

## Chromatography of Proteins on Diethylaminoethyl-cellulose in Concentrated Ammonium Sulfate<sup>1</sup>

STEPHEN G. MAYHEW AND LARRY G. HOWELL<sup>2</sup>

*Department of Biological Chemistry, The University of Michigan,  
Ann Arbor, Michigan 48104*

Received November 6, 1970

Two techniques which are widely used in protein purification are salting-out by high concentrations of ammonium sulfate and ion-exchange chromatography on a column of a resin such as diethylaminoethylcellulose (DEAE-cellulose). When a fractionation with ammonium sulfate precedes ion-exchange chromatography in a purification procedure, it is often necessary to remove excess salt before application of the protein mixture to the column. This is done to ensure that the ionic strength of the solution is sufficiently low for adsorption of at least some of the proteins in the mixture. However, Mortenson (1) and Lovenberg and Williams (2) observed that the bacterial iron-proteins rubredoxin and ferredoxin are retained by a column of DEAE-cellulose when they are applied in a solution that contains a high concentration of ammonium sulfate. Both proteins are eluted from the column when the salt concentration is decreased. Since they are eluted by different concentrations of salt, rubredoxin and ferredoxin can be separated by this method (2).

We have found that a number of other proteins also show this behavior on DEAE-cellulose equilibrated with high concentrations of ammonium sulfate, and in some cases the phenomenon has been used for enzyme purification. This paper describes conditions under which proteins are retained by and eluted from DEAE-cellulose equilibrated with ammonium sulfate; we feel that the method may be of general use in protein purification.

### METHOD

The following method was used to test a number of pure proteins for their retention by DEAE-cellulose equilibrated with concentrated am-

<sup>1</sup>Supported by USPHS Grant GM-11106 to Dr. V. Massey.

<sup>2</sup>Postdoctoral fellow of the United States Public Health Service. Present address, Department of Chemistry, University of South Florida, Tampa, Florida 33620.

monium sulfate. A weighed amount of solid ammonium sulfate (enzyme grade from Mann) was added to a solution of protein (1 mg/ml) in 0.03 *M* sodium phosphate buffer, pH 6.3, at 4°. The final concentration of ammonium sulfate depended on the protein used, but in general it was 5–10% lower than the salt concentration needed to salt-out the protein. The mixture was stirred for several hours and then centrifuged for 20 min at 23,500*g*. This centrifugation was done to ensure that the solution did not contain a precipitate of protein. Then 2 ml of clear solution was applied to a column (8 × 1.6 cm) of DEAE-cellulose (Whatman DE 22) equilibrated with 0.03 *M* sodium phosphate buffer, pH 6.3, containing ammonium sulfate at the same concentration as that in the protein solution. The column was washed with several column-volumes of the same buffer and ammonium sulfate, before connecting a linear gradient of decreasing ammonium sulfate concentration. The gradient was made by continuous dilution of 100 ml of 0.03 *M* sodium phosphate, pH 6.3, containing ammonium sulfate at the concentration in the original protein solution, with 100 ml of 0.03 *M* sodium phosphate, pH 6.3. Fractions (approximately 2 ml) were collected from the column at 5 min intervals, and monitored for protein (absorption at 280 *mμ*) and salt (conductivity measurements made with a conductivity bridge supplied by Industrial Instruments Incorporated). The concentration of ammonium sulfate at the protein peak was determined by plotting absorption at 280 *mμ* and salt concentration against the fraction number.

#### MATERIALS

Horse heart cytochrome *c* and beef pancrease ribonuclease A were obtained from Sigma, ovalbumin was from Worthington, bovine hemoglobin was from Pentex, and bovine plasma albumin from Armour Pharmaceutical Company, Kaukaee, Illinois. Flavodoxin (3), ferredoxin (1), and rubredoxin (4) from *Peptostreptococcus elsdenii*, ferredoxin (1) and rubredoxin (2) from *Clostridium pasteurianum*, and lactate oxidase from *Mycobacterium smegmatis* (5) were prepared as described elsewhere. Butyryl CoA dehydrogenase from *P. elsdenii*<sup>3</sup> and *p*-hydroxy benzoate hydroxylase from *Pseudomonas fluorescens*<sup>4</sup> were isolated by unpublished procedures. Ammonium sulfate (enzyme grade) was from Mann. DEAE-cellulose (Whatman DE 22) was prepared for use by cycling through acid and alkali washes as described by the manufacturer. The washed gel was suspended in 0.03 *M* sodium phosphate, pH 6.3, containing ammonium sulfate at the appropriate saturation. Dissolved gases were removed from the suspension by evacuation at a water

<sup>3</sup> P. C. Engel, private communication.

<sup>4</sup> L. G. Howell, unpublished.

pump immediately before use. Solutions of ammonium sulfate used to elute columns of DEAE-cellulose were also freed of dissolved gases.

### RESULTS AND DISCUSSION

The association of protein with DEAE-cellulose equilibrated with concentrated ammonium sulfate can be readily demonstrated with cytochrome *c*. Cytochrome *c* is not precipitated from dilute solutions by 90% saturated ammonium sulfate. However, when DEAE-cellulose (about 2 gm of damp resin equilibrated with 90% saturated ammonium sulfate), is mixed with a solution (5 ml) containing 5 mg cytochrome *c*, 0.03 *M* sodium phosphate, pH 6.3, and 90% saturated ammonium sulfate, and the mixture is centrifuged, over 90% of the cytochrome *c* is found in the precipitate. The protein can be extracted from the resin with 50% saturated ammonium sulfate.

The ammonium sulfate concentrations at which several proteins are retained by and eluted from DEAE-cellulose are shown in Table 1. With the exception of ovalbumin and bovine plasma albumin, all the proteins tested remained on the column after washing with several column-volumes of starting buffer. They were eluted only when the concentration

TABLE 1  
Concentrations of Ammonium Sulfate Used to Apply Proteins to DEAE-cellulose.  
Concentrations at the Peak Fraction in the Elution Profile

Protein	Ammonium sulfate in sample applied to column	Ammonium sulfate in peak fraction from column
	% saturation	
<i>P. elsdonii</i> ferredoxin	60	20
<i>P. elsdonii</i> rubredoxin	60	32
<i>P. elsdonii</i> flavodoxin	60	40
<i>C. pasteurianum</i> ferredoxin	50	30
<i>C. pasteurianum</i> rubredoxin	50	40
Lactate oxidase	50	44
<i>p</i> -Hydroxybenzoate hydroxylase <sup>a</sup>	42	36
Hemoglobin	60	51
Butyryl CoA dehydrogenase	50	30
Cytochrome <i>c</i>	90	77
Ribonuclease A	65	58
Ovalbumin	60	Retarded <sup>b</sup>
Bovine plasma albumin	60	Retarded <sup>b</sup>

<sup>a</sup> Results given for Whatman DE 32. This enzyme is greatly retarded by DE 22, but DE 32 was found to be more effective.

<sup>b</sup> Protein was eluted after washing column with several column-volumes of starting buffer.

of ammonium sulfate in the buffer was decreased. Although ovalbumin and bovine plasma albumin were not strongly retained by the column, their elution was retarded, and several column-volumes of buffer were required to elute them. In the case of bovine plasma albumin, the elution profile showed a number of peaks, suggesting that this protein was slowly leached from the column.

In experiments with colored proteins, visual inspection showed that these proteins remained in the top half of the column until the ammonium sulfate concentration was decreased. This observation was made with both negatively charged proteins (ferredoxin, rubredoxin, and flavodoxin) and a positively charged protein (cytochrome *c*).

In the experiments of Table 1, the columns of DEAE-cellulose were eluted with a linear gradient of ammonium sulfate, and the mobility of the protein band on the column increased as the ammonium sulfate concentration decreased. However, the ammonium sulfate in the eluting buffer can be adjusted to a concentration at which a particular protein moves very slowly. Under these conditions two proteins which are difficult to separate by ammonium sulfate fractionation in solution may be readily separated on the column (e.g., refs. 2 and 6).

This technique has been used in our laboratory as a convenient and rapid method for concentration of protein from solutions too dilute for a classical ammonium sulfate precipitation, and also to separate enzymes from crude cell-free extracts. A sequence of operations that has been useful for the purification of a number of proteins is as follows: ammonium sulfate is added to a cell-free extract to a concentration just below that required to precipitate the desired protein. The mixture is centrifuged and the precipitate discarded. The supernatant solution is applied directly to a column of DEAE-cellulose equilibrated with ammonium sulfate. Fractions from the column are monitored for enzyme activity while eluting either batchwise or with a gradient of ammonium sulfate. This sequence combines a classical ammonium sulfate fractionation with a chromatographic procedure, and it eliminates the need for a second centrifugation and an intermediate dialysis step.

The explanation for the observed behavior of proteins on DEAE-cellulose in concentrated ammonium sulfate is not known. It is unlikely that ionic bonding between protein and resin is a major factor, since both negatively charged proteins (e.g., ferredoxin) and positively charged proteins (cytochrome *c* and ribonuclease) show this behavior. It is possible that the effective ionic strength within the resin matrix is sufficient to cause proteins to be salted-out. By using the data published by the manufacturer (exchange capacity 1 milli equivalent per gram and bed volume 7.7 ml per gram), it can be calculated that, in

Whatman DE 22 completely equilibrated with ammonium sulfate, the effective ionic strength contributed by the resin would be about 0.39. This is equivalent to an increase of 3-4% in the ammonium sulfate saturation. However, this calculation is based on the over-all exchange capacity of the DEAE-cellulose, and it includes the void volume of the gel. When the void volume and the increase in gel density which occurs in high salt concentrations are taken into consideration, the local charge concentration within the gel matrix will be higher than that calculated with the data above. As pointed out by Dixon and Webb (7), the change in ammonium sulfate saturation necessary to precipitate 90% of the protein from a saturated protein solution is usually between 5 and 10%. It is clear that an increase of this magnitude could be provided by the effective ionic strength of DEAE-cellulose.

Proteins might be expected to show a similar behavior when other ion-exchange resins are substituted for DEAE-cellulose. Preliminary experiments with flavodoxin have shown that, in 60% saturated ammonium sulfate, this protein is retarded by carboxymethylcellulose (Whatman CM 22). However, cytochrome *c* in 90% saturated ammonium sulfate is not retarded by guanidoethylcellulose (Cellex GE from Bio-Rad laboratories), Bio-Rad AGI-X8, or amberlite CG-50 (from Mallinckrodt). These negative results can be attributed in some cases to low exchange capacity of the gel. However, it is possible that factors other than an increase in ionic strength provided by the charged group on the resin are involved. In particular, accessibility of the protein to the exchanger matrix and protein-matrix interactions may have a significant role in the retardation observed with DEAE-cellulose.

#### REFERENCES

1. MORTENSON, L. E., *Biochim. Biophys. Acta* **81**, 71 (1964).
2. LOVENBERG, W., AND WILLIAMS, W. M., *Biochemistry* **8**, 141 (1969).
3. MAYHEW, S. G., AND MASSEY, V., *J. Biol. Chem.* **244**, 794 (1969).
4. MAYHEW, S. G., AND PEEL, J. L., *Biochem. J.* **100**, 80P (1966).
5. SULLIVAN, P. A., *Biochem. J.* **110**, 363 (1968).
6. MAYHEW, S. G., *Anal. Biochem.*, submitted.
7. DIXON, M., AND WEBB, E. C., *Advan. Protein Chem.* **16**, 167 (1961).