USE OF DEAE-CELLULOSE IN THE SEPARATION OF PROTEINS FROM EGG WHITE AND OTHER BIOLOGICAL MATERIALS

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Cellulose ion-exchange chromatography has emerged recently as a powerful tool for separation and identification of proteins in simple and complex biological mixtures. Successful application of this method has been made in the analysis of horse and human sera[1,2], purification of various enzymes and hormones[3-7], and characterization of egg white proteins[8]. Since the appearance of these early reports, a large amount of information has accumulated on the application of cellulose ion-exchange chromatography to the purification of specific proteins[9]. In most instances, the anion-exchange celluloses used were diethylaminoethyl cellulose (DEAE-cellulose) and triethylaminoethyl cellulose (TEAE-cellulose), and the cation exchange cellulose used was carboxymethyl cellulose (CM-cellulose).

During the course of an investigation of the protein constituents of chicken egg white and yolk, we have employed both DEAE-cellulose and CM-cellulose. The former was more useful in separating the components of fresh unaltered egg white because elution could be largely accomplished near neutrality at low ionic strength. We have also found that the proteins of other complex biological materials of either plant, animal, or bacterial origin may be separated by DEAE-cellulose chromatography, although for egg yolk, a few preliminary purification steps are necessary. Our experiences with DEAE-cellulose in the separation of proteins from such mixtures will be described in this report.

MATERIALS AND METHODS

DEAE-cellulose, type 20 (coarse grade), 0.7 mequiv./g, was purchased from Brown Company, Berlin, New Hampshire. Before use, the DEAE-cellulose was washed on a Büchner funnel with fritted glass disc successively with 0.1 N HCl–1 M NaCl, water, 1 M $NaHCO_3$, water, 1 M Na_2CO_3, water, 0.1 N NaOH, water, ethanol and water. The cellulose was then suspended in water overnight to permit trapped air bubbles to escape. After the suspension had settled, any fines which remained were decanted.

Preparation of columns

The washed cellulose was suspended in 0.1 N NaOH and portions were poured into

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glass chromatography columns of appropriate size for the intended use, usually, 2.5 cm diameter and 40 cm long. When the cellulose had settled to the desired height, further additions were discontinued and the column was washed to neutrality with water. This was followed by a wash with 0.02 *M* glycine until a blue band, which appears to be a Schiff's base, was washed out of the column. The symmetry of this blue band as it migrated down the column served as an effective indicator of the levelness of packing of the column.

Elution schedules

The majority of the separations described below were carried out with a non-linear gradient elution schedule adapted from SPEERS *et al.*[2]. With egg white, for example, 5 ml of carefully blended[10] material were mixed with an equal volume of 0.02 *M* glycine and the mixture was entered on a 2.5 × 40 cm column. The diluted egg white was then permitted to sink almost entirely into the column before being washed with two 10-ml portions of glycine solution. Gradient elution was then begun with 750 ml of 0.02 *M* glycine in the mixing flask and a reservoir containing 0.02 mole each of KH_2PO_4, K_2HPO_4, and glycine in 1 l of solution. The flow rate was adjusted to 2 ml/min. When the solution in the reservoir was exhausted, it was replaced by 1 l of solution containing 0.1 mole KH_2PO_4, 0.1 mole NaCl, and 0.02 mole glycine. After this volume was used, the solution was replenished with 1 l of solution containing 0.1 mole KH_2PO_4, 0.1 mole NaCl, 0.03 mole HCl, and 0.02 mole glycine.

In the chromatography of egg white for preparative purposes, the white of an entire egg was carefully blended, diluted with an equal volume of 0.02 *M* glycine, entered on a column 5.0 cm in diameter and 60 cm long, and followed by two washes with equal volumes of glycine solution. Elution of the egg white protein was accomplished by changing the composition of glycine–phosphate buffer added to the column in discrete steps.

The course of the elution of proteins was followed by measuring the optical density of the fractions collected at 280 mμ. It was found, however, that modification of the elution schedule could be assessed more easily when the eluant was monitored by a UV absorption monitor and recording meter*. In this manner, a continuous graphic record of the absorption at 254 mμ of the emerging solutions was obtained without necessity of collecting and analyzing individual fractions.

Assays for egg white proteins

In experiments with egg white, ovalbumin was assayed for content of free SH groups by the method of BOYER[11]; lysozyme was identified and estimated by its lytic activity on *Micrococcus lysodeikticus*[12]; conalbumin was measured by its iron binding capacity[13]; and ovomucoid was detected by its antitrypsin activity[14]. The biotin-binding capacity of avidin was measured by the HERTZ modification of the EAKIN method[15].

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* Gilson Medical Electronics, Middleton, Wisconsin. Mention of specific products does not constitute endorsement by the Department of Agriculture.

Muscle extract

Chicken breast muscle extract was prepared by blending freshly excised chicken breast muscle with an equal weight of cold distilled water in a Waring Blendor. The homogenate was centrifuged at 15,000 *g*, and the supernatant was dialyzed overnight against running distilled water. After dialysis, the supernatant was clarified by centrifugation and chromatographed. Assays were made for phosphorylase[16], phosphoglucose isomerase[17], and aldolase[18]. Adenylate kinase[19] was detected by following the disappearance of ADP and the concomitant appearance of ATP and AMP on paper chromatograms.

Cabbage extract

Cabbage extract was prepared by blending fresh cabbage with an equal volume of 0.5 *M* sucrose. The homogenate was centrifuged at 23,000 *g*, and the supernatant was examined chromatographically with no prior dialysis. Assays[20] were made for apyrase, inorganic pyrophosphatase and acid phosphatase activity*.

Bacterial extracts

Escherichia coli ATC 11246 and *E. coli* 6-204-55** were grown under identical conditions to approximately the same turbidity in a 3 % trypticase-soy broth. Cells from each of the cultures were harvested, washed with water, and disrupted by ultrasonic vibration. The sonicate was clarified by centrifugation at 23,000 *g* and dialyzed against distilled water overnight in order to remove low molecular weight UV-absorbing material. After dialysis, the sonicates were examined successively with two columns of DEAE-cellulose, 1 cm diameter and 30 cm long, which had been made at the same time. The fractions obtained from the chromatographic analysis of *E. coli* ATC 11246 were assayed for presence of L-glutamic acid decarboxylase[21].

RESULTS

Egg white

When 5 ml of fresh egg white containing approximately 600 mg of protein were chromatographed on DEAE-cellulose according to the gradient elution schedule described above, the pattern shown in Fig. 1 was obtained. One tube from each peak was assayed for lysozyme, conalbumin, ovalbumin, and ovomucoid. Lysozyme activity was found in peaks A, B and C, conalbumin in peak D, ovalbumin in peaks G and H, and after dialysis, ovomucoid activity in peak E. The flavoprotein component previously described by RHODES *et al.*[8] was identified with peak J. Although not discernible as a distinct peak, avidin activity was found in fractions 52 and 53. Peaks F, I and K were not characterized.

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* Cabbage extract as well as the apyrase and phosphatase assays were generously provided by Dr. M. MAZELIS of this Laboratory.

** *E. coli* 6-204-55 was obtained from the laboratories of Dr. MAX LEVINE, Territorial Dept. of Health, Honolulu, Hawaii, and is reported to contain the antibiotic colicin.

An ultraviolet absorption spectrum was determined for peaks A, D and H, and the results are shown in Fig. 2a. For purposes of comparison, the ultraviolet absorption spectra of crystalline lysozyme, conalbumin and ovalbumin are shown in Fig. 2b. It can be seen that the spectra of peaks A, D and H are indistinguishable from those of the corresponding purified crystalline proteins. In addition, it was found that the

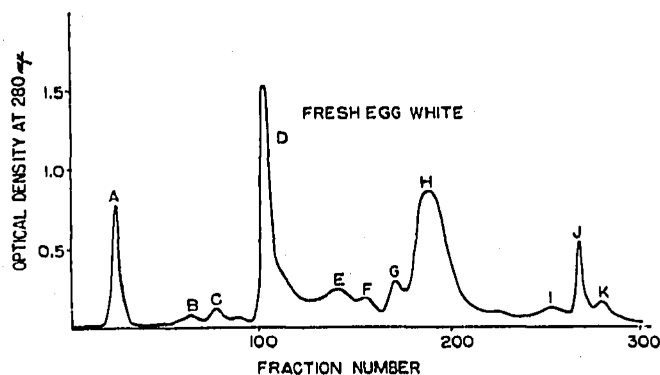

Fig. 1. Chromatography of egg white. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 M glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of K_2HPO_4, and 0.02 mole of KH_2PO_4. Reservoir changed at fraction No. 100 to 1 l solution containing 0.02 mole of glycine, 0.1 mole of KH_2PO_4, 0.1 mole of NaCl, and 0.03 mole of HCl. Volume of each fraction: 10 ml.

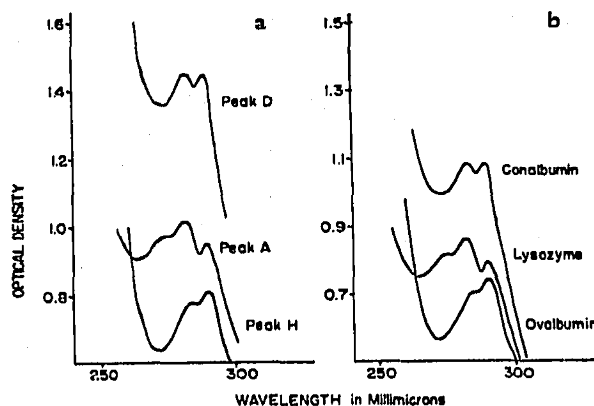

Fig. 2. Ultraviolet spectra of egg white proteins. Spectra of proteins determined in Cary Recording Spectrophotometer. Protein solutions adjusted to pH 13 with 1 M NaOH. Proteins in Fig. 2b were prepared in classical manner and recrystallized several times. No purification of proteins in Fig. 2a was attempted.

ultraviolet absorption spectra of peaks B and C were also similar to that of lysozyme. Whether these components represent a molecular species separate from the main lysozyme or the result of lysozyme interacting with other proteins or the cellulose is not known at present. Some preliminary evidence indicates that peaks B and C have a 20% higher specific activity than ordinary lysozyme, and in addition are more easily lost during dialysis. Similarly, a comparison of the ultraviolet absorption spectra of peaks G and H shows them to be indistinguishable. It can be seen from the pattern in Fig. 1 that separation of individual peaks is not complete. We have found that by judicious selection of the eluting buffers, complete separations can be effected, for example: elution with 0.02 M glycine will separate peak A from B and C completely; 0.01 M phosphate pH 6.8 will separate peaks B and C from D; and 0.02 M phosphate pH 6.8 will separate peak D from peak E, and so on. Considerably longer periods of time are required to achieve complete resolution in this manner, however. Recovery of protein absorbed on the column was determined by submitting material from each of a number of peaks obtained previously by chromatography to an additional chromatographic separation and comparing the optical densities at 283 mμ and pH 6 before and after rechromatography. Results are shown in Table I. It was found that each of the components tested emerged as a single peak at the same position as the original material, and that only lysozyme and ovomucoid were not

TABLE I

PERCENT RECOVERY OF PROTEINS

Peak	O.D./ml × total volume		% Recovery
	before rechromatography	after rechromatography	
A* (lysozyme)	5.86	4.45	76
D (conalbumin)	13.24	13.20	99
E (ovomucoid)	8.2	6.9	85
F**	14.9	14.8	99
G (ovalbumin-2)	2.43	2.36	97
H (ovalbumin-1)	8.60	8.15	95

Optical density of each sample was measured at 283 mμ, pH 6. Samples were dialyzed before and after chromatography.

* Dialysis omitted.

** Optical density determined at 265 mμ, pH 6.5.

recovered quantitatively. We were unable to detect the presence of possible breakdown products of lysozyme anywhere else in the chromatographic pattern.

Reproducibility

The patterns obtained by gradient elution were reproducible to within 5 % provided that the columns used in the comparison were made at the same time from the same batch of washed DEAE-cellulose, and that the material being chromatographed was the same for each column. Variations in the position of peaks under these conditions could be attributed to differences in flow rate, buffer concentration, and temperature. These differences could be minimized, however, by operating comparative columns simultaneously. We have found that patterns obtained with egg white on columns operated in this manner are practically indistinguishable from each other.

Separation of proteins in chicken breast muscle extract

When 30 ml of chicken muscle extract, containing approximately 10 mg protein/ml were chromatographed on DEAE-cellulose, the pattern shown in Fig. 3 was obtained. The optical densities of only the first 100 fractions are shown in this figure because no additional peaks were eluted beyond this point when the analysis was continued in the same manner as described above for egg white. However, when the pH of the eluting fluid was lowered to 2, fractions consisting mainly of nucleic acid appeared. These fractions were not characterized any further.

Assays for phosphorylase, phosphoglucose isomerase, aldolase, and adenylate kinase were performed on one tube from each of the seven peaks lettered in Fig. 3. Although each of the above enzyme activities was present in the original extract, only adenylate kinase activity could be found after chromatography. This enzyme appeared to be localized wholly within peak F of Fig. 3. In addition, peak A was identified by spectrophotometric examination as cytochrome *c*.

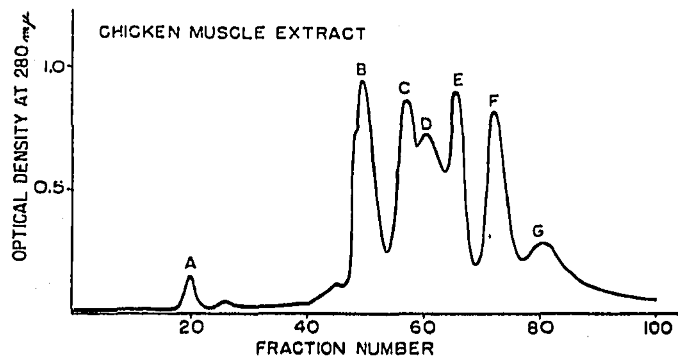

Fig. 3. Chromatography of chicken breast muscle extract. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml of 0.02 M glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of K_2HPO_4 and 0.02 mole of KH_2PO_4. Volume of each fraction: 10 ml.

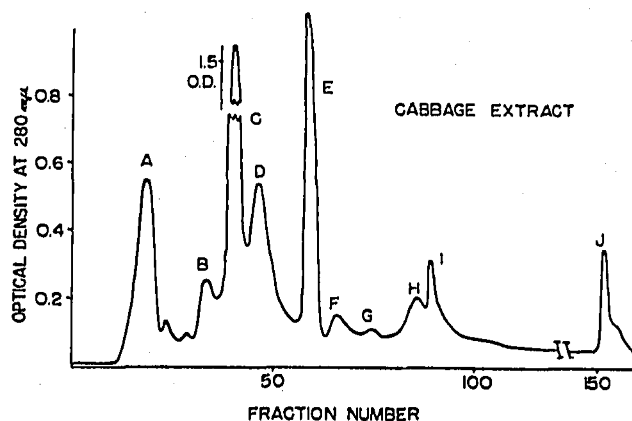

Fig. 4. Chromatography of cabbage extract. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 M glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.02 mole of K_2HPO_4, and 0.02 mole of KH_2PO_4. Reservoir changed at fraction No. 100 to 1 l solution containing 0.02 mole of glycine, 0.1 mole of KH_2PO_4 and 0.1 mole of NaCl. Volume of each fraction: 10 ml.

Chromatography of cabbage extract

Forty ml of undialyzed cabbage extract, containing approximately 10 mg protein/ml were chromatographed and the pattern obtained is shown in Fig. 4. One tube from each of the nine peaks lettered in Fig. 4 was assayed for apyrase, inorganic pyrophosphatase and acid phosphatase activities. Only peak E showed any significant activity, and it contained all three activities. In addition, peak E was characterized by an intense yellow color which, upon spectrophotometric examination at various pH's revealed a spectrum reminiscent of pyridoxal phosphate. Experiments are now in progress to characterize this component.

Chromatography of two strains of E. coli

In some early trials, an attempt was made to isolate L-glutamic acid decarboxylase from extracts of *E. coli* ATC 11246[21] and L-lysine decarboxylase from extracts of *Bacterium cadaveris*[22] by chromatography on DEAE-cellulose. The results of these attempts were largely negative. It was noted, however, that the chromatography patterns of these two organisms were strikingly different. An attempt was made, therefore, to compare the chromatographic patterns of different strains of the same organism that grow at approximately the same rate in a complex medium. Accordingly, cultures of *E. coli* ATC 11246 and *E. coli* 6-204-55 were grown and extracts prepared as described above. The samples used in the chromatography contained 100 mg of protein each, and were chromatographed on columns 1 cm diameter and 30 cm long. The volumes of buffer used in the elution schedule were altered to compensate for these smaller capacity columns. It may be seen that the pattern obtained from *E. coli* ATC 11246 in Fig. 5a is similar to the pattern obtained from *E. coli* 6-204-55 in Fig. 5b in only a few respects, namely the fractions obtained near fraction

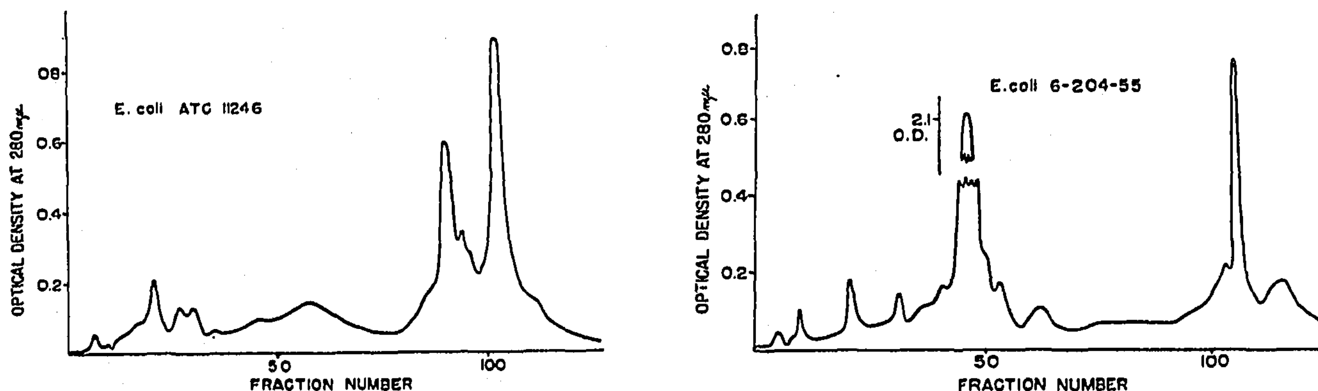

Fig. 5. Chromatography of *E. coli*. Column size: 1.0 cm diameter, 30 cm long. Mixing volume: 400 ml 0.02 *M* glycine. Reservoir: 250 ml solution containing 0.005 mole of glycine, 0.005 mole of K_2HPO_4, and 0.005 mole of KH_2PO_4. Reservoir changed at fraction No. 50 to 250 ml solution containing 0.005 mole of glycine, 0.025 mole of KH_2PO_4 and 0.025 mole of NaCl. At fraction No. 100, reservoir changed to 250 ml solution containing 0.005 mole of glycine, 0.025 mole of KH_2PO_4, 0.025 mole of NaCl, and 0.01 mole of HCl. Volume of each fraction: 5 ml.

6, 20 and 105. Other areas of similarity may exist but they are obscured by the components that are obviously different chromatographically.

Chromatography of the livetin fraction of egg yolk

After some preliminary trials with whole egg yolk, it became clear that an initial purification was required in order to remove the bulk of the lipid. Accordingly, a separation was made of whole egg yolk into three protein fractions by the method of MARTIN *et al.*[23]. The water-soluble or livetin fraction was chromatographed on DEAE-cellulose, and the pattern shown in Fig. 6 was obtained. A more acidic gradient elution schedule was used for the chromatography of the livetin fraction so that the major portion of the proteins could be eluted in a convenient time period. It can be seen that, as in the case with egg white, the number of components that can be separated by DEAE-cellulose chromatography is greater than the number that can be

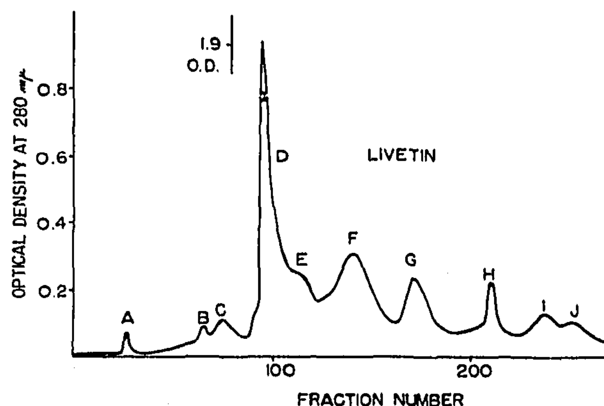

Fig. 6. Chromatography of livetin. Column size: 2.5 cm diameter, 40 cm long. Mixing volume: 750 ml 0.02 *M* glycine. Reservoir: 1 l solution containing 0.02 mole of glycine, 0.05 mole of KH_2PO_4, 0.05 mole of NaCl. Reservoir changed at fraction No. 100 to 1 l solution of 0.02 mole of glycine, 0.1 mole of KH_2PO_4, 0.1 mole of NaCl. At fraction No. 200, reservoir changed to 1 l solution containing 0.02 mole of glycine, 0.1 mole of KH_2PO_4, 0.1 mole of NaCl, and 0.03 mole of HCl. Volume of each fraction: 10 ml.

visualized by electrophoresis[24, 25]. Work is now in progress on the characterization of these proteins.

DISCUSSION

The early electrophoretic studies of LONGSWORTH *et al.*[24] indicated that egg white contained seven protein components. We have found that separation of egg white by DEAE-cellulose chromatography yielded at least 12 components that were easily visualized in the chromatographic patterns. Similarly, SHEPARD AND HOTTLE[25] and MARTIN, VANDEGAER AND COOK[23] found four components in the water-soluble or livetin fraction of yolk by electrophoretic analysis. In our preliminary trials with this material, at least 9 distinct components were found by DEAE-cellylose chromatography. Because of its high resolving power, it seems clear that DEAE-cellulose chromatography is a useful analytical adjunct.

With respect to the effectiveness of DEAE-cellulose chromatography in the separation of enzymes from crude mixtures, our attempts were partially successful in that only one of the 4 enzymes assayed for in muscle extract was found after chromatography, and the assays for L-glutamic acid decarboxylase in extracts of *E. coli* were negative in all the chromatographic fractions tested. It must be pointed out, however, that only the maximum tube from each peak was tested for enzyme activity in these assays. It is quite possible that an enzyme that represents a small fraction of the total protein in a crude biological mixture may be eluted at a point which does not correspond to a peak on the chromatographic patterns. An enzyme eluted in this manner would have been overlooked, therefore, in the selection of samples for assay. It would appear that this difficulty might be overcome by assaying all of the fractions collected.

The observation that two strains of *E. coli*, when chromatographed, yielded different patterns suggests a taxonomic use for DEAE-cellulose chromatography. One might even speculate on its usefulness in genetic investigations where a mutation may be reflected in a change of chromatographic pattern. However, if a mutation resulted in a change in a quantitatively minor component in the cellular constituents, alterations in the chromatographic patterns would not be readily observed.

SUMMARY

1. A procedure is described for the use of DEAE-cellulose chromatography in the separation and analysis of proteins in complex biological mixtures.

2. Examples are given of the chromatographic analysis of egg white, livetin fraction of egg yolk, chicken breast muscle extract, cabbage extract, and extracts of two different strains of *E. coli*.

3. It was found that, in the case of egg white and livetin fraction, patterns obtained by DEAE-cellulose chromatography show more detail than the patterns obtained by electrophoretic examination.

4. Some limitations of the procedure are discussed.

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