2. Jones, H. B., Chaikoff, I. L., and Lawrence, J. H., *Am. J. Cancer,* 1940, v40, 243.
3. Kenney, J. M., *Cancer Research,* 1942, v2, 130.
4. Tuba, J., Rawlinson, H. E., Shaw, L. G., Fraser, M. S., and Jeske, I., *Canad. J. Med. Sci.,* 1953, v31, 400.

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Hydrolysis of Arginine Esters by Male Accessory Sexual Tissues. (22384)

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(Introduced by Charles Huggins.)
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During the course of studies on coagulation of rodent semen(1) it was observed that extracts of the coagulating gland of the guinea pig hydrolyzed tosyl-L-arginine methyl ester (TAMe) at very rapid rates. This TAMe hydrolyzing enzyme could be separated from vesiculase, the enzyme responsible for the coagulation of seminal vesicle proteins, by fractionation with ammonium sulfate and with acetone. In this paper it will be shown that certain esters of L-arginine, but not those of a number of other amino acids, are hydrolyzed by extracts of the prostate gland of some species. Factors affecting the activity of this arginine ester hydrolyzing enzyme, and its intracellular and tissue distribution are discussed.

Methods. The esters of free or substituted amino acids were of commercial origin except for L-lysine ethyl ester, which was synthesized according to Werbin and Palm(2). The melting points of all these substances agreed well with those in the literature. Five times recrystallized soy bean trypsin inhibitor was obtained from the Nutritional Biochemical Corporation. Manometric measurements were made with conventional Warburg flasks fitted with one sidearm. Protein was determined spectrophotometrically(3). Dry weights were determined by heating to constant weight at 100°. The hydrolysis of casein and of hemoglobin at pH 7.5 was measured by the methods of Kunitz(4) and Anson(5) respectively. Canine prostatic fluid was obtained from two dogs which had undergone the prostatic isolation operation of Huggins *et al.*(6). The animals were castrated and had received 50 mg of testosterone propionate per day for some months. The fluid was obtained by the administration of pilocarpine(6), centrifuged to remove small amounts of debris, and stored in the frozen state until used. The accessory sexual organs of various rodents were dissected carefully in order to avoid contamination by secretions of neighboring tissues. We are grateful to Dr. Evelina Ortiz for dissection of the accessories of the mouse and the hamster. In all animals the secretion of the seminal vesicles was removed by expression. The hydrolysis of free or substituted amino acid esters was followed by manometric determination of the carbon dioxide liberated from 0.026 M sodium bicarbonate buffer in equilibrium with 95% nitrogen-5% carbon dioxide at 38°. The reaction was initiated by the addition of the substrate from the sidearm after a temperature equilibration of 10 minutes. In every case suitable blank vessels were set up to correct for any nonenzymatic hydrolysis of the substrate, and also for any acid formation by the tissue extract. All values are given in terms of micromoles of carbon dioxide liberated by the hydrolysis of a given substrate. With crude saline extracts of the coagulating gland of the guinea pig, the rate of hydrolysis of TAMe was strictly proportional to the amount of extract added and to the time of incubation provided that the activity was not greater than 30 μmoles per hour.

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Results. Guinea pig coagulating gland. Aqueous or isotonic saline homogenates of guinea pig coagulating gland hydrolyze 100-300 μmoles TAMe per mg protein per hour. The bulk of the activity remained in the supernatant fluid after centrifugation at 3,500 x *g* for twenty minutes at 2°. The same preparations attacked benzoyl-L-arginine ethyl ester (BAEe) at about 50% of the rate of TAMe. However, benzoylglycine methyl ester, and the ethyl esters of the following amino acids were not hydrolyzed: L-leucine, L-cystine, N-phenylglycine, acetyl-DL-tryptophan, benzoyl-DL-α-alanine, L-lysine, DL-α-alanine and DL-tyrosine. At pH 7.5, guinea pig coagulating gland extracts barely hydrolyzed either casein or hemoglobin. The Michaelis constant for TAMe hydrolysis was estimated by the method of Lineweaver and Burk(7) from values for the rate of hydrolysis at different substrate concentrations, and was found to be 0.007 M. The hydrolysis of TAMe (0.03 M) was found to be unaffected by the following substances: ethylenediamine tetraacetic acid (6 x 10^{-4} M), $HgCl_2$ (4 x 10^{-5} M), $CuSO_4$ (9 x 10^{-5} M) $MnCl_2$ (4 x 10^{-4} M), $CaCl_2$ (2 x 10^{-3} M) and crystalline soy bean trypsin inhibitor (0.3 mg per ml). These concentrations of ethylenediamine tetraacetic acid and of $HgCl_2$ abolish the action of the coagulating enzyme vesiculase(1). The partial purification of the arginine ester hydrolyzing enzyme and its separation from vesiculase in guinea pig coagulating gland is shown in Table I.

TABLE I. Separation of Arginine Ester Hydrolyzing Enzyme from Vesiculase.

Fraction	Vesiculase activity	TAMe-splitting activity
(a) Ammonium sulfate fractionation of crude extract		
.3-.5 saturation	.81	210
.5-.8 "	0	1610
(b) Acetone fractionation of .3-.5 ammonium sulfate fraction		
0-20% acetone	2.44	21
20-30% "	1.93	23
30-50% "	.2	191

Crude extract was prepared by homogenization of the tissue in 0.9% NaCl, followed by centrifugation at 3,500 × *g* for 20 min. All operations carried out between 0° and 2°. All values expressed as units per mg protein. Vesiculase in arbitrary units. TAMe hydrolysis as μmoles split per hr.

TABLE II. Hydrolysis of TAMe by Rodent Accessory Sexual Tissues.

Organ	Guinea pig	Rat	Mouse	Hamster
Ventral prostate		14.8	.04	1.4
Dorsal "		.7	.06	.03
Lateral "	.7			.02
Coagulating gland	55.8	.02	.1	.02
Seminal vesicle	.3	.04	.2	.03

Tissues homogenized in 0.15 M KCl at 0°. Substrate concentration 0.03 M. Values represent μmole TAMe hydolyzed per mg wet weight of tissue per hour. Two experiments with mouse and hamster , in each of which tissue was pooled from 4 to 5 animals. Three experiments with guinea pig and 5 experiments with rat. The figures recorded are means of the values observed, which did not differ by more than 30%.

Distribution of arginine ester hydrolyzing activity in rodent accessory sexual tissues. The hydrolysis of TAMe by isotonic saline homogenates of the accessory reproductive organs of sexually mature male rodents is shown in Table II. In the guinea pig, only the coagulating gland hydrolyzes TAMe at significant rates. In the rat the coagulating gland exhibits negligible activity, but in this species TAMe is split rapidly by the ventral prostate gland. A similar situation is apparent in the hamster, whereas in the mouse none of the accessory sexual tissues examined hydrolyzed TAMe at more than negligible rates.

Intracellular distribution. Rat ventral prostate tissue was homogenized in 0.25 M sucrose-0.001 disodium ethylenediamine tetraacetate-0.003 M sodium bicarbonate, and various cellular components isolated by differential centrifugation according to Hogeboom, Schneider and Pallade(16). The fraction obtained after removal of nuclei and mitochondria was the only one which hydrolyzed TAMe at significant rates. The TAMe hydrolyzing activity of this fraction, which was not separated further into microsomal and "soluble" fractions, accounted for virtually all of the activity of the original homogenate.

Effect of castration. Table III shows that 8 days after castration the ability of rat ventral prostate homogenates to hydrolyze TAMe

TABLE III. Effect of Castration upon Hydrolysis of TAMe by Rat Ventral Prostate Gland.

Group	Wet wt of ventral prostate (mg)	μmole TAMe hydrolyzed/mg wet wt tissue/hr
Normal	333 (257-380)	13.9 (11.6-14.9)
Castrated 8 days	76 (65- 95)	5.9 (4.1- 8.4)

Substrate concentration 0.03 M. Figures in parentheses depict range of values observed. Five animals in each group.

was diminished to 46% of the normal level, whereas the wet weight of the glands had fallen to 23% of normal.

Canine and human prostatic fluids. Extremely rapid rates of TAMe hydrolysis by dog prostatic fluids were observed, 1 cc hydrolyzing from 10,000 to 30,000 μmoles per hour. These rates are equivalent to approximately 1,000 to 3,000 μmoles TAMe split per mg protein per hour. Most of the enzyme was recovered in the proteins precipitated by the addition of ammonium sulfate at 0° between 0.5 and 0.8 saturation. In contrast to the enzyme present in guinea pig coagulating gland and in the ventral prostate gland of the rat, canine prostatic fluid hydrolyzed BAEe at about twice the rate of TAMe. Dog prostatic fluids failed to hydrolyze the ethyl esters of either L-leucine or L-lysine. The arginine ester hydrolyzing enzyme of canine prostatic fluid was unaffected by heating to 60° for 10 minutes, but 90% of the activity was destroyed by heating to 70° for the same period of time. Centrifuged isotonic saline homogenates of whole prostatic glands removed within 3 hours post mortem from adult men without evidence of prostatic pathology hydrolyzed 0.2 to 0.4 μmole TAMe per mg protein per hour.

Discussion. The arginine ester hydrolyzing enzyme of rodent and canine prostatic fluid bears a strong resemblance to the human seminal enzyme which hydrolyzes BAEe described by Lundquist *et al.*(8). Thus both enzymes are unaffected by ethylenediamine tetraacetic acid, and appear to be quite distinct from trypsin-like enzymes which degrade casein. Although both TAMe and BAEe are substrates for trypsin(9), the latter enzyme also attacks L-lysine ethyl ester, which is not hydrolyzed by the prostatic enzyme. The lack of identity of the prostatic enzyme with trypsin is further established by the failure of crystalline soy bean trypsin inhibitor to inhibit its activity. Sherry and his coworkers(10,11) have shown that both thrombin and plasmin will hydrolyze TAMe. The latter enzyme also hydrolyzes L-lysine ethyl ester, and would thus appear to be different from the prostatic enzyme described here. Moreover, canine prostatic fluid, which is practically devoid of fibrinolysin(12), splits TAMe at extremely rapid rates, whereas human prostatic fluid, which is rich in fibrinolysin(12,13) hydrolyzes TAMe rather slowly. Canine prostatic fluids have little ability to clot fibrinogen(12), which suggests that the prostatic arginine ester hydrolyzing enzyme is not identical with thrombin. Moreover, crude extracts of the guinea pig coagulating gland, which hydrolyze TAMe rapidly, will not clot fibrinogen(1). Lundquist *et al.*(8) concluded that the human seminal enzyme which splits BAEe is not identical with trypsin, thrombin or seminal fibrinolysin, and a similar conclusion can be reached concerning the enzyme investigated here, which is probably the same as that described by Lundquist.

The ready separation of vesiculase from the arginine ester hydrolyzing enzyme in the guinea pig coagulating gland shows that these two enzymes are not identical. The distribution of these two enzymes in the accessory reproductive organs of other rodents also negates the possibility that the arginine ester hydrolyzing enzyme is primarily concerned with the coagulation process. Thus in the rat and in the hamster the ventral prostate gland is the only accessory sexual tissue which splits TAMe, while vesiculase activity is confined to the coagulating gland(1,14). It has been pointed out elsewhere(1) that TAMe itself, which inhibits the clotting activity of the thrombin(11), does not affect vesiculase activity.

The extremely rapid rates of TAMe hydrolysis by only some lobes of the prostate gland of a given species, and the remarkable differences in the distribution of this arginine

ester hydrolyzing enzyme among the accessory reproductive organs of various animals is reminiscent of similar tissue and species differences in the manufacture of other components of the seminal plasma such as fructose, citric acid, inositol and acid phosphatase (15). The immense rates of arginine ester hydrolysis by some accessory sexual tissues, and the fall in activity induced by castration, adds this arginine ester hydrolyzing enzyme to the already imposing list of chemical accessory sexual characteristics in the male.

Summary. Certain esters of arginine, but not those of a number of other amino acids, are hydrolyzed rapidly by some lobes of the prostate gland of a number of species. Marked differences in the activity of this arginine ester hydrolyzing enzyme in different lobes of the prostate gland of various rodents have been observed. Dog prostatic fluid is extremely rich in this enzyme, whereas the human prostate hydrolyzes arginine esters rather slowly. The activity of the enzyme in rodent prostatic tissue is depressed after castration. Some properties of this enzyme, and its relationship to other proteolytic enzymes in prostatic secretion as well as to the process of semen coagulation are discussed.

1. Gotterer, G., Ginsburg, D., Schulman, T., Banks, J., and Williams-Ashman, H. G., *Nature,* 1955, v176, 1209.

2. Werbin, H., and Palm, A., *J. Am. Chem. Soc.,* 1951, v73, 1382.

3. Warburg, O., and Christian, W., *Biochem. Z.,* 1941, v310, 384.

4. Kunitz, M., *J. Gen. Physiol.,* 1947, v30, 291.

5. Anson, M. M., *ibid.,* 1938, v22, 79.

6. Huggins, C., Masina, M. H., Eichelberger, L., and Wharton, J. D., *J. Exp. Med.,* 1939, v70, 543.

7. Lineweaver, H., and Burk, D., *J. Am. Chem. Soc.,* 1934, v56, 658.

8. Lundquist, F., Thorsteinsson, Th., and Buus, O., *Biochem. J.,* 1955, v59, 69.

9. Neurath, H., and Schwert, G. W., *Chem. Rev.,* 1950, v46, 69.

10. Troll, W., Sherry, S., and Wachman, J., *J. Biol. Chem.,* 1954, v208, 85.

11. Sherry, S., and Troll, W., *ibid.,* 1955, v208, 95.

12. Huggins, C., and Neal, W., *J. Exp. Med.,* 1942, v76, 527.

13. Huggins, C., *The Harvey Lectures,* 1947, v42, 148.

14. Walker, G., *Bull. Johns Hopkins Hosp.,* 1910, v21, 182.

15. Mann, T., and Lutwak-Mann, C., *Physiol. Revs.,* 1951, v31, 27.

16. Hogeboom, G. H., Schneider, W. C., and Pallade, G. E., *J. Biol. Chem.,* 1948, v172, 619.

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Influence on Hepatic Ferritin Systems of Tertiary Amine, G-D 131, with Beneficial Effects in Shock.* (22385)

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This report deals with effects and mode of action, in experimental shock, of a group of synthetic agents not previously explored, since they possessed no known properties suggestive of potential value as protective agents in shock. They are tertiary amines, chemically related to β-chloroalkylamines, adrenergic blocking agents, of which N, N-dibenzyl-β-chloroethylamine (Dibenamine) is the prototype. These compounds are structurally altered so that their adrenergic blocking properties have been lost. Several of these compounds were utilized by Nickerson and Gump (1) in their study of the relation between chemical structure and adrenergic blocking activity; others have been synthesized for the current study. This group of compounds was

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