Chromatographic Studies of Rat Kidney Lysozyme.* (24060)

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Experiments in this laboratory indicated that lysozyme activity of some tissues, particularly kidney of rat, may be altered under conditions of hypophysectomy and hormone administration(1). The large inhibition of enzyme activity after administration of thyroid hormone involved a change of state of the enzyme(2). Because of the stability of egg white lysozyme (EWL), it appeared that the kidney enzyme might be a convenient system for biosynthetic studies. It was, therefore, necessary to investigate a procedure for purification of the enzyme. Ion-exchange chromatography of the enzyme from egg white has been reported(3,4). Isolations of lytic enzymes from rabbit spleen and spleen and kidney of dog by chromatographic technics have been carried out by Jollés and Fromageot(5,6). Another lysozyme from papaya latex has recently been isolated as the mercury derivative(7). This paper reports chromatographic studies of rat kidney lytic enzyme (KLE) by use of ion-exchange methods. A partial preliminary report of this work has appeared(8).

Methods. Lytic activity in crude extracts or eluate fractions was determined photometrically as previously described(9). *Micrococcus lysodeikticus,* cultured and prepared as before(10), was used as the test organism. Enzyme activity is expressed as μg equivalent to crystalline EWL (Armour) or in terms of specific activity where activity is based on Folin nitrogen(1). Protein content was estimated by use of the Folin-Ciocalteu reagent (11) or extinction at 280 mμ. Fresh rat kidneys were obtained from animals of Rutgers stock colony (male albino rats of Wistar strain weighing 200 to 400 g). These tissues were immediately frozen until ready for use. When extracts were to be made, the kidneys were allowed to thaw at 5°C for 30 minutes and teased free of all surrounding tissue. *Preparation of resin.* Two volumes of water were added to the unused resin and the suspension immersed in boiling water bath 5 minutes. The resin was centrifuged and washed with water until the supernatant fluid remained clear. The resin was washed successively with N HCl, water and N NaOH. NaOH was decanted and a slurry was made by adding water. To increase rate of eluant flow through Amberlite IRC-50 (XE-64), the larger particles were separated by sedimentation. The slurry was poured into 1000 ml graduated cylinder and stirred vigorously. After particles settled for 7 min., the supernatant was removed by siphon. This process was repeated several times. The larger particles were then recycled with acid, water and base and buffered to pH 6.4 with 0.2 M phosphate buffer. A column was packed by gravitation and the bed measured 1.2 x 57 cm in all experiments. The same resin was used for all work reported here. Ten ml fractions were collected using an automatic fraction collector. Concentrations of cytochrome c have been expressed as μg equivalents to standard solutions of oxidized beef heart cytochrome c(12). (Wyeth and Co.) Absorption of reference cytochrome c at 415 mμ was linear with concentrations from 0 to 0.2 mg/ml. This wavelength was selected to increase the sensitivity of the assay. Closed-strip paper electrophoresis (200 volts; field strength about 4 v/cm; 8 to 15 hrs) was carried out with Whatman No. 3 paper strips (3 x 52 cm) with 0.2 M phosphate buffer at pH 6.4 as conductor, using CCl_4 as insulator in a 20°C chamber. The apparatus was similar in principle to that described by Markham and Smith(13). Densitometry was carried out according to Block, *et al.*(14) using a Photovolt Corporation densitometer. *pH gradient elution* was carried

* Supported in part by res. grant (E-1119C) from the Nat. Inst. of Allergy, N.I.H., U.S.P.H.S. Paper of Journal Series, N. J. Ag. Exp. Sta. Abbreviations used in this paper are: EWL, egg white lysozyme (Armour); KLE, rat kidney lysozyme, and EEWL, equivalents of egg white lysozyme (Armour).

out using an apparatus which was essentially a modification of a mixing system described by Bock and Ling(15). This apparatus consisted of two rectangular chambers, a reservoir and a mixing chamber. A constant volume was maintained in the mixing chamber throughout the elution. The column with the buffered resin was first connected to the mixing chamber (closed system). The reservoir contained 500 ml 0.2 M NaH_2PO_4 at pH 4.5 while the mixing chamber held 500 ml 0.2 M Na_2HPO_4 at pH 9.3. The elution was carried out so that the drop rate from the column was 4 drops per minute and the volume in the mixing chamber was maintained at 500 ml. The enclosed system was under air pressure which was kept constant by a capillary device (T tube) immersed in a column of mercury. Direct elution was carried out with an identical column by standard procedures. The dropping rate was maintained at 4 drops per minute for which no air pressure was required.

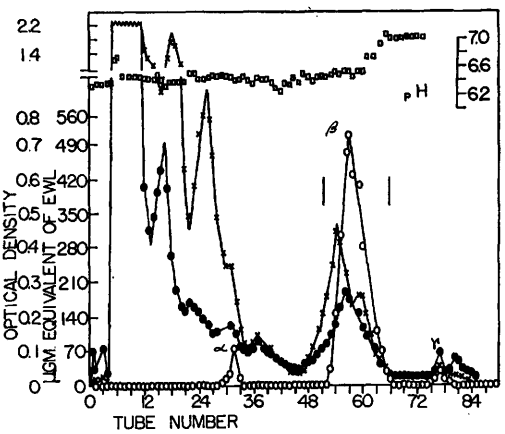

FIG. 1. Gradient elution chromatography of an extract of rat kidneys partially purified by heat coagulation and centrifugation, followed by dialysis and volume reduction. Open circles represent lytic activity expressed as equivalents of EWL. Solid circles represent optical density at 280 mμ (protein). Crosses represent optical density at 415 mμ (cytochrome pigments), and the open squares represent pH of tube contents. Each tube contained 10 ml eluate. Column load = 3.0 mg EEWL.

Results. Effects of pH upon KLE activity. Initial fractionations were made from chilled, 1:6 aqueous homogenates of thawed kidney tissue. After adjusting the pH of aliquots of the homogenate to different values, the fractions were centrifuged at 20,000 x g for 20 min. at 0°C. The clear supernatants were readjusted to pH 5.0, which appeared to be the pH of maximum stability of the enzyme, and assayed for activity(9) and Folin-Ciocalteu reaction. The pH values of 4-4.5 yield the greater specific activities. When a homogenate was heat-treated (90 seconds at 100°C) prior to centrifugation, the pH range of maximum stability was found to be 4.5-5.. Aqueous homogenates, adjusted to pH 4.5 ± 0.2 could be immersed in a boiling water bath for 90 seconds with negligible inactivation of the enzyme. This pH range was also effective for supernatant preparations. The pH optimum for supernatant enzyme stability was 5.0-5.5 during heating at 38°C for 90 minutes. The optimal buffer pH for the assay system after lysis was 6.2 which is similar to results previously reported for EWL(16). The starting preparation for chromatography experiments was subsequently obtained by heating a 1:6 aqueous homogenate at pH 4.5 for 90 seconds at 100°C, centrifuging and retaining the supernatant and one washing with an equal volume of H_2O. The combined supernatant and washings were dialyzed for 24 hours at 5°C against distilled water. *Volume reduction.* After removal from the dialysis tubing, the dialysate was adjusted to pH 5.0 and the volume was reduced by blowing air on a dialysis sac containing the enzyme at 5°C. The volume was reduced to 5-8 ml in this manner. Some loss of KLE activity inside the sac was experienced due to leakage of KLE through the membrane. A clear, pink solution was obtained which was then assayed and applied to the buffered column. *Gradient elution analysis* of 3 mg equivalents of EWL is shown in Fig. 1. Three peaks of enzyme activity have been resolved by this procedure. Absorption at 280 mμ and 415 mμ show peaks of inert proteins. A concentrated inert protein fraction was rapidly eluted. This gave rise to a large purification. The first peak contained a yellow-brown chromoprotein, probably similar to that seen by Pâleus and Neilands(17) with beef heart. The inert fractions also reacted strongly with the Folin reagent and absorbed strongly at 415 mμ. The 2 peaks of pigment material (415 mμ) in the region of the α and the first part of the β peaks of enzyme activity proved to be reduced

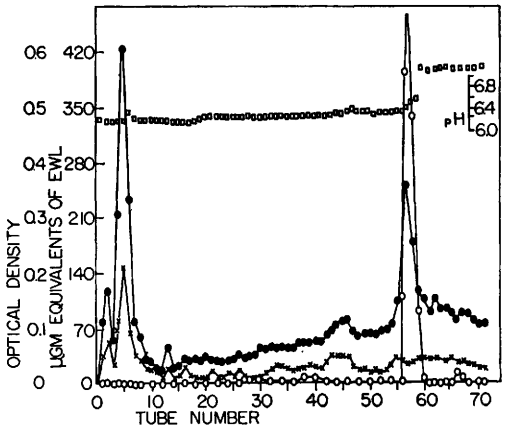

FIG. 2. Gradient elution rechromatography of the beta KLE peak of enzyme activity (tubes 52-66, shown in Fig. 1, which were combined, dialyzed and reduced in volume. Symbols are identical to those in Fig. 1. Column load = 500 μg EEWL. Each tube contained 10 ml eluant.

and oxidized forms of cytochrome c similar to the observations of Pâleus and Neilands(17) with beef heart cytochrome c. The eluant pH was alkaline during elution of the inert protein fraction, however, pH 6.4 ± 0.3 was maintained during elution of the enzyme activity up to tubes 58-60 where a rise in pH occurred. Fig. 2 shows a gradient elution rechromatography of the β peak of KLE activity. The contents of tubes 52-66 were combined, dialyzed and reduced in volume before rechromatography on freshly regenerated resin. By rechromatographing the major peak of enzyme activity, further purification of the enzyme is obtained signified by elution of inert protein in tubes 1-10. The major peak of enzyme activity is relatively free from cytochrome c and an overall purification of enzyme specific activity of about 100-fold has been achieved. Closed-strip paper electrophoresis of combined cytochromes and 3 peaks of enzyme activity failed to separate these components (mobility = 4 x 10^{-5} cm^2 v^{-1} sec^{-2}). Densitometry was carried out before color development to assay the pigments and after bromphenol blue development for total protein. Frequent tailing of basic proteins has been avoided using the closed-strip method.

Fig. 3 shows results of a direct elution of KLE extract using 0.2 M phosphate buffer at pH 6.4. Three peaks of KLE activity were also resolved by this procedure (Fig. 3A). The β peak represents about 30 times the activity of the α or γ peaks. In general, 75 to 85% of total original activity was recovered. Fig. 3B shows the Folin reaction. It was evident, since the Folin reaction produced greater equivalents than activity measurements, that rechromatography might result in further purification as was found in the gradient elution procedure (Fig. 2). In Fig. 3C, 4 pigments have been partially resolved compared to 2 pigment peaks from gradient elution analysis. This demonstrates a greater resolving power of direct elution analysis. These are also indicated by the Folin reaction in 3B. Each peak was in the oxidized form. The visible absorption spectrum for each peak has been measured (reduction of pigments by sodium hydrosulfite); maxima = Cl oxidized, 410, and 530 mμ; Cl reduced, 415, 520 and 550 mμ; C2 oxidized, 410 and 530 mμ; C2 re-

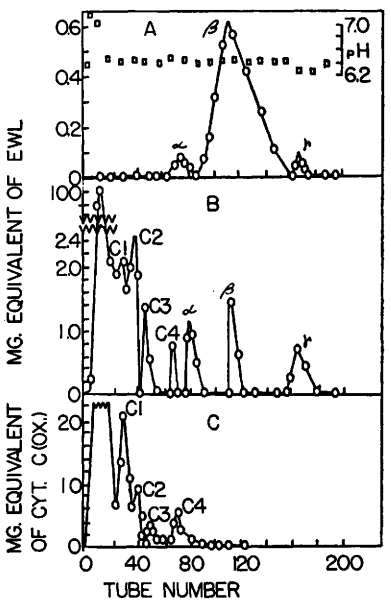

FIG. 3. Direct elution of supernatant, heat-treated, crude KLE. Column load = 13 mg EEWL. A. KLE activity (open circles) and eluant pH (open squares). B. Folin reaction for protein. C1, C2, C3 and C4 correspond to cytochromes; α, β and γ peaks correspond to KLE. C. Equivalents of beef heart cytochrome c measured by extinction at 415 mμ. C1, C2, C3 and C4 correspond to Folin protein peaks in B. Each tube contained 10 ml eluant. Fraction size = 10 ml. Column bed = 53 × 0.9 cm. Buffers as indicated in text.

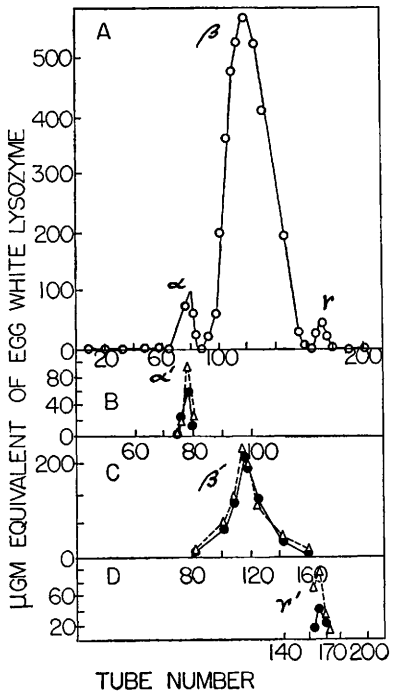

FIG. 4. Separate rechromatography by direct elution of individual KLE peaks obtained from a direct elution experiment. A. KLE activity of a direct elution experiment. Column load = 13 mg EEWL. B. Rechromatography of α KLE activity (solid circles) and protein measured by extinction at 280 mμ (open triangles). Column load = 352 μg EEWL. C. Rechromatography of β peak of KLE (symbols as in B). Column load = 7.72 mg EEWL. D. Rechromatography of γ peak of KLE (symbols as in B). Column load = 48 μg EEWL. Each tube contained 10 ml of eluant.

duced, 415, 520 and 550 mμ; C3 oxidized, 409 and 528 mμ; C3 reduced, 417, 520 and 550 mμ and C4 oxidized, 409 and 525 mμ. While comparisons have not been made on an equiprotein basis, some qualitative differences in the shapes of the curves, particularly in the ascending portion of the first peak, were seen. Previously, separation of KLE from pigments by classical fractionation or dialysis procedures was not obtained.

Separate direct elution rechromatography of each enzyme peak is shown in Fig. 4. Figs. 4B, 4C and 4D show separate rechromatography of each peak. Tube contents representing each peak were combined, dialyzed and reduced in volume prior to rechromatography. Protein was estimated by absorption at 280 mμ. The α peak appeared to contain more protein than measured by enzyme activity. This was due to a small amount of pigment which remained with this fraction. The β fraction was highly purified and probably free of pigment. The γ fraction may have contained more tyrosine or tryptophan than EWL or was still contaminated, to explain the higher values obtained from extinction measurements. Occasionally a very small fourth peak has been observed, however, the KLE concentration of such a fraction always fell at the lower limit of the enzyme assay.

Discussion. By direct or gradient elution analyses, 3 peaks of KLE activity have been resolved. Each of these peaks, found after direct elution, appears not to be an artifact since rechromatography of each peak under identical conditions produced peaks with similar chromatographic properties. The pH values of eluted fractions were very similar in gradient or direct elution experiments. During elution of the inert chromoprotein fraction, the pH rose sharply regardless of method of elution. The increase in pH seemed to be proportional to concentration of the inert fraction. After the chromoprotein fraction had been eluted, pH dropped to 6.4 and remained in the range of 6.4 $\pm$ 0.3 during elution of KLE activity until about 600 ml where the pH began to rise again.

While the presence of cytochrome c has not been reported for lysozymes isolated from other sources including the dog kidney, it has been found to occur with KLE rather consistently during several modes of fractionation. KLE and cytochrome c exist in free form and are difficult to separate because of strikingly similar properties, however, a separation may be accomplished by the methods described.

Summary. Chromatography of rat kidney lysozyme with Amberlite XE-64 ion-exchange resin by direct and gradient elution procedures have been described. Three peaks of enzyme activity have been resolved by either procedure. Separate rechromatography of each peak by direct elution substantiated that none of the peaks was an artifact. The central peak contains the major portion of enzyme activity and after rechromatography the enzyme is spectrophotometrically free of cytochrome c. Cytochrome c appeared, after direct elution, as 4 peaks

closely associated with the enzyme due to similar physical-chemical properties. Two peaks of reduced and oxidized cytochrome were found by gradient elution. Total purification of the specific enzyme activity was about 100-fold over the original supernatant.

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Effects of Gaseous Ions on Tracheal Ciliary Rate.* (24061)

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Recent experiments have shown that (+) and (−) air ions have marked effects on ciliary rate and other properties of the mammalian trachea, both *in vitro*(3) and *in vivo* (4). It seems reasonable to conclude that these effects stem from physiologically significant alterations in state of gases present in ambient atmosphere and that deliberately produced changes in composition of this atmosphere would be reflected in the response of the tracheal tissue. The following experiments were performed to test this assumption.

Methods. Anesthetized rabbits were tracheotomized and placed in humid-air ionizing chamber according to technic already described(4). Gases used were warmed to 25-28°C, bubbled through water, and blown continuously into the chamber. Since earlier work(2) had shown that presence of smog or cigarette smoke in ambient atmosphere altered the action of air ions on bacterial suspensions, cigarette smoke was employed in some of these experiments. It was produced in mechanical device operating under negative pressure, and then treated in the same way as gases. For N_2 and CO_2 experiments it was necessary to sacrifice the rabbits with an overdose of nembutal; experiments with O_2 and cigarette smoke permitted exposure of living as well as sacrificed animals. As in previous work(1-4): (a) ions were generated by β radiation from sealed tritium foils, a rectifying circuit allowing selection of (+) or (−) ions, (b) the same stroboscopic method was employed to determine rate of ciliary beat (accuracy ± 50 beats/min.), and (c) atmospheric temperature in exposure chamber varied from 25-28°C on different days, while relative humidity remained close to 90%.

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