cuts were suspended, respectively, in 250 ml of 50% and 70% saturated ammonium sulfate solutions containing 0.05 M Tris-HCl (pH 7.97), and were packed into separate columns (22 × 3.7 cm). The antigen K and E columns were eluted with 1-l. gradients from 50 to 0% saturation and from 70 to 20% saturation, respectively. Antigens K and E were recovered from the fractions in the saturation ranges of 21–27% and 38–44%, respectively, and their yields were determined by immunodiffusion to be 240 and 420 mg after concentration by salting out and dialysis against 0.05 M Tris-HCl (pH 7.97). Each of the solutions (about 26 ml) was purified to yield the desired antigen by two successive chromatographies on a column (22 × 1.6 cm) of triethylaminoethylcellulose as described before (King *et al.*, 1964, 1967), with the slight modification that the column was first eluted with 150 ml of 0.05 M Tris-HCl (pH 7.97) then with a 1-l. gradient from 0 to 0.20 M NaCl in 0.05 M Tris-HCl. Two successive chromatographies were required to remove fully the bound pigments. The yield of the purified antigen E was 163 mg; 47 mg in the B form and 116 mg in the C form. The yield of antigen K in A form was 56 mg. These yields were the same as those from a separate experiment using the previously published procedures.

The homogeneity of the antigen preparations was studied by isoelectric focusing as described below.

Isoelectric Focusing in Polyacrylamide Gel. The procedure has been described previously (Spencer and King, 1971). When focusing was carried out in gels containing pH 3–10 Ampholine (LKB Produkter), there was a marked shrinkage of the gel in the pH region 5–6 probably as a result of local heating caused by high electrical resistance (Davis, 1969). Incorporation of 31% by volume of glycerin into the gel markedly reduced the shrinkage as glycerin was found to increase the conductance of the gel. This also has the possible advantage that glycerin is known to stabilize most proteins against heat denaturation.

Results

Bovine Plasma Albumin. Seven separate experiments were carried out with albumin as a test protein to study the different parameters involved in the salt gradient solubilization procedure. These parameters include the sample load, the amount of carrier Celite, the steepness, and the pH of the ammonium sulfate gradient as listed in Table I. In all cases, with the exception of expt 7, albumin was solubilized to yield a nearly symmetrical peak as shown in Figure 1 for expt 1–3 and in Figure 2 for expt 3, 6, and 7. The results of expt 4 and 5 are not shown in the figures, and they are similar to those of experiments 1 to 3.

The recoveries of the applied albumin in a salt saturation range of 7% were 60% for expt 1 and 85% for expt 2–6. The low recovery for expt 1 is caused by the fact that the fraction of the sample solubilized prior to the peak became significant with the small amount of sample used (26 mg). The observed peak widths of these experiments are in accord with the solubility property of albumin wherein a 6% change in the ammonium sulfate saturation produces a tenfold change in the albumin solubility as determined under the same conditions used for the solubilization studies. The results indicate that the flow rate used was such as to establish equilibrium of albumin between the liquid and the solid phases. This was confirmed by a separate experiment in which the flow rate was reduced to one-half of that used for the above experiments.

The salt saturations at the peak positions were not identical

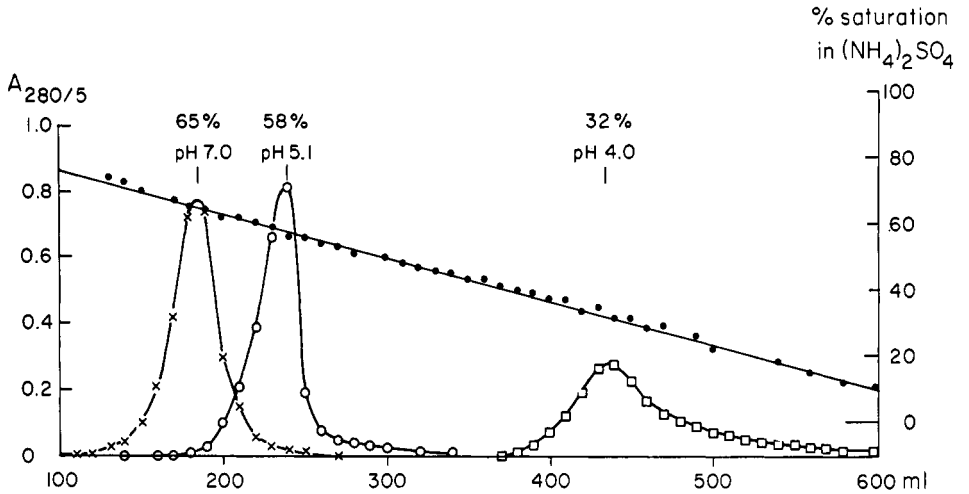

FIGURE 2: Ammonium sulfate gradient solubilization of bovine plasma albumin on a column of Celite 545. The results of expt 3, 6, and 7 are denoted by crosses, open circles, and open squares, respectively. The result of expt 3 is shown here again for the purpose of comparison. The experimental details are given in Table I. The pH values given were determined after a 20-fold dilution of the peak tubes with water. The salt saturation at the peak position is given above each curve.

in these seven experiments. In expt 1 and 2 the gradient was kept constant at 0.23% salt saturation decrease per milliliter, and the peak positions for the 26-mg samples were at 69 and 63% salt saturations, respectively. In experiments 2, 3, and 4 the amount of sample was kept constant at 260 mg, and the peak positions solubilized with gradients of 0.23, 0.13, and 0.077% salt saturation decreases per milliliter were at 63, 65, and 70% salt saturations, respectively. Keeping the sample size and the gradient constant but varying the amount of Celite used produced a small difference of the peak positions 63 and 62% salt saturations, respectively, in expt 2 and 5 but the difference is within experimental error.

The observed dependence of the peak position on the amount of sample and the gradient is to be expected from the solubility properties of proteins where the critical salt saturation, which is required to precipitate a protein, depends on the protein concentration. With the solubilization procedure the solvent volume is determined by the steepness of the gradient. Accordingly the salt saturation at the peak will depend on the gradient and the amount of sample used.

In expt 3, 6, and 7 the amount of sample and the gradient were constant but the pH of the gradient was varied. The peak position of albumin corresponds to 65% salt saturation at pH 7.97, 58% at pH 5.26, and 31% at pH 4.01 (Figure 2). The solubility of most proteins in salt solutions shows a minimum near the isoelectric point (Dixon and Webb, 1961). Therefore the peak position for the pH 5 gradient is at a lower salt saturation as compared to that for the pH 7 gradient, in accord with the isoelectric point of albumin being about 5.3 as determined by isoelectric focusing (Spencer and King, 1971). For the pH 4 gradient the further decrease in salt saturation of the peak as well as the broadness of the peak are both unusual findings which may be related to the fact that albumin is known to undergo conformational changes in acid solutions (Wong and Foster, 1969) and to exhibit isoelectric heterogeneity (Spencer and King, 1971). Therefore, four cuts were taken from the broad peak and were examined by isoelectric focusing in polyacrylamide gel containing 6 M urea after prior conversion of the single cysteinyl residue of albumin into an *S*-half-cystinyl residue. All four cuts were found to contain the same distribution of isoelectric components.

Ragweed Pollen Antigens. The solubilization procedure was applied to the isolation of two antigens E and K from ragweed pollen. These two antigens are the major allergens of ragweed pollen. Both are acidic proteins with molecular weight of about 38,000. They are separable with difficulty by chromatography on Sephadex G-100 and not separable by chromatography on cellulose anion exchangers (King *et al.*, 1964, 1967).

The results in Figure 3 show that antigens E and K were easily separated by gradient solubilization of a 2.3-g mixture of proteins as obtained from 100 g of ragweed pollen. The peak positions of antigens E and K were at 41 and 25% salt saturations, respectively, and the recoveries of the two antigens were 85% in the indicated cuts which covered a salt saturation range of 5%. In another experiment where 4.6 g

TABLE I: Ammonium Sulfate Gradient Solubilization of Bovine Plasma Albumin under Different Conditions.[a]

Expt No.	Albumin (mg)	Celite (g)	$(NH_4)_2SO_4$ Gradient (% Satn Decrease/ml)	pH of Gradient[b]	% (NH_4)-SO_4 Satn at Peak Position
1	26	22	0.23	7.97	69
2	260	22	0.23	7.97	63
3	260	22	0.13	7.97	65
4	260	22	0.077	7.97	70
5	260	10	0.23	7.97	62
6	260	22	0.13	5.26	58
7	260	22	0.13	4.01	31

[a] A 200-ml solution of albumin was brought to 80% $(NH_4)_2SO_4$ saturation in the presence of Celite. The albumin–Celite mixture was packed into a column of 2.2 cm i.d. then it was eluted with a decreasing salt gradient at 25° and 30 ml/hr. [b] The values given refer to the pH of the buffer in the absence of ammonium sulfate.

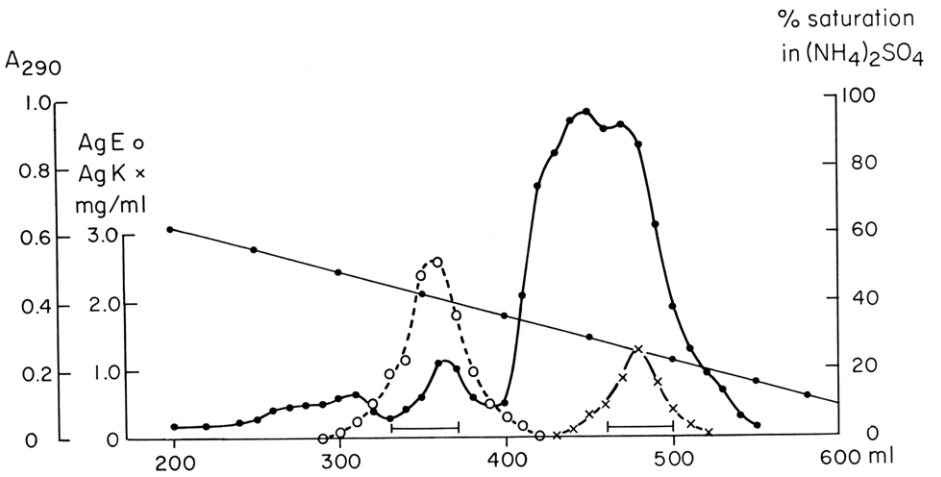

FIGURE 3: Separation of ragweed pollen antigens E and K by ammonium sulfate gradient solubilization. A column containing 2.3 g of ragweed pollen proteins and 22 g of Celite 545 was eluted at 25° with a gradient at a flow rate of 30 ml/hr. The gradient had a decrease of 0.13% salt saturation per milliliter and it was buffered with 0.05 M Tris and 0.03 M HCl. The total protein concentration is indicated by solid circles and the antigen E and K concentrations are indicated by open circles and crosses, respectively. The indicated cuts were found to contain 88 and 38 mg of antigens E and K, respectively.

of ragweed pollen proteins was used with the other conditions same as those in Figure 3, the peak positions of antigens E and K were found to be at 38 and 24% salt saturations, respectively. Apparently increasing the amount of proteins has shifted the peaks to lower salt saturations with an accompanying decrease of resolution.

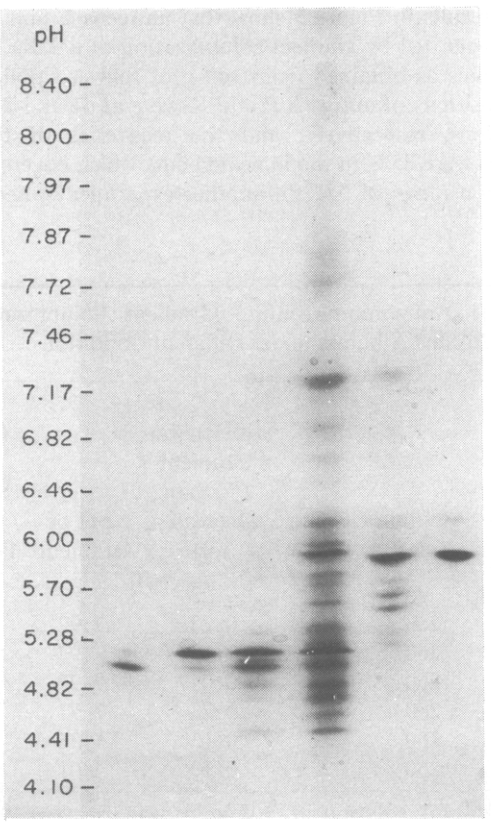

FIGURE 4: Isoelectric focusing on polyacrylamide gel containing 31% glycerin. From left to right: antigen E in C form (12 μg), antigen E in B form (20 μg), antigen E cut of Figure 3, ragweed pollen extract, antigen K cut of Figure 3, and antigen K in A form (30 μg). The amounts of pollen extract, antigen E and K cuts applied were equivalent to 100 mg of pollen. Incorporation of glycerin into the gel was found to reduce the gel shrinkage in the pH 5 region (see Methods).

The crude antigen cuts from Figure 3 were examined by isoelectric focusing on polyacrylamide gel together with the purified antigens and the whole pollen extract. The results in Figure 4 show that the pollen extract contained a number of heavily stained bands but the two cuts contained mainly the bands corresponding to those of the purified antigens. The purified antigens were obtained from the cuts by chromatographies on triethylaminoethylcellulose as described in Methods. Both antigens are known to be isolable in multiple isoelectric forms. Only the major forms B and C for antigen E and A for antigen K are shown in Figure 4.

For the isolation of antigens from 500 g of pollen it was found convenient to make a preliminary salt precipitation of the extract to give antigen E and K enriched fractions. The enriched fractions were then fractionated separately on columns 60 g of Celite with a gradient of 0.055% salt saturation decrease per milliliter. The details of these experiments are given in the Methods section.

Discussion

The above results clearly indicate that the gradient solubilization procedure is a useful addition to the standard precipitation procedure for ammonium sulfate fractionation of proteins. The application of this procedure has simplified the isolation of the two major allergens from ragweed pollen thus making them more accessible for studies.

Several of the parameters involved in the use of this procedure were studied. These parameters are the amounts of proteins and of the carrier Celite to be used, the steepness of the decreasing salt gradients, and the control of pH. The effect of temperature was not studied but it can be expected that this is also an important parameter. The experiences gained suggest that when applying this procedure to other problems one should use about 2 g of a protein mixture and 20 g of Celite with a gradient of about 0.1% saturation decrease in ammonium sulfate per milliliter. When the desired protein is less than 1% of the mixture, a preliminary enrichment by the precipitation procedure is desirable. This is because when the initial amount of protein is low, the recovery may be poor due to the trace solubilization prior to the main peak. Using too large an amount of protein for the above

conditions can lead to poor resolution. As noted in the Results section, the peak positions are dependent on the amounts of proteins, and increasing the amounts of proteins compresses the peaks into the lower salt saturation range. The procedure can be scaled up or down easily to meet the required amounts. The choice of pH and temperature conditions will of course depend on the stability of the protein. Repetition of the separation under a different pH or temperature condition should give an improved selectivity as the solubility dependence of proteins on pH and temperature changes are different.

The present procedure bears a close resemblance to another procedure termed "zone precipitation" (Porath, 1962). With zone precipitation a small volume of protein solution is applied to a column of Sephadex which is equilibrated with an ammonium sulfate solution of high saturation then the column is eluted with a decreasing salt gradient. The protein solution migrates faster than the salt gradient due to the sieving effect of Sephadex, and it soon reaches a region of high salt saturation and precipitation occurs. As the gradient advances, the protein is again in an environment of low salt saturation and it redissolves and migrates. This process of precipitation and dissolution is repeated through the entire length of the column.

The above mechanism is probably operating in the gradient solubilization procedure, as suggested by the nearly symmetrical curves shown in the figures. If the present procedure were a simple process of solubilization in a decreasing salt gradient, one would expect from the solubility properties of proteins that the curves would be highly unsymmetrical. While Celite may not serve as a sieve like Sephadex, it is possible that some type of surface phenomenon on the Celite particles serves to retard the migration of the salt gradient relative to that of the protein solution.

The present procedure is also similar to the reported procedure of chromatography of proteins on diethylaminoethylcellulose in concentrated ammonium sulfate solution (Mahew and Howell, 1971). A solution of proteins is adjusted to an ammonium sulfate saturation of 5 to 10% lower than required for precipitation and it is applied to a column of diethylaminoaminoethylcellulose containing ammonium sulfate solution at the same saturation. The proteins are absorbed to the column presumably as a result of salting out on account of the higher effective ionic strength within the cellulose ion-exchanger matrix, then the proteins are eluted with a decreasing salt gradient.

It is reasonable to suggest from the above discussion of the three different procedures that they all operate on the basis of the same principle involving the repeated process of precipitation and dissolution. The three procedures differ in practice of how the sample is initially precipitated onto the carrier and of the carrier used. Therefore the present observations on the proper use of the gradient solubilization procedure will also be applicable to the other two procedures. It also follows that the carriers used in the other two procedures will be applicable in this procedure.

In this paper, only ammonium sulfate gradients were studied for the separation of proteins, but other gradients based on a change in pH or in the concentrations of other salts or organic solvents may also be used. With the zone precipitation procedure, the use of sodium sulfate gradient (Sargent and Graham, 1964) and pH gradient (Hoffmann, 1969; Björklund, 1971) has been reported.

Acknowledgment

I thank Mrs. Anita Bohensky for her technical assistance.

References

Björklund, B. (1971), *Anal. Biochem. 41*, 287.
Davis, H. (1969), Protids of Biological Fluids, Proceedings of the 17th Colloquim, Pergamon Press, New York, N. Y., p 389.
Dixon, M. and Webb, E. C. (1961), *Advan. Protein Chem. 16*, 167.
Hoffmann, L. G. (1969), *J. Chromatogr. 40*, 39.
Keil, B., Keilová, H., and Bartŏsck, I. (1962), *Collect. Czech. Chem. Commun. 27*, 2940.
King, T. P., Norman, P. S., and Connell, J. T. (1964), *Biochemistry 3*, 458.
King, T. P., Norman, P. S., and Lichtenstein, L. M. (1967), *Biochemistry 6*, 1992.
Mayhew, S. G. and Howell, L. G. (1971), *Anal. Biochem. 41*, 466.
Porath, J. (1962), *Nature (London) 196*, 47.
Sargent, R. N., and Graham, D. L. (1964), *Anal. Chim. Acta 30*, 101.
Spencer, E. M., and King, T. P. (1971), *J. Biol. Chem. 246*, 201.
Taylor, J. F. (1953), *Proteins 1*, 2.
Wong, K. P., and Foster, J. F. (1969), *Biochemistry 8*, 4104.
Zahn, R. K., and Stahl, I. (1953), *Z. Physiol. Chem. 293*, 1.
Zittle, C. A., and Della Monica, E. S. (1955), *Arch. Biochem. Biophys. 58*, 31.