Lysozyme is a basic protein of low molecular weight (1) which may be prepared readily in crystalline form as the isoelectric protein, the chloride, or the carbonate by the methods of Fevold and Alderton (2). According to the electrophoretic analyses and the sedimentation and diffusion data reported by Alderton, Ward, and Fevold (3), the material isolated by adsorption on bentonite appears to be homogeneous. Electrophoretic homogeneity of the directly crystallized enzyme is indicated by the work of Smith and Stocker (4) and of Wetter and Deutsch (5). Small amounts of a second component have been noticed in some cases, however, by Alderton et al. (3), Thompson (6), and by Anderson and Alberty (7), although the nature of the contaminant could not be ascertained. Furthermore, lysozyme is not homogeneous by the reversible spreading and steady state boundary criteria (5, 7) and inhomogeneity has also been detected by immunological methods (5).

The fact that lysozyme may be chromatographed successfully on a column of the carboxylic acid ion exchange resin IRC-50 (XE-64) has been reported briefly in recent communications (8, 9). The possibility of evaluating the homogeneity of lysozyme by an additional criterion thus became evident. The differences in chromatographic behavior exhibited by various preparations of lysozyme furnish the basis for the present report.

**Methods**

*Isolation of Lysozyme*—The procedures of Fevold and Alderton (2) were followed. In the present paper, "isoelectric lysozyme" refers to material prepared by direct crystallization from egg white (procedure B (2)) and recrystallized once isoelectrically (pH 9.5 to 10 in the presence of 5 per cent NaCl); "lysozyme carbonate" refers to material also isolated directly but recrystallized in the presence of 5 per cent NaHCO₃; "lysozyme chloride" refers to material isolated by adsorption on bentonite (procedure A (2)) and recrystallized at pH 4.5 to 5.0 in the presence of 5 per cent NaCl. In every case, after filtration and washing, residual water was removed.

* A preliminary report of this work was given at the meeting of the Federation of American Societies for Experimental Biology, New York, April 14–18, 1952 (Federation Proc., 11, 297 (1952)).
Chromatography of Lysozyme

from the moist filter cake by lyophilization. The preparations were stored at room temperature.

Chromatographic Separations on Analytical Scale—The 0.9 X 30 cm. columns were prepared and operated in the manner described by Hirs et al. (10), except that the eluent was a sodium phosphate buffer of pH 7.18 (0.2 M in respect to phosphate). About 5 mg. of lysozyme in 0.5 ml. of buffer were chromatographed in most cases. Insoluble material, if present, was removed by centrifugation before the sample was added to the column. The effluent was collected in 0.5 ml. fractions, each of which required 0.05 ml. (1 drop) of N HCl to bring it to pH 5 before ninhydrin analysis.

Measurement of Lysozyme Activity—Enzymatic activity was determined in the effluent fractions by a modification of the method of Smolelis and Hartsell (11), with Micrococcus lysodeikticus. A suspension of the dried bacteria was prepared in 0.066 M phosphate buffer (Sorensen) of pH 6.24 such that, on a Coleman junior spectrophotometer, the percentage transmittance of a portion of the suspension diluted with an equal volume of buffer had an arbitrarily chosen value of 25.0. Readings were taken at a wave-length of 570 m\(\mu\). An aliquot of the effluent fractions containing 5 to 10 \(\gamma\) of enzyme (usually less than 0.05 ml.) was diluted to 3 ml. with the buffer of pH 6.24, followed by the addition of 3 ml. of the bacterial suspension. The mixture was shaken and kept, with frequent shaking, at 25° for 40 minutes. The percentage transmittance was again read, and the amount of enzyme present was determined by comparison with a standard curve relating transmittance to enzyme concentration. This curve was obtained by use of a sample of lysozyme purified chromatographically in the manner described in the next section (see Fig. 4). The activity curve is S-shaped, and only that portion up to 10 \(\gamma\) of enzyme was used. A unit of enzyme activity, as employed in this paper, is equivalent to 1 \(\gamma\) of the purified preparation.

Chromatography on Preparative Scale—The apparatus employed was of the type described by Hirs et al. (12), appropriately scaled down for use with a 4 X 30 cm. column of resin. As much as 350 mg. of lysozyme dissolved in 20 ml. of buffer was chromatographed in a single run. Any undissolved material was removed by centrifugation prior to the addition of the sample to the column. The effluent fractions were 10 ml. in volume and were collected at a rate of three fractions an hour, maintained by a pressure of 5 cm. of mercury. Photometric ninhydrin analyses and determinations of enzymatic activity were performed on aliquots removed from

1 When a synthetic mixture of amino acids (10) is chromatographed under the conditions employed in these experiments, the acidic and neutral amino acids move rapidly as a group and appear on the effluent curve between 10 ml. and 20 ml., followed by lysine and histidine at about 65 ml.

2 It is a pleasure to acknowledge the generous assistance of Dr. M. McCarty and Dr. C. A. Stetson, Jr., who supplied the microorganisms.
every third tube. For the recovery of the purified enzyme, appropriate fractions were combined, freed of buffer salts, and lyophilized.

Removal of Salts by Dialysis—Lysozyme passes through Visking cellophane casing, enzymatic activity being readily detectable in the dialysate; as much as 90 per cent of the enzyme was lost in one experiment. Dialysis in collodion sacs (13) against running distilled water in a rocking dialyzer at 4° was therefore employed. After 20 hours dialysis, the ash content of the lyophilized preparations generally exceeded 30 per cent; the average recovery of the enzyme was 83 per cent. A second dialysis through a new collodion sac was necessary to reduce the ash content to less than 1 per cent; the loss in this step averaged 10 per cent.

Removal of Salts by Ion Exchange—Studies were made of possible methods for the removal of the buffer salt by means of a column of the mixed ion exchange resins Amberlite IR-120 and IRA-400 (analytical grade, 16 to 50 mesh). The resins were separately washed with distilled water, and the exchange capacity of the air-dried materials was determined to be 5.0 m.eq. per gm. for IR-120 and 1.56 m.eq. per gm. for IRA-400. Allowing a 25 per cent excess, the resins were mixed in proportions calculated to deionize a given volume of the phosphate buffer. When 290 ml. of a lysozyme solution (combined effluent fractions of a preparative chromatogram) were passed through a 4 X 33 cm. column consisting of 139 gm. of IRA-400 and 26 gm. of IR-120, at a rate of 60 ml. per hour, the recovery of activity was only 75 per cent and the ash content was 13.6 per cent. Moreover, chromatographic analysis of the material thus obtained revealed that the desalting process had produced alterations in the enzyme. The peak was broader than usual and the preparation contained 18 per cent of faster running material. When the desalting was carried out as a batch process, the lysozyme solution and the mixed resins being stirred for 25 minutes at a moderate speed and then filtered through a 0.9 X 15 cm. column of mixed resin, the yield was again 75 per cent; the ash content was 0.66 per cent and 7 per cent of faster running material was noted. When a lysozyme solution was first dialyzed in collodion sacs (yield 84 per cent, ash content 37 per cent) and then passed through a 0.9 X 15 cm. column of mixed resins, the ash was completely removed and the yield was 87 per cent, but the chromatographic pattern was extensively altered. Thus, in contrast to the findings with ribonuclease (10), it does not appear possible to remove salts from solutions of lysozyme by mixed bed deionization without alteration of some of the enzyme.

Electrophoretic Analyses—The electrophoretic measurements were made at 0.5° in the apparatus described by Longsworth (14) with single section cells of either 11.0 or 2.5 ml. capacity.  

3 It is a pleasure to acknowledge the assistance of Dr. L. G. Longsworth and Dr. G. E. Perlmann in the performance of the electrophoretic analyses.
Results

Homogeneity of Lysozyme Carbonate—The chromatographic behavior of different samples of lysozyme appears to depend upon the exact method of preparation and the previous history of the material. For example, a sample of lysozyme carbonate, freshly prepared in the manner indicated in the experimental section, gave on chromatography the results shown by the
solid line in Fig. 1. About 93 per cent of the material appears as a peak (A), the position of which corresponds to that observed for lysozyme when whole egg white was chromatographed (9). A small amount of a faster moving (Peak B) material is also present. When the fresh preparation was allowed to stand in the lyophilized state at room temperature, a progressive transformation occurred, increased quantities of Peak B and Peak C material being formed at the expense of Peak A (see also Tallan and Stein (9)).

![Fig. 3. Electrophoretic patterns of lysozyme preparations. Electrophoresis was carried out with 1 per cent protein solutions in a diethyl barbiturate buffer of pH 7.82 and ionic strength 0.1 (0.02 N diethyl barbituric acid, 0.02 N sodium diethyl barbiturate, 0.08 N NaCl). a, isoelectric lysozyme after 20,000 seconds at a potential gradient of 4.7 volts per cm.; mobility (descending) $4.64 \times 10^{-4}$. b, lysozyme carbonate (stored at room temperature for 300 days) after 10,000 seconds at a potential gradient of 9.2 volts per cm.; mobilities (descending) $2.43 \times 10^{-5}$, $3.24 \times 10^{-5}$, and $4.17 \times 10^{-5}$]

The rate of this transformation is shown in Fig. 2. At the end of 258 days storage, lysozyme carbonate gave the chromatographic pattern shown by the dash line in Fig. 1. Some insoluble material was present. At this time, the composition of the soluble portion of the preparation was about 41 per cent of Peak A, 41 per cent of Peak B, and 18 per cent of Peak C. It is of interest that the ninhydrin and activity curves are parallel to one another throughout. The inhomogeneity of the stored preparation was also demonstrated by electrophoretic analysis, the results of which are given in Fig. 3, b. For comparison, Fig. 3, a shows the electrophoretic pattern of a sample of isoelectric lysozyme found chromatographically to contain 93 per cent of Peak A material.
Taken together, the chromatographic and electrophoretic data suggest that more acidic, enzymatically active molecular species are formed during storage of lysozyme carbonate at room temperature. Loss of ammonia, as a result of the hydrolysis of amide linkages, would, of course, produce more acidic molecules, but it has not yet been possible to obtain unequivocal evidence in support of this mechanism for the transformation.

An attempt was made to reproduce rapidly in solution the effects which occurred slowly in the solid state. For this purpose, a 1 per cent solution of freshly prepared lysozyme carbonate was brought to pH 9 to 9.5 with NaOH and kept at 25°. At the end of 9 days, the composition of the material remaining in solution was 67 per cent Peak A, 25 per cent Peak B, and 8 per cent Peak C, indicating that some transformation to more acidic species had occurred. The major part of the lysozyme had been rendered insoluble, however, and could not be chromatographed, a reaction that did not occur to nearly the same extent in the solid state. The amount of precipitate formed during the first 7 days could be minimized if the solution was deaerated and saturated with nitrogen at the beginning of the experiment, though almost complete precipitation still occurred after about 10 days. These changes could also be observed with a solution of isoelectric lysozyme. It would appear, therefore, that it was not possible to duplicate by storage in alkaline solution the transformations undergone by lysozyme carbonate in the solid state.

Isoelectric Lysozyme—Isoelectric lysozyme, prepared by “direct crystallization,” would appear to be the most homogeneous chromatographically of any of the preparations examined. The several samples analyzed were usually found to contain 95 per cent or more of the Peak A enzyme. In most cases, Peak B occurred in amounts less than 5 per cent, as determined by ninhydrin analysis, though as much as 12 per cent of inactive, fast moving, ninhydrin-positive material was encountered in some instances. In general, preparations that have been recrystallized several times contain more Peak B. For example, a sample furnished by Dr. W. A. Schroeder, that had been crystallized twice isoelectrically, once as the carbonate, once as the chloride, and again twice isoelectrically, showed 78 per cent of Peak A, 16 per cent of Peak B, and 6 per cent of Peak C. A preparation that, on chromatographic analysis, contained 93 per cent Peak A gave homogeneous electrophoretic patterns at pH 4.6 and 7.8 (Fig. 3, a), but showed a small rear shoulder at pH 9.2. In contrast to lysozyme carbonate, isoelectric lysozyme is stable on storage at room temperature.

Lysozyme Chloride—The chromatographic behavior of lysozyme isolated as the chloride after adsorption on bentonite is slightly different from either of the two types of preparation already discussed. In this case, each step in the isolation procedure was followed chromatographically.
The picture obtained with whole egg white has already been given (9). After elution from the bentonite with 5 per cent aqueous pyridine adjusted to pH 5, followed by dialysis, pervaporation, and lyophilization, the crude preparation was grossly contaminated with an inactive fast moving component. Peak A, which amounted to about 40 per cent of the ninhydrin-positive material present, exhibited a large shoulder. Peak B was present to the extent of about 2 per cent. A single recrystallization as the chloride removed most of the fast moving material, leaving a preparation which consisted of 93.5 per cent of Peak A (including the shoulder, all of which was not active) and 3.4 per cent of Peak B. There still remained 3.1 per cent of an inactive contaminant not seen in the other preparations of lysozyme. Further recrystallization did not alter the chromatographic picture significantly. Electrophoretic analysis of lysozyme chloride gave results identical with those obtained with the isoelectric preparation. Like isoelectric lysozyme, the chloride is also stable on storage at room temperature.

**Chromatographically Purified Lysozyme**—In view of the chromatographic inhomogeneity of all the lysozyme preparations examined, the enzyme was further purified by chromatography on a preparative scale. For this purpose, 280 mg. of the isoelectric protein, representing 335,000 units of activity, were chromatographed on a 4 X 30 cm. column, yielding the results shown in Fig. 4. The fractions within the bracket were combined and freed of buffer salts by dialysis through collodion, followed by a second
dialysis through new sacs. After lyophilization, 114 mg. of enzyme were obtained which contained 0.35 per cent ash and 18.7 per cent N (moisture-and ash-free) and which had a ninhydrin color value of 0.356 μM of leucine equivalents per mg., corresponding to about 5 per cent as much color per mg. as is given by leucine. Upon chromatography on an analytical column, 0.9 × 30 cm., the dotted curve shown in Fig. 4 resulted, indicating that the preparation was homogeneous chromatographically.

SUMMARY

Lysozyme has been chromatographed by elution analysis on columns of IRC-50 (XE-64) with 0.2 M sodium phosphate buffer of pH 7.18 as the eluent. The chromatographic patterns of lysozyme chloride, lysozyme carbonate, and the isoelectric protein are described, and it is shown that three chromatographically distinct, active components may be found. The most homogeneous preparation is the isoelectric precipitate recrystallized once. Recrystallization as the carbonate should be avoided. From preparations containing more than one component, chromatography on a larger scale has permitted the isolation of a chromatographically homogeneous lysozyme.

BIBLIOGRAPHY


4 It may be calculated that lysozyme contains seven amino groups per mole, arising from 6 residues of lysine (cf. (1, 15)), of which 1 residue is on the amino end of the chain (16). The intensity of color observed in the ninhydrin reaction would arise from five amino groups per mole, provided each reacted with ninhydrin to the same extent as does the amino group of leucine. It would seem that the amino groups in the protein are on the average less reactive and have an average color yield of about 0.7 compared to leucine as 1.0.
CHROMATOGRAPHIC STUDIES ON LYSOZYME
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