Fig. 1 is a radioautograph of the ionogram. The free phosphate band has been allowed to run off the anode end of the paper. The exact correspondence of the radioactive bands from the three di-isopropyl phosphoryl-enzymes indicates that the hydrolysates contain identical peptides of phosphoserine which are entirely different from those derived from ovalbumin. The structure of the different peptide bands is not certain at present but is under investigation. From the number present and their identity in the three enzymes it is probably safe to conclude that elastase, like trypsin and chymotrypsin contains the sequence Gly·Asp·Ser·Gly· around its reactive serine residue.

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A simplified spectrophotometric determination of ester groups in lipids

Our laboratory's need for an extremely simple yet highly sensitive and accurate assay of lipid ester groupings in large numbers of chromatographic samples prompted the development of the method reported here. It is based on a hydroxylaminolysis in which an ester reacts with alkaline hydroxylamine to form a hydroxamic acid; the latter forms a purple iron-chelate complex in the presence of acid ferric perchlorate. This method is a modification of more laborious procedures[1-3], and its range extends to 4.00 μequiv. ester.

A standard curve was determined on samples of tristearin, tripalmitin, methyl stearate, or methyl palmitate (California Foundation for Biochemical Research). The weighed lipid was dissolved in Fisher reagent-grade chloroform. Aliquots of the standards were pipetted into test tubes (85 × 15 mm), and the solvent removed under infrared lamps. Acetone (about 0.5 ml) was routinely added to all samples at this point to insure complete removal of the chloroform. The acetone was again evaporated under infrared lamps.

Reagents: *Stock Ferric Perchlorate*[1]. 5 g ferric perchlorate (nonyellow) were dissolved in 10 ml 70 % $HClO_4$ and 10 ml water, then diluted to 100 ml with cold absolute ethanol (stored in refrigerator); *Reagent Ferric Perchlorate*. 4 ml stock ferric perchlorate and 3 ml 70 % $HClO_4$ were diluted to 100 ml with cold absolute ethanol; *Alkaline Hydroxylamine*. Equal vols. of a 4 % ethanolic hydroxylamine solution (2.0 g

dissolved in 2.5 ml H_2O, diluted to 50 ml with absolute ethanol) and of an 8% ethanolic NaOH (4 g dissolved in 2.5 ml H_2O, diluted to 50 ml with absolute ethanol) were mixed in a stoppered cylinder. The NaCl was separated by centrifugation and the supernatant was decanted for use.

Procedure: (1) 1 ml alkaline hydroxylamine reagent (fresh daily) was added to each of the dry lipid samples. (2) The samples were placed in a water bath at 65° for 2 min. (3) The samples were removed from the water bath and allowed to cool for 5 min. (4) The ferric perchlorate reagent (2.5 ml, fresh daily) was added to the tubes, mixed, and after 30 min the purple color was read in a 1-cm cuvette against a reagent blank in a Beckman B spectrophotometer at 530 mμ. The color was stable for more than 1 h.

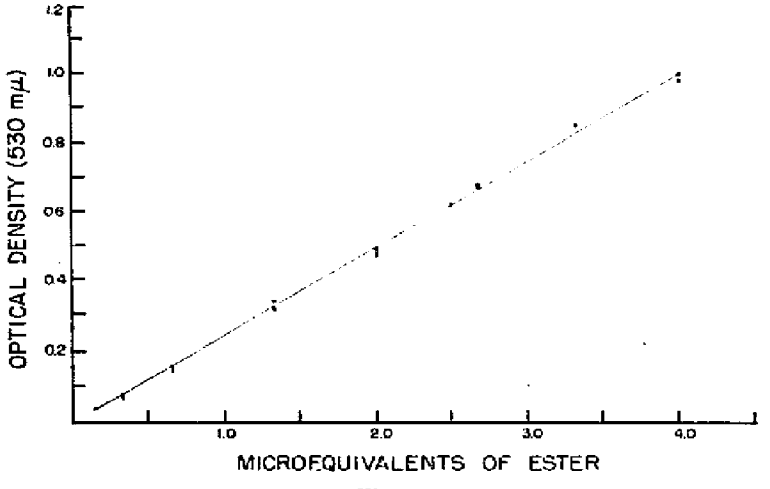

Fig. 1.

The precision and accuracy of this method can readily be seen from the curve in Fig. 1, which shows the result of duplicate determinations of ester in tristearin samples as measured on 3 different days. Ester equivalents of tripalmitin, methyl palmitate, or methyl stearate gave curves identical to that obtained with tristearin. We have also used this method successfully with lecithin samples. The actual time required to develop the purple color in 20 to 30 samples is between 15 and 20 min. The method described avoids the time consumed in treating each sample individually[2] and at the same time provides a means of determining extremely small quantities of lipid esters with excellent reproducibility.

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The purification of renin by use of ion-exchange chromatography

Recent advances in the preparation and uses of cellulose ion-exchange agents for the purification of proteins[1,2] suggested their possible use in the purification of hog-kidney renin.

The adsorbent chosen was DEAE-cellulose prepared according to PETERSON AND SOBER[3], with a capacity of 0.76 mequiv./g. Columns with dimensions of 1.2 × 55 cm and hold-up vol. of 11 ml were prepared from 4 g of the ion exchanger. Semi-pure renin was prepared from fresh hog kidneys and from desiccated hog-kidney powder by a procedure described by HAAS *et al.*[4,5]. These preparations were carried through five of the ten steps of their isolation procedure. The fractionations were performed in the cold room (6°), and the precipitate obtained from Step 5 was dissolved in water and dialyzed. After dialysis the dry weight of enzyme was obtained by drying to constant weight by heating to 105° and cooling in a desiccator over P_2O_5. Pressor activity of the preparation was measured by injection of solutions of suitable strength intravenously into a dog anesthetized with Na Pentobarbital (35 mg/kg). A unit[6] of renin is the quantity required to raise the mean femoral blood pressure of a dog 30 mm Hg. The specific activity of the renin is expressed as the number of dog units/mg protein.

The equilibrated enzyme preparation was washed into a glass column containing the exchanger which had been adjusted to pH 7.0 by addition of NaH_2PO_4 solutions and packed with air pressure (10 lb./in.^{2}) until a constant column height was obtained. The column would not run dry under gravity flow and maintained an effluent flow of two drops per minute when a total hydrostatic head of 27 in. was employed. Gradients of salt (NaH_2PO_4) and pH were employed for elution, with the addition of NaCl in the later stages of the elution to raise the salt concentration. Gradients were established by introducing from a separatory funnel into a constant-volume (50 ml) mixing chamber buffer having the composition of the gradient limit. All column operations were carried out in the cold room. Individual tube collections of about 6 ml were determined by a drop counter but the volume of each tube was measured directly.

The effluent fractions were examined for protein in a Beckman DU spectrophotometer by measuring the absorbance at 280 mμ. Paper-electrophoretic determinations were made on 10 μl of material which was obtained by concentrating the effluent against polyvinylpyrrolidone. Gross sampling was necessary to provide sufficient protein for these analyses which were made with veronal buffer (μ, 0.075; pH, 8.6) at room temperature for 16 h at a constant current of 6 mA. The paper strips were stained with bromophenol blue.

Fig. 1 shows the elution diagram obtained when the dialyzed semi-pure renin was chromatographed on DEAE-cellulose. The starting buffer was 0.005 *M* NaH_2PO_4 at pH 7.0, after which decreasing pH and increasing salt gradients were employed.

All effluent fractions were assayed for protein content and enzyme activity. Fractions 41–60 which contained activity were combined and concentrated against polyvinylpyrrolidone. This active fraction was approximately ten times as pure as the material placed on the column *i.e.*, 9 mg protein was recovered containing 85 % of the original activity.

The original semi-pure renin gave an absorption spectrum atypical for a protein

i.e., no maximum in the region of 280 mμ (tyrosine and tryptophan), but did give a strong absorption band between 180 and 230 mμ (CO–NH) corresponding to the β-form proposed by HAAS *et al.*[7]. These authors report that these aromatic amino acids appear in high content as compared with that of other proteins. On the other hand, renin eluted from the column gave a typical u.v. spectrum with a maximum at 280 mμ. These differences were found to be due to acetone contamination and not to differences in protein configuration, as proposed by HAAS *et al.*, since 1 ml of semi-pure enzyme contains 3.6 μmoles acetone. Furthermore, serum albumin treated in like manner can be made to yield two different curves depending upon whether acetone is present in the preparation. This study then indicates that the differences observed with u.v. spectroscopy are due to the strong absorption of the acetone-carbonyl function in the region of 260 mμ.

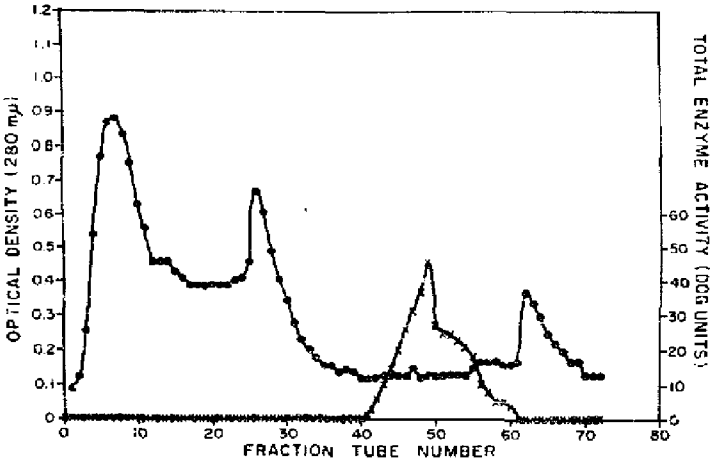

Fig. 1. Effluent diagram of semi-pure hog-kidney renin; 100 mg dialyzed protein (430 units) in 10 ml applied to 4 g adsorbent; effluent collected in 5- to 6-ml fractions. Buffers: I, 0.005 *M* NaH_2PO_4, pH 7.0; II, 0.02 *M* NaH_2PO_4, pH 6.0; III, 0.05 *M* NaH_2PO_4; IV, 0.02 *M* NaCl–0.05 *M* NaH_2PO_4; V, 0.05 *M* NaCl–0.05 *M* NaH_2PO_4; VI, 0.1 *M* NaCl–0.05 *M* NaH_2PO_4; VII, 0.2 *M* NaCl–0.05 *M* NaH_2PO_4; VIII, 0.5 *M* NaCl–0.05 *M* NaH_2PO_4; IX, 1.0 *M* NaCl–0.05 *M* NaH_2PO_4; X, 1.5 *M* NaCl–0.05 *M* NaH_2PO_4; XI, 2.0 *M* NaCl–0.05 *M* NaH_2PO_4; XII, Satd. NaCl–0.5 *M* NaH_2PO_4. Mixing chamber vol., 50 ml. Shaded area is the enzyme distribution.

The semi-pure renin preparation gave a positive nitroprusside reaction for a sulfhydryl-containing compound while the chromatographed renin gave a negative test. The extraneous proteins in the semi-pure preparation may therefore be proteins having sulfhydryl groups and some of the renin present may be in the denatured form.

Paper electrophoresis at pH 8.6 revealed the presence of two components in the eluted renin whereas the semi-pure material was composed of at least four bands.

The column charge in these studies was 100 mg protein for 4 g adsorbent. This was decreased to 3 g exchanger and packed under (14.7 lb./in.^{2}) air pressure with a flow rate of one drop per 2 min. Under both conditions with different samples of enzyme the reproducibility of the positions and magnitudes of the chromatographic peaks were good. In another study the enzyme was not equilibrated to pH 7.0 but run at pH 5.1, the pH of step 5. The effluent pattern of this was similar to that obtained with the equilibrated samples.

An ion-exchange chromatographic technique for the purification of renin has been described. The specific activity of the preparation which was applied to the column was 4.3. The eluted renin had a specific activity of 43.2. This constitutes a 10-fold purification of renin by use of cellulose ion-exchange chromatography, with a recovery of 85 % of the enzyme activity.

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A simplified method for the purification of mushroom polyphenol oxidase

Purification of mushroom polyphenol oxidase has been sought in this laboratory in order to pursue structural studies on this enzyme[1] and to explore the role of tyrosine in the secondary and tertiary structure of proteins[2–5]. While this work was in progress, KERTESZ AND ZITO[6] reported the preparation of homogeneous mushroom polyphenol oxidase. Taking advantage of the excellent early steps of this procedure*, we have used chromatography on diethylaminoethyl-cellulose (DEAE-cellulose)[7] as the basis for a highly simplified method to prepare the purified enzyme in good yield. A similar chromatographic procedure for the preparation of a soluble mammalian tyrosinase was also recently reported by BROWN AND WARD[8].

The yields and steps in the method are summarized in Table I and in the experimental section. After extraction and preliminary purification through Step 4, the crude enzyme is prepared for direct chromatography by an extensive dialysis

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