and adrenals from both aldosterone-treated groups. These findings are interpreted to indicate the indirect role of aldosterone in regulating renin production. Aldosterone failed to influence the vascular lesions of renal hypertension.

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Pepsinogen I in Semen. Chromatographic Separation of Pepsinogen I from Human Semen.* (29858)

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Investigation of proteolytic enzymes in human semen led Lundquist and Seedorff to the discovery of a proteolytic enzyme active at low pH in seminal fluid(1). This enzyme was thought to be a pepsinogen and could be activated with acid to a pepsin with kinetics analogous to those described by Herriott(2) for porcine pepsinogen. The activity of the resulting pepsin resembled the peptic activity of human gastric juice very closely in proteolytic activity *vs* pH curve, in milk clotting activity, in lability towards weak alkali, and in stability at acid reaction. The unactivated pepsinogen in semen showed no milk clotting activity; such activity was readily elicited after prior incubation with acid. Lastly, from semen the above investigators crystallized a material which was indistinguishable from crystalline swine pepsin. Investigation pointed to the seminal vesicles as the organ of secretion(3).

Until recently it was generally assumed that peptic activity in human gastric juice was ascribable to a single pepsin, derived from a single pepsinogen. However, column chromatographic fractionation on DEAE (diethylaminoethyl)-cellulose has enabled us to show recently that at least 3 closely related pepsins(4), derived from separate pepsinogens(5), account for the proteolytic activity in human gastric juice. In view of the reported closely similar behaviour of the proteolytic enzymes secreted by gastric mucosa and by seminal vesicles(4) it was of interest to determine whether this resemblance could be confirmed by chromatographic separation of the pepsinogens from seminal plasma. This report presents a comparison of the chromatographic behaviour of human seminal pepsinogen with that of human gastric mucosal pepsinogens and of human gastric pepsins with that of seminal pepsin, respectively.

Methods. Human semen was obtained by masturbation from 5 healthy unmarried individuals, aged 22-24 years. The five specimens were pooled and diluted to 150 ml with 0.1 M acetate buffer pH 5.3. This solution was dialyzed *vs* 0.1 M acetate buffer pH 5.3 at 4°C and thereafter filtered through filter paper (Eaton and Dikeman #615). The filtrate was applied to a DEAE-cellulose column (v. infra).

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Human gastric musosal extract was made from fundic mucosa of a partially resected stomach specimen obtained at surgical operation as described previously in detail(4). This extract will be referred to as unacidified extract. Acidified musocal extracts were prepared from unacidified pyloric and fundic musocal extract as described elsewhere in detail(6). Both acidified and unacidified mucosal extracts were dialyzed *vs* 0.1 M acetate buffer pH 5.3 prior to chromatography.

Column chromatographic separation of pepsins and pepsinogens. DEAE (diethylaminoethyl)-cellulose—batch 1410 (Brown Co.) was equilibrated with 0.1 M acetate buffer pH 5.3 and the slurry pressed under 10 p.s.i. nitrogen in a column of 20 × 2 cm. Dialyzed human semen solution and gastric mucosal extracts were applied to the DEAE column by gravity. These crude enzyme solutions were applied in such quantity that assay for proteolytic activity would give measurable activity in the linear or almost linear range for the method. Assay for proteolytic activity of the effluent of the crude enzyme solutions applied to the DEAE-cellulose columns showed that all proteolytically active material had been adsorbed.

Gradient elution of unacidified mucosal extract and the semen solution (pepsinogens) was achieved by connecting the DEAE-cellulose column with a Varigrad(7) having nine cylinders which contained sodium chloride dissolved in 250 ml 0.1 M acetate buffer pH 5.3. The concentration of sodium chloride in cylinder one through nine was 0.11 M; 0.11 M; 0.12 M; 0.12 M; 0.12 M; 0.13 M; 0.13 M; 0.14 M and 0.16 M, respectively. Fifteen ml fractions of eluate were collected with an automatic fraction collector at a rate of 2 to 3 fractions per hour. Temperature was kept between 10-15°C during elution.

Elution of acidified mucosal extracts (pepsins) was achieved by passing through the column 100 ml 0.1 M NaCl in 0.1 M acetate buffer at pH 5.3, followed by 200 ml 0.2 M NaCl in 0.1 M acetate buffer pH 5.3 after which the column was connected with a Varigrad which contained sodium chloride in 250 ml 0.1 M acetate buffer pH 5.3 in each of its cylinders. The concentration of NaCl in cylinder one through nine was 0.230 M; 0.230 M; 0.230 M; 0.230 M; 0.235 M; 0.240 M; 0.245 M; 0.255 M and 0.260 M, respectively. Otherwise conditions for elution were exactly as described above for elution of pepsinogens.

Rechromatography of acidified semen eluate was carried out as follows: the eluate fractions with proteolytic activity resulting from chromatography of the semen solution were pooled after assay. These pooled fractions containing semen pepsinogen were acidified with 2 N HCl to give a final pH of 2.0 and incubated for 5 minutes at 20°C. Thereafter, for reasons detailed previously(8) 2 M sodium acetate was added until a pH of 4.0 was reached; incubation for 60 minutes at pH 4.0 and 20°C was performed prior to dialysis against 0.1 M acetate buffer pH 5.3 at 4°C. The dialyzed solution was applied to a DEAE-cellulose column by gravity and eluted as described for acidified mucosal extract (v. supra).

Assay of proteolytic activity with acid hemoglobin was based on the method of Anson and Mirsky(9). Hemoglobin powder (Fischer Scientific Co.) was heated at 60°C for 3 hours to destroy inherent proteolytic activity. Substrate for assay of proteolytic activity was acid hemoglobin consisting of 1 part 0.29 N HCl and 4 parts of 2½% (w/v) hemoglobin. Five ml of acid hemoglobin and 1 ml portions of alternate eluate fractions were incubated along with appropriate blanks at 37°C for 60 minutes in the case of acidified and unacidified gastic mucosal extract. Alternate eluate fractions from chromatography of the semen solution were incubated for 2 hours; and eluate fractions obtained from elution of the acidified semen eluate were incubated for 3 hours. The pH of incubation mixtures was between 2.2-2.4. Over this pH range conversion of pepsinogen to pepsin is almost instantaneous, and no significant change in proteolytic activity occurs. In all instances proteolysis was terminated by addition of 9 ml 0.3 M trichloroacetic acid (TCA). A tyrosine standard consisting of 5 ml acid hemoglobin and 1 ml of

500 μg per ml tyrosine was incubated during the same period, and served as standard after addition of 9 ml TCA. The resulting TCA supernatants were fed into an Autoanalyzer (Technicon Instrument Co.) which completed the analysis for tyrosine-like substance. Results were expressed as micrograms of tyrosine-like substance released by 1 ml enzyme solution from 5 ml acid hemoglobin during 60 minutes incubation at 37°C in reference to the tyrosine standard of 500 μg tyrosine. All determinations were performed in the linear or nearly linear range of the method. Unless otherwise qualified the terms 'proteolytic activity' or 'assay of proteolytic activity at pH 2.2-2.4' refer to proteolytic activity of pepsinogens and pepsins at pH 2.2-2.4 with acid hemoglobin as substrate.

Assay of proteolytic activity by milk clotting. Unactivated: 0.1 ml of eluate fractions was pipetted into 2 ml 0.1 M acetate buffer pH 5.3 and the reaction started by adding 0.5 ml of a milk-buffer-calcium chloride mixture (5 parts fresh homogenized milk, 5 parts 0.1 M acetate buffer pH 5.3 and 1 part 0.1 M calcium chloride) and incubating the reaction mixture at 37°C. The pH of the final incubation mixture was 5.3. This assay will show no milk clotting activity if only proteolytically inactive proenzyme (pepsinogen) is present. Activated: 2 ml of alternate eluate fractions were acidified ('activated') by adding 0.15 ml 1.0 N HCl and incubated at 37°C for 60 minutes (pH of activation mixture 3.35). One-tenth ml of the activation mixture was thereafter assayed for milk clotting activity as described for the unacidified enzyme solution. This assay will reveal milk clotting activity if pepsinogen is present, as a result of the conversion of pepsinogen to pepsin at pH 3.35 ('activation'). The reaction was followed for 60 minutes. Clotting time expressed in minutes is the time elapsed between addition of milk and appearance of grossly visible aggregates of protein.

Chloride concentration in eluate was assayed by titration with silver nitrate; potassium chromate served as indicator.

Results and discussion. Fig. 1 depicts chromatographic separation of seminal (A)

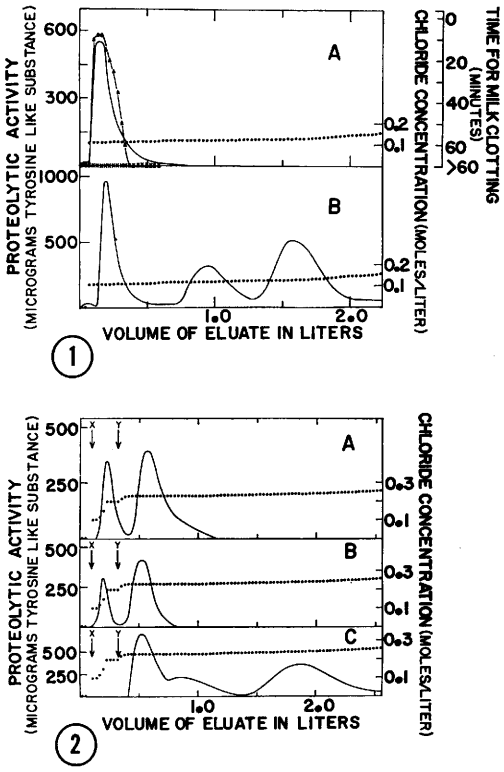

FIG. 1. Chromatographic fractionation of proteolytic enzymes (activity at low pH) of gastric mucosal extract and semen.

A. Fractionation of unacidified semen.

B. Fractionation of unacidified gastric fundic mucosal extract.

——— Proteolytic activity of eluate fractions as measured with the acid hemoglobin method (v. supra).

▲—▲ Proteolytic activity of acidified eluate estimated by time of coagulation of milk protein in the milk clotting method (v. supra).

X---X Proteolytic activity of unacidified eluate assayed by time of coagulation of milk protein.

● ● Chloride concentration in eluate.

FIG. 2. Chromatographic fractionation of proteolytic enzymes (activity at low pH) of gastric mucosal extracts and semen.

A. Fractionation of acidified semen.

B. Fractionation of acidified gastric pyloric mucosal extract.

C. Fractionation of acidified gastric fundic mucosal extract.

Proteolytic activity of eluate fractions as measured with the acid hemoglobin method (v. supra).

● ● Chloride concentration of eluate.

Arrow marked X denotes replacement of eluent containing 0.1 M NaCl by an eluting solution containing 0.2 M NaCl; arrow Y indicates the point at which the DEAE-cellulose column was connected with the Varigrad for continuous gradient elution with chloride.

and fundic mucosal (B) pepsinogens, respectively. In these chromatograms proteolytic activity and chloride concentration of eluate fractions have been plotted *vs* volume of eluate. Alternate fractions of the first 40 fractions of the semen eluate have been assayed for milk clotting activity with and without prior activation with acid.

The proteolytic activity of the semen solution was recovered as a single peak of proteolytic activity, (Fig. 1A), and was similar to the first of the 3 peaks of proteolytic activity in the chromatogram of unacidified fundic mucosal extract (Fig. 1B); these 3 have been attributed, in order of their elution to human pepsinogen I, pepsinogen II and pepsinogen III, respectively(4). Peaks of proteolytic activity attributable to human gastric pepsinogen II or III were notably absent in semen. No additional protelytic activity could be eluted by the subsequent passing of a strong NaCl solution (1.0 M NaCl in 0.1 M acetate buffer pH 5.3) through the column. Milk clotting activity was absent in the untreated eluate fractions assayed (no milk clotting activity observed within 60 minutes), while strong milk clotting activity was elicitable in the same eluate fraction after activation with acid.

Pooling of fractions with proteolytic activity in the semen eluate, subsequent acidification and elution as described above resulted in the elution chromatogram depicted in Fig. 2A. Fig. 2B and 2C depict elution chromatograms of acidified pyloric mucosal extract and acidified fundic mucosal extract, respectively. In these chromatograms (Fig. 2) proteolytic activity and chloride concentration of eluate fractions have been plotted against volume of eluate. The chromatogram of acidified semen eluate in Fig. 2A is very similar to that of acidified pyloric mucosal extract in Fig. 2B. The latter yields a single pepsin peak (after arrow Y), corresponding in place of elution to the first pepsin peak seen in chromatograms of acidified fundic mucosal extract. The proteolytic activity of this pepsin peak is therefore, as discussed elsewhere (6), attributable to human pepsin I. The peak of proteolytic activity before arrow Y in chromatograms of acidified dilute mucosal extracts has been referred to as a prepepsin peak and is ascribable to human pepsin-pepsin-inhibitor (HPPI)-complex(6,5). HPPI-complex is under otherwise similar conditions not demonstrable in concentrated acidified fundic mucosal extracts(6); this accounts for the absence of a prepepsin peak in the chromatogram in Fig. 2C.

Three peaks of proteolytic activity appear in chromatograms of acidified fundic mucosal extract (Fig. 2C); they have been called first, second and third pepsin peaks in order of their elution(6). The proteolytic activity of the first and second pepsin peak have been attributed to human pepsin I and pepsin IIA, respectively. The proteolytic activity of the third pepsin peak as ascribable to a mixture of pepsin IIB and pepsin III(6).

The above results have corroborated the existence(1) of human seminal proteolytic enzyme activity at low pH. Human gastric pepsinogens have no milk clotting activity at pH 5.3, but such activity is readily elicitable after prior conversion to pepsin by acid(5). Since the human seminal proteolytic enzyme activity had chromatographic mobility identical to gastric mucosal pepsinogen I and had milk clotting properties typical for pepsinogens (Fig. 1) we have presumptively designated this activity as seminal pepsinogen I. The pepsinogen I-like character of seminal pepsinogen has been further substantiated by formation from it after acidification of a pepsin-pepsin-inhibitor complex and a pepsin I (Fig. 2).

Seedorff has adduced convincing evidence for the secretion of seminal pepsinogen by the epithelium of the seminal vesicles(3). We have previously shown that gastric mucosal pepsinogens II and III are probably secreted by the fundic chief cell, while pepsinogen I has been presumed to originate from unknown and less differentiated cell types in duodenal, pyloric and fundic mucosa(5). Since the highly differentiated fundic chief cell is characteristically restricted to fundic gastric glands it is not difficult to accept the absence of pepsinogen II and pepsinogen III from semen. On the other hand pepsinogen I is secreted by fundic mucosa, pyloric mucosa and duodenal mucosa, and therefore is

presumably associated with cells of lesser differentiation in a variety of loci. The presence of pepsinogen I (rather than pepsinogen II and III) in the embryologically different epithelium of the seminal vesicles is therefore not so surprising.

We have reported small but significant differences in the characteristics of different human gastric pepsins(10) which must have escaped detection by others(1,3); otherwise it is difficult to account for the reported similarity(3) between gastric and seminal pepsinogen with respect to alakli resistance and activity *vs* pH curves.

Although other proteolytic enzymes active around pH 7 are known to be present in semen, the presence of pepsinogen is, teleologically at least, somewhat puzzling. The near neutral pH of semen precludes conversion of the inactive proenzyme (pepsinogen I) to proteolytically active pepsin I. Seedorff has discussed the problem of activation of seminal pepsinogen; he speculated that activation most likely occurred on the cellular surface of the spermatozoa(3). It is, however, also possible that conversion of pepsinogen to pepsin may take place in the vagina after coitus. The weakly acidic pH (4.0-5.0) of normal adult vaginal secretions(11) would make this feasible. If this is the case, it may be that proteolytic activity from seminal pepsinogen plays a role in the complex process of reproduction.

Summary. Fractionation of human semen on DEAE (diethylaminoethyl)-cellulose has shown that the seminal proteolytic activity at low pH is eluted as a single peak of proteolytic activity. This peak of proteolytic activity exhibits no milk clotting activity unless it is first activated by acidificaton. The chromatographic mobilities of the proteolytic activity at low pH of unacidified and acidified semen are similar to those of unacidified and acidified gastric mucosal pepsinogen I, respectively. The possible conversion of seminal pepsinogen to pepsin by weakly acidic vaginal secretions posed the question of a possible role for pepsin in human reproduction.

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In vitro Reinitiation of Pituitary Somatotropin Release by an Acid Extract of Hypothalamus.*† (29859)

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Rat anterior pituitary loses most of its capacity to release somatotropin (STH) after culture for 6 days *in vitro*(1). This appears to be true for the release of all tropic hormones of the anterior pituitary except prolactin(2), which is inhibited by the hypo-

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