scious dog. This action of glucagon, which is independent of its hyperglycemic effect, appears to be due to a direct action on the kidney tubules. The physiologic significance of these effects remains to be clarified.

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Some Factors Which Influence Vesiculase Action. (22855)

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A previous publication(1) described coagulation of proteins of the seminal vesicle secretion by vesiculase, an enzyme of prostatic origin. In experiments conducted at room temperature, coagulation was prevented by ethylenediamine tetraacetic acid (versene), and certain other metal chelating agents. Versene inhibition could be reversed by addition of Mn^{++} ions, and somewhat higher concentrations of Ca^{++} ions, but not by Mg^{++} ions. Heavy metal ions, *e.g.*, Hg^{++}, also abolished coagulation. This paper describes experiments which give further insight into the role of metal ions in the coagulation of semen. The influence of temperature and of a number of substances on the over-all coagulation process has also been studied.

Methods. Turbidity measurements were made with a Coleman Junior spectrophotometer using cuvettes of 0.8 cm light path. Other spectrophotometric measurements were made with Beckmann model D.U. spectrophotometer using quartz cells of 1 cm light path. Protein was determined spectrophotometrically according to Warburg and Christian(2) or by the method of Lowry *et al.*(3). Nitrogen was estimated by the Kjeldahl technic. Dry weights were determined by heating to constant weight at 105°. Total phosphorus was estimated after digestion with sulfuric acid, subsequent clarification with H_2O_2 and hydrolysis in 1 N acid for 10 minutes at 100°, by determination of inorganic phosphorus according to Gomori(4).

Materials. Crystalline proteolytic enzymes, takadiastase and hyaluronidase were of commercial origin. Five times recrystallized soy bean trypsin inhibitor was obtained from Nutritional Biochemicals Corp. 2,3 dimercaptopropanol was purchased from Mann Research Laboratories. Spermine hydrochloride was generously donated by Dr. E. A. Evans, Jr. Heparin (60 units/mg) was obtained from Parke, Davis and Co. Acetone-desiccated proteins of seminal vesicle secretion were prepared as described elsewhere(1). For coagulation studies, the acetone powders were extracted with isotonic NaCl, and a small insoluble residue removed. Ultraviolet absorption spectra of these saline extracts were identical in either phosphate or tris (hydroxymethyl) aminomethane (Tris) buffers, and also in 6.4 M urea, at pH 7. The ratio of absorbancy at 280 mμ to that at 260 mμ ranged from 1.24 to 1.53 (mean 1.38). Mean absorbancy at 280 mμ was 2.64/mg nitrogen/ml and 0.43/mg dry weight/ml. Phosphorus content of acetone dried preparations varied from 0.02% to 0.03%. Crude preparations were obtained by homogenizing fresh coagulating glands in isotonic NaCl, followed by centrifugation at 5,000 x g for 20 minutes at 2°. Partially purified preparations were obtained by fractionation of crude extracts

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with ammonium sulfate between 0.3 and 0.5 saturation, followed by fractionation with acetone between 0% and 30%(5). All fractionations were carried out at less than 2°, and the fractions were dissolved in and dialyzed against isotonic NaCl. Ultraviolet absorption spectra of these partially purified preparations showed a distinct peak at 280 mμ; the ratio of absorbancy at 280 mμ to that at 260 mμ was in the neighborhood of 1.75. Both crude and partially purified preparations of guinea pig vesiculase retained their activity for many weeks when stored frozen, and for several days when stored at 2°. Repeated freezing and thawing, however, was deleterious to the enzyme. *Optical Determination of Coagulation.* The coagula formed by action of vesiculase formed a firm gel only when initial concentration of seminal vesicle proteins was very high. For this reason it was not feasible to determine the end point of coagulation by inversion of the tube. Rate and extent of coagulation was followed by measurement of change in turbidity at 660 mμ. The reactions were carried out in a final volume of 1.0 to 1.5 ml. Unless stated otherwise, the reaction was initiated by addition of vesiculase in 0.1 ml or less. After all components were added, the end of each tube was covered with a sheet of "parafilm" and contents mixed by slow inversion 3 times. Optical density readings were taken after mixing, and at suitable time intervals thereafter. In all experiments blank tubes were set up in which either seminal vesicle proteins or vesiculase were omitted, to correct for any turbidity changes in absence of these components. Vesiculase preparations by themselves never resulted in turbidity formation. The experiments were carried out at pH 7.0-7.4 in either Tris or phosphate buffers. Final ionic strength was adjusted to approximately 0.12 with NaCl, and the final concentration of seminal vesicle proteins was approximately 20 mg/ml. The term "standard conditions" describes reaction mixtures of this type. The final optical density in such systems was in the vicinity of 1, and the lag period was defined arbitrarily as time (in minutes) required to reach an optical density of 0.1. Under these conditions changes in turbidity reflected the formation of a uniform, opaque coagulum. Turbidity changes in duplicate tubes invariably agreed within 5% of one another. Change in light absorption at 400 mμ was 1.5 to 1.8 times greater than that observed at 660 mμ, but followed the same rate of change with time. All experiments were performed with vesiculase and seminal vesicle proteins prepared from guinea pigs weighing between 450 and 600 g.

Results. Temperature and coagulation. In experiments of less than 4 hours duration, no insoluble material was formed from seminal vesicle proteins without addition of vesiculase below 30°. However, at 38°, a slow formation of insoluble material occurred in the absence of this coagulating enzyme under standard conditions. This "spontaneous" coagulation of seminal vesicle proteins occurred more rapidly, and to a greater extent, if the ionic strength was less than 0.10. However, final turbidity, observed without addition of vesiculase was, under standard conditions,

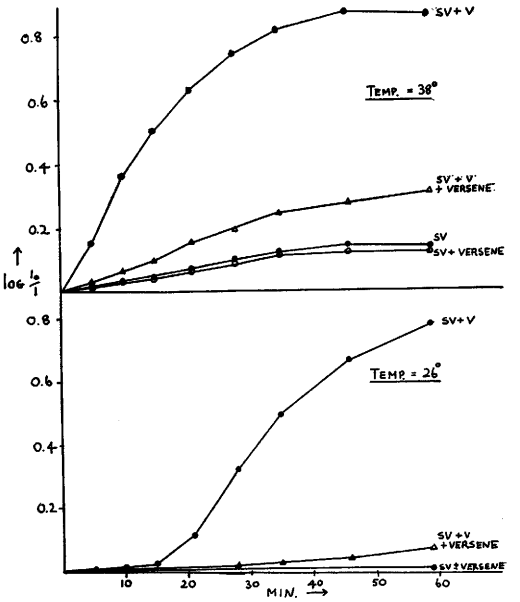

FIG. 1. Influence of temperature on vesiculase activity. Each vessel contained 50 μM Tris buffer of pH 7.4; 90 μM NaCl; 25 mg seminal vesicle protein and 0.7 mg vesiculase protein in a final vol of 1.2 ml. SV = seminal vesicle protein added; V = vesiculase protein added. Disodium versenate, if added: 0.3 μM. No change in optical density occurred at either temperature when vesiculase was added in the absence of seminal vesicle protein.

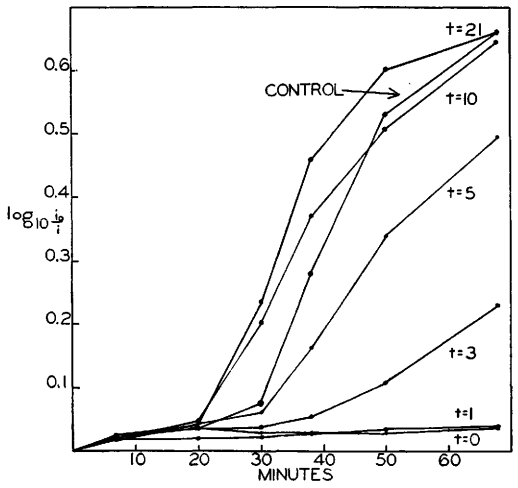

FIG. 2. Delayed addition of versene. Each cuvette contained 50 μM Tris buffer of pH 7.45; 105 μM NaCl; 25 mg seminal vesicle protein and 0.17 mg semi-purified vesiculase, in total vol of 1.42 ml. Disodium versenate (0.5 μM) added in vol of 0.02 ml. t = time in min. elapsing between mixing of all components and addition of versene. Temp. 25°.

usually less than one-tenth of that observed in the presence of vesiculase. Fig. 1 shows that the "spontaneous" coagulation at 38° was quite insensitive to versene, whereas, the vesiculase-induced coagulation was sensitive to versene at either 26° or 38°. Raising the temperature between these limits decreased the lag period, but had much less influence upon the rate of turbidity change once coagulation was initiated.

Inhibition by versene and by Hg^{++} ions. Previous experiments(1) demonstrated that the inhibitory action of versene on coagulation could be counteracted by Mn^{++} or Ca^{++} ions, neither of which affected vesiculase action in the absence of this chelating agent. Further insight into the role of divalent metal ions in the coagulation process was afforded by experiments in which vesiculase was allowed to react with seminal vesicle proteins for short periods of time, after which inhibitors were added to the reaction mixture. These experiments were conducted at ionic strengths such that no coagulation occurred during the period of preincubation of vesiculase (*cf.* 1). Typical experiments illustrated in Fig. 2 show that preincubation with vesiculase in the absence of versene for one minute did not permit coagulation to occur. However, preincubation with the enzyme for as little as 3 minutes resulted in coagulation even after addition of versene in concentrations sufficient to abolish coagulation when added prior to vesiculase. Fig. 3 shows that Hg^{++} ions, which also inhibited the over-all coagulation process, act in a manner different from versene. Thus, even after 10 minutes preincubation of seminal vesicle proteins with vesiculase, no coagulation took place following addition of Hg^{++} ions. Clearly, two distinct reactions are involved in the coagulation process. The first of these is sensitive to versene and does not necessarily involve preincubation of insoluble material, whereas the second, versene-insensitive stage leads to formation of a coagulum and is inhibited by Hg^{++} ions.

Miscellaneous agents. The following substances were without influence upon vesiculase-induced coagulation determined under standard conditions at 28°: spermine hydrochloride (0.001 M); glucose (0.04 M); glycerol (0.08 M) (*cf.* 6); 2,4-dinitrophenol (0.0007 M); crystalline soy bean trypsin inhibitor (0.07 mg/ml) and heparin (0.6 unit/ml). Higher concentrations of heparin caused precipitation of seminal vesicle proteins in the absence of vesiculase. Although cysteine (0.002 M) was without influence

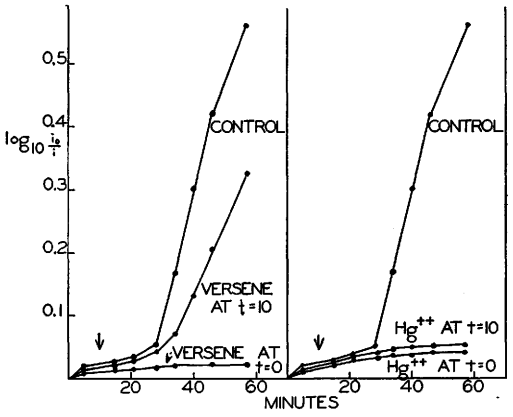

FIG. 3. Delayed addition of versene and of Hg^{++} ions. Each vessel contained 50 μM Tris buffer of pH 7.4; 25 mg seminal vesicle protein; 120 μM NaCl and 0.17 mg semi-purified vesiculase, in total vol of 1.42 ml. Disodium versenate (0.5 μM) or $HgCl_2$ (0.6 μM) added in vol of 0.02 ml. t = time in min. elapsing between mixing of all components and addition of inhibitors. Temp. 26°.

upon coagulation, and reversed the inhibitory action of Hg^{++} ions (0.00003 M)(1), low concentrations of 2,3-dimercaptopropanol (0.0002 M) abolished coagulation. At a final concentration of 0.0006 M, coagulation was unaffected by sodium citrate, adenosine triphosphate and sodium pyrophosphate. Higher concentrations of the latter substances inhibited coagulation, but it was not possible to ascertain whether this inhibitory action was due to their metal chelating properties, or to an increase in ionic strength resulting from addition of these multivalent ions. Profound inhibitory effects of high ionic strengths on vesiculase-induced coagulation of seminal vesicle proteins have been recorded elsewhere(1).

Attempts to induce coagulation with proteolytic and other enzymes. Many attempts were made to induce coagulation of seminal vesicle proteins in Tris buffer of pH 7.4 at high and at low ionic strengths by addition of crystalline or highly purified proteolytic enzymes. Over a concentration range of 0.005 to 1 mg/ml, the following enzymes failed to induce coagulation over a period of 4 hours at 25°: pepsin, trypsin, chymotrypsin, carboxypeptidase and papain. Moreover, addition of either hyaluronidase or takadiastase failed to induce coagulation. It has been reported previously(1) that thrombin will not clot seminal vesicle proteins. The coagulated material formed by action of vesiculase upon seminal vesicle proteins, after many washes with isotonic NaCl, was readily digested by trypsin and by chymotrypsin, and more rapidly by a mixture of these 2 proteolytic enzymes. Ultraviolet absorption spectra of these proteolytic digests (corrected for small absorption of the added proteolytic enzymes) showed distinct peaks at 280 mμ, and were fully consonant with the original protein nature of the coagula. Hyaluronidase was devoid of action upon the insoluble material produced by vesiculase action.

Discussion. The above experiments agree with previous reports(1,5) that coagulation of seminal vesicle proteins by vesiculase is an enzymatic denaturation of protein which bears little resemblance to the clotting of fibrinogen by thrombin. It is well established that in the latter reaction, the type of clot formed is dependent, *inter alia,* upon the composition of the medium employed. The ionic strength, pH and temperature affect in a most striking manner the opacity, tensile strength and electron microscopic structure of fibrin clots(6,7). Thus, it must be emphasized that under different experimental conditions, the turbidity changes resulting from vesiculase action upon seminal vesicle proteins may well reflect differences in the physicochemical state of the denatured proteins formed rather than differences in the primary action of vesiculase itself.

There is good evidence that the primary action of thrombin upon fibrinogen is proteolytic in nature. The ability of thrombin to hydrolyze tosyl-L-arginine methyl ester (TAMe) parallels its clotting activity, and TAMe itself inhibits coagulation of fibrinogen by thrombin(8). Vesiculase is not inhibited by either TAMe(1) or by soy bean trypsin inhibitor, which suggests that its primary action is different from thrombin or fibrinogen. Other studies(5) have emphasized that prostatic enzyme(s) capable of hydrolyzing TAMe are not identical with vesiculase. There is evidence that the action of thrombin upon fibrinogen leads to formation of acid-soluble peptides(9). Attempts to demonstrate liberation of acid-soluble nitrogen containing or ninhydrin reacting substances during coagulation of seminal vesicle proteins by vesiculase have been unsuccessful. However, the methods employed may not have been sufficiently sensitive to detect such materials. It is of interest that all of the proteolytic enzymes tested failed to induce clotting of the seminal vesicle proteins.

The first stage of vesiculase action appears to require a metal ion which is effectively chelated by versene, α, α′-dipyridyl and o-phenanthroline, and which can be replaced by Mn^{++} or Ca^{++} ions, but not by Mg^{++} ions. The nature of the "natural" activating metal cannot be ascertained from these studies. The substance bound by the metal chelating agents which inhibit coagulation appears to be present in the preparations of

seminal vesicle proteins rather than in the vesiculase preparations used, since coagulation induced by partially purified vesiculase, which had undergone repeated precipitation and dialysis, is not influenced by the addition of Mn^{++} or Ca^{++} ions to the reaction mixture (1).

Summary. Studies are presented on the influence of temperature and of a number of chemical substances upon coagulation of proteins of the seminal vesicle secretion by the prostatic enzyme vesiculase. Experiments involving delayed addition of metal chelating agents and of heavy metal ions have shown that the coagulation process can be separated into two distinct stages.

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Comparative Metabolism of Sr^{89} and Ca^{45} by Bone Grown *in vitro*.* (22856)

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The comparative metabolism of calcium and strontium has been of recent interest because of the possible hazards arising from ingestion of radioactive strontium. Calcium has been shown to be preferentially absorbed from the digestive tract(1) and strontium to be excreted to a greater extent than calcium by the kidney(1,2); the net result of both processes favors the retention of calcium over strontium. Evidence is lacking, however, that bone metabolism distinguishes between these two alkaline earth metals. The large discriminations of the intestinal tract and the kidney could mask any selective capacity of bone when the intact animal is used as the experimental subject. Therefore, embryonic chick bones were cultured *in vitro* in order to study the comparative metabolism of Sr^{89} and Ca^{45} by bone uninfluenced by the action of the other organs.

Most experiments have compared calcium to carrier-free radiostrontium. It seemed advisable for present purposes to include the uptake of radiostrontium in the presence of significant levels of carrier strontium. This would also have clinical aspects since stable strontium salts have been advocated as therapeutic agents to aid in the remineralization of bone(3).

Methods. The procedure used was essentially that of Fell(4) except that the bones, rather than resting in a plasma clot, were bathed by a liquid medium while in a roller-tube apparatus. Tibias and femurs were dissected from 7-day-old chick embryos and adhered to the walls of 18 x 150 mm pyrex test tubes in a chicken plasma clot. The medium consisted of 0.5 ml chick embryo extract, 2. ml of dog serum, 50 μl of Tyrode's solution containing about 0.1 and 0.05 μc of Ca^{45} and Sr^{89}, respectively, 100 units of penicillin, and 100 μg of streptomycin. The total amount of medium was increased by 50 μl in the experiments testing the effects of carrier strontium. The additions were made to the medium from

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