THE BASIC PROTEIN RESPONSIBLE FOR THE CLOTTING OF GUINEA PIG SEMEN*

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In many mammals, the clotting of semen which occurs after ejaculation is due to coagulation of protein derived from the seminal vesicles by the action of vesiculase, an enzyme secreted by the anterior prostate (coagulating) gland.[1-4] In certain rodents the clotted semen forms a "copulation plug" which occludes the vagina, while in man the coagulated semen quickly liquifies due to the presence of proteolytic enzymes in the seminal plasma.[5] Previous experiments showed that four major and additional minor protein constituents could be separated from crude guinea pig vesicular secretions by chromatography on Sephadex CM-50 ion exchangers.[6] One of these bulk proteins was basic in character, and devoid of tyrosine, proline, and cyst(e)ine. This basic protein appeared to be a precursor of the seminal clot formed by the action of vesiculase on vesicular secretion. We describe here a simple method for the preparation of the basic clottable protein of guinea pig vesicular secretion in a homogeneous form. Some physiocochemical properties of this protein are discussed in relation to the mechanism of its coagulation by vesiculase. Mányai and his co-workers have recently described the isolation and characteristics of a similar basic protein present in the seminal vesicle secretions of rats, mice, and guinea pigs.[7-9]

Methods and Materials.—Isolation of basic vesicular secretion protein: Guinea pig vesicular secretion was expressed manually from ligated glands into an iced medium containing 0.15 *M* NaCl, 0.002 *M* disodium ethylenediaminetetraacetate (EDTA), 0.04 *M* Tris-HCl of pH 7.9. After stirring, the solution was centrifuged at 12,000 × *g* for 20 min and insoluble material discarded. Two volumes of redistilled acetone (−20°) were added slowly and with stirring to the supernatant fluid. The precipitate of protein was collected by centrifugation at 2° and washed three times with acetone (−20°). The proteins were dried *in vacuo* over NaOH at 23°, and stored at −20° in the presence of dessicant. The acetone powder was extracted with 10–20 vol of 0.01 *M* Tris-HCl of pH 7.5 for 1 hr at 23°. The solution was dialyzed overnight at 4° against 4 liters of the same buffer. Insoluble material was removed by centrifugation. The basic vesicular secretion protein was isolated by chromatography on *O*-(diethylaminoethyl) cellulose (DEAE-cellulose); a typical protocol is as follows. The dialyzed solution (62 ml containing approximately 800 mg of protein) was layered on a column (5 × 48 cm) of DEAE-cellulose. (The DEAE-cellulose was previously washed successively with 0.5 *N* NaOH, water, 0.5 *N* HCl, water, and finally equilibrated against 0.01 *M* Tris-HCl of pH 7.5.) An additional 400 ml of 0.01 *M* Tris-HCl of pH 7.5 was passed over the column. A linear gradient varying from 0 to 0.25 *M* NaCl in 0.01 *M* Tris-HCl of pH 7.5 was then applied. The column was operated at 23°, and 10-ml fractions were collected in a refrigerated apparatus. The first major and symmetrical peak of protein material eluted was found in fractions 80–105, which contained the basic protein. Additional solid NaCl was added to the pooled fractions 84–101, inclusive, to give a final concentration of 0.5 *M*. To this solution (180 ml) was added 540 ml of acetone (−20°) with stirring at 4°. After standing for 30 min, the precipitate was removed by centrifugation at 12,000 × *g* for 30 min. The insoluble protein was washed three times with cold acetone, dried *in vacuo*, and stored at −20° over dessicant. The yield of protein was 120 mg. It may be mentioned that two other major peaks of material containing protein were later eluted from the DEAE-cellulose column, following application of the gradient of NaCl, in fractions 130–160 and 170–194.

Amino acid analyses: Samples of protein (0.3–0.7 mg) were hydrolyzed in 3 ml of constant

boiling HCl in sealed tubes under vacuum. The tubes were heated to 110 ± 5° for 22, 48, and 70 hr. The samples were evaporated to dryness under a stream of nitrogen and dissolved in 0.05 citrate buffer at pH 2.88. After addition of 0.2 μmole of norleucine as reference standard, the mixtures were subjected to automatic amino acid analysis using a Technicon apparatus according to the procedures described by Boyer and Talalay;[10] corrections for hydrolytic losses were made according to the latter authors. Amide nitrogen was estimated by the method of Stegemann[11] using duplicate samples of approximately 2 mg by weight.

Gel filtration: Bio-Gel P-100 (Calbiochem) was suspended in 0.01 *M* Tris-HCl of pH 7.5 containing 0.1 *M* NaCl, and allowed to swell for at least 48 hr before packing on a column 2.5 × 42 cm). The molecular Stokes' radius of the basic clottable protein was determined by the method of Ackers.[12] The inverse error function complement of the column partition coefficient was plotted as a function of the molecular Stokes' radius (r) of the standards. Beef hemoglobin, type I , 2 times crystallized (Sigma); α-chymotrypsin, 3 times crystallized (Worthington); horse heart cytochrome *c*, type III (Sigma); and ribonuclease, type I-A (Sigma) were used as standards. From the experimentally determined molecular Stokes' radius (r) and the sedimentation coefficient ($s_{20,w}$), the molecular weight (M) of the protein was calculated according to the following combination of the Stokes-Einstein and Svedberg equations:

$$M = \frac{6\pi\eta\, rNs_{20,w}}{(1 - \bar{v}\rho)},$$

where η is the solvent viscosity, N is Avogadro's number, $\bar{v}$ the apparent partial specific volume of the protein, and ρ the density of the solvent.

Ultracentrifugation was carried out in a Spinco model E analytical ultracentrifuge equipped with a Schlieren optical system. The protein was exhaustively dialyzed against 3 liters of 0.03 *M* NaCl-0.01 *M* Tris-HCl of pH 7.4 for 72 hr at 4°. Ultracentrifugations of protein solutions (3–6 mg of protein per ml) were conducted at 20° at 52,640 rpm. Photographs were taken at 4-min intervals after maximal velocity was attained. We are grateful to Mr. S. Orrell for these determinations.

Polyacrylamide gel electrophoresis was carried out in glass tubes (4 × 70 mm) using gels containing 9.0% of acrylamide and 0.45% of N,N'-methylenebisacrylamide. The gel was polymerized by the addition of 0.03% of N,N,N',N'-tetramethylethylenediamine and 0.1% ammonium persulfate. The electrophoresis buffer contained 0.03 *M* Tris base, 0.0014 *M* disodium EDTA, and 0.012 *M* boric acid pH 8.7. The gels were exposed to a constant 150 volts for 1 hr prior to the application of the protein samples to remove the catalyst and other impurities. The samples of protein were layered onto the gel surface in a 1:3 dilution of the electrophoresis buffer containing 20% sucrose. The protein (10–100 μg) was applied in volumes of 5–25 μl. Electrophoresis was carried out for 1 hr at a constant 150 volts (approximately 2 ma per tube). The protein bands were fixed and stained with a 0.5% solution of Amido Black in 7% acetic acid.

Other methods: Anthrone-reactive carbohydrate was determined by a modification of the procedure of Scott and Melvin.[13] Sialic acids were estimated by the method of Warren.[14] Protein was determined by the biuret method with bovine serum albumin as standard.[15] A solution of the purified basic vesicular protein at a concentration of 1 mg per ml (in 0.01 *M* Tris-HCl of pH 7.3) exhibited an absorbancy of 0.230 at 280 mμ and 0,179 at 289 mμ in cells of 1-cm light-path. Tryptophan in the basic protein was determined either by direct ultraviolet spectrophotometry[16] or by the xanthydrol reaction.[17]

Results and Discussion.—Bulk proteins of guinea pig vesicular secretion: Previous studies showed that four major protein bands were obtained when crude, acetone-insoluble proteins from guinea pig vesicular secretions were subjected to electrophoresis on paper in barbital buffer at pH 8.6.[6] More discrete and reproducible separations of the bulk proteins in this fluid were achieved by electrophoresis on polyacrylamide gels in Tris-EDTA-borate buffer at pH 8.7. Figure 1 shows that, by the latter procedure, three major and five minor protein constituents migrated towards the anode, whereas a single protein band traveled to the cathode. When the crude mixture of vesicular proteins was coagulated by addition of vesiculase

and then centrifuged, the basic protein migrating towards the cathode at pH 8.7 was removed. The basic protein isolated by chromatography on DEAE-cellulose travelled to the same position as the material that was rendered insoluble when the vesicular secretion proteins were treated with vesiculase. The purified basic protein was not contaminated by any other proteins that migrated to the anode at pH 8.7.

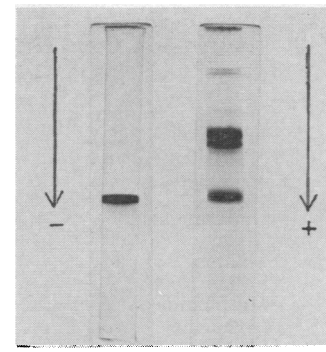

FIG. 1.—Gel electrophoretic separation of the guinea pig seminal vesicle proteins in a Tris-EDTA-borate buffer of pH 8.7. The gel on the right shows the proteins migrating from the origin at the top, toward the anode at the bottom. The gel on the left shows the protein migrating toward the cathode.

Characteristics of the basic coagulable protein: The purified basic protein was colorless, and formed viscous 2 per cent solutions in water or 0.15 *M* NaCl that were not coagulated by heating to 100° for 30 minutes. The protein was insoluble in 5 per cent solutions of trichloracetic or perchloric acids. The ultraviolet absorption spectrum of the protein in 0.15 *M* NaCl-0.001 *M* Tris-HCl of pH 7.0 was similar to that of the basic protein previously isolated by chromatography on Sephadex-CM-50.[6] The protein exhibited absorption peaks at 251, 258, 264, and 268 mμ (due to phenylalanine) and at 278 and 288 mμ (due to tryptophan). The spectrum in the range of 240–300 mμ was hardly perturbed by adjustment of the solution to pH 12 with NaOH, tyrosine being absent from the protein. The protein migrated as a single band on gel electrophoresis over the range of pH 4–9. The isoelectric point was in the vicinity of pH 9.3. The purified protein sedimented as a single symmetrical peak on ultracentrifugation. The sedimentation coefficient ($s_{20,w}$) was computed to be 1.56*S*. Gel filtration on Bio-Gel P-100 by the method of Ackers[12] suggested that the molecular Stokes' radius (r) of the protein was 2.73 mμ. The apparent partial specific volume ($\bar{v}$) was reckoned to be 0.731 mg per ml from the amino acid composition described below, and the known partial specific volumes of the individual amino acids.[18] The molecular weight calculated from physical measurements by the method described above was 17,890.

The amino acid analysis of the basic clottable protein is depicted in Table 1. The protein was devoid of tyrosine, proline, cyst(e)ine, hydroxyproline, and hydroxylysine. Tryptophan was the amino acid present in lowest amounts. Division of the concentration of each amino acid by the quantity of tryptophan (39 mμmoles per mg of protein) gave the molar ratio of each amino acid. When the latter figures were adjusted to the nearest integral value, the empirical formula is: Asp_{10}, Thr_6, Ser_{22}, Glu_{26}, Gly_{23}, Ala_6, Val_{12}, Met_5, $Ileu_2$, Leu_{14}, Phe_6, Lys_{20}, His_2, Arg_{10}, Trp_1, with a total of 165 residues. Direct analyses[11] indicated the presence of 21 amide residues. The calculated minimal molecular weight was 17,892, a value in remarkable agreement with that computed from physical measurements. The basic protein contained less than 0.05 per cent of anthrone-reactive carbohydrate, and was devoid of sialic acids.

Mányai and co-workers[8] have shown that the basic clottable protein of the seminal vesicle secretion of the rat is characterized by high lysine and glutamate content, a low tryptophan and methionine content, and the absence of proline

TABLE 1

AMINO ACID COMPOSITION OF PURIFIED BASIC VESICULAR PROTEIN AND OF PROTEIN IN VESICULAR SECRETION CLOTTED BY VESICULASE

Amino acid residue	Basic Vesicular Protein (Mμmole per mg of protein*)	Basic Vesicular Protein (Nearest integral number)	Washed seminal clot protein (mμmoles per mg of protein†)
Aspartic acid	390	10	389
Threonine	217	6	200
Serine	863	22	807
Glutamic acid	998	26	1010
Glycine	880	23	836
Alanine	218	6	221
Valine	474	12	470
Methionine	210	5	197
Isoleucine	60	2	74
Leucine	554	14	534
Phenylalanine	247	6	224
Lysine	775	20	671
Histidine	70	2	109
Arginine	376	10	464
Amide-NH_2	810	21	460
Tryptophane	39‡	1	39§
Proline	0	0	0
Tyrosine	0	0	0
Cyst(e)ine	0	0	0
Hydroxyproline	0	0	0
Hydroxylysine	0	0	0

* Most probable value obtained by acid hydrolysis of samples for 22, 48, and 70 hr.
† Corrected values obtained after acid hydrolysis for 22 hr.
‡ Spectrophotometric determination.
§ Determined by xanthhydrol reaction.

and cyst(e)ine. In marked contrast to the basic protein from guinea pig vesicular secretion, however, the clottable protein isolated by Mányai *et al.*[8] contained tyrosine. A molecular weight of 43,700 was reported for the basic protein of the rat seminal vesicle secretion.[8]

Role of the basic vesicular secretion protein in formation of seminal clot: If the basic protein obtained by chromatography on DEAE-cellulose was dissolved in 0.075 *M* NaCl-0.005 *M* $MnCl_2$-0.04 *M* Tris-HCl at pH 7.5 at a final concentration of 5 mg per ml, the material was rapidly coagulated on addition of a crude soluble extract of guinea pig coagulating gland. Other protein fractions obtained from DEAE-cellulose chromatography were not clotted upon exposure to crude vesiculase preparations. That the basic protein present in seminal vesicle secretion is the principal if not sole precursor of the coagulum formed by vesiculase action is suggested by the following simple experiment. Fresh seminal vesicle secretion was collected in 0.075 *M* NaCl-0.04 *M* Tris-HCl of pH 7.4. Insoluble material was removed by centrifugation. The soluble proteins were clotted by addition of coagulating gland secretion. The clot was separated by centrifugation, and washed successively five times with 0.15 *M* NaCl, five times with 8 *M* urea, five times with water, and three times with acetone at room temperature. The amino acid analysis of the washed coagulum is shown in Table 1. It is evident that there is a similarity between the amino acid composition of the washed clot and that of the purified basic protein. The complete absence of tyrosine, proline, and cyst(e)ine from both materials is particularly noteworthy. Direct measurements indicated that clotting by vesiculase reduces the amide-NH_2 of the basic protein from a value of about 21 residues per 18,000 molecular weight to a value of about 12 amide-NH_2 residues. The possibility that the action of vesiculase on the basic

protein involves transamidase reactions analagous to those occurring during the fibrinase reaction[19, 20] is under investigation.

The dissimilarity between semen and blood coagulation mechanisms has been discussed previously.[3, 4, 8, 9] This and other[9] studies emphasize that the purified basic clottable proteins of rodent vesicular secretions bear no resemblance to fibrinogen.[21]

Summary.—A basic protein has been isolated in a homogeneous form from the seminal vesicle secretion of guinea pigs. This protein is devoid of tyrosine, proline, and cyst(e)ine, and can assume a molecular weight of about 17,900 in solution. Evidence is presented that this protein is the major if not sole precursor of the seminal clot formed by the action of the coagulating gland enzyme vesiculase on seminal vesicle secretions.

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