small intestine, cerebrospinal fluid and brain.

In the chest and peritoneal exudates produced by aleuronat-starch injection, the titres of bacitracin one hour after an intravenous administration approximated those in the blood.

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Production of an Inactive Derivative of Purified Prothrombin by Means of Purified Thrombin.* (17395)

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The blood clotting mechanism is of such complexity that the reactions which the several clotting factors can participate in may be followed with certainty only when these factors are isolated and obtained in purified form. With the use of such purified preparations, it has been shown that, depending upon the conditions of the experiment, thrombin may have entirely different effects on prothrombin. When small amounts of thrombin are added to prothrombin dissolved in 25% sodium citrate solution, the rate of activation of the prothrombin to thrombin is increased;[1,2] when thrombin and prothrombin react in physiological saline solution the prothrombin is changed to an inactive form in which it does not react to the addition of calcium, Ac-globulin and thromboplastin.[3,4] These effects are apparently produced consecutively when relatively large amounts of thrombin are used. There first appears a decrease in sensitivity to calcium, Ac-globulin and thromboplastin, then a reappearance of the original sensitivity. These experiments might be interpreted to mean that prothrombin is inactivated only to be changed back to its original active form again. It appears more reasonable, however, that thrombin acts first to form a slightly modified prothrombin which is not reactive to thromboplastin, and that this modified prothrombin is then changed to another active form of prothrombin which yields thrombin. The sequence may be described by the following: Prothrombin (sensitive to calcium + Ac-globulin + thromboplastin) → Prothrombin-derivative I (not sensitive to calcium + Ac-globulin + thromboplastin) → Prothrombin-derivative II (sensitive to calcium + Ac-globulin + thromboplastin) → Thrombin and possibly other reaction products. In this paper we shall show that the first prothrombin-derivative, a protein insensitive to calcium + Ac-globulin + thromboplastin, can be identified by electrophoresis.

Experimental procedures. A large homogeneous supply of prothrombin was obtained by pooling several preparations made as previously described.[4-6] Many experiments were performed but all of those described in this paper were performed on this pooled sample of prothrombin.

When dissolved in saline solution it retained at least 95% of its activity for 6 hours at room temperature. On electrophoresis the patterns (Fig. 1) showed that 95% of the total protein was present in one component in the descending boundary, while 87% was present in the ascending boundary. The impurities present in the prothrombin sample were thus shown to be sufficiently reduced to

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1 Seegers, W. H., and Ware, A. G., *Fed. Proc.*, 1949, **8**, 249.

2 Seegers, W. H., *Am. Heart J.*, in press.

3 Mertz, E. T., Seegers, W. H., and Smith, H. P., PROC. SOC. EXP. BIOL. AND MED., 1939, **41**, 657.

4 Ware, A. G., and Seegers, W. H., *J. Biol. Chem.*, 1948, **174**, 565.

5 Seegers, W. H., Loomis, E. C., and Vandenbelt, J. M., *Arch. Biochem.*, 1945, **6**, 85.

6 Seegers, W. H., *J. Biol. Chem.*, 1940, **136**, 103.

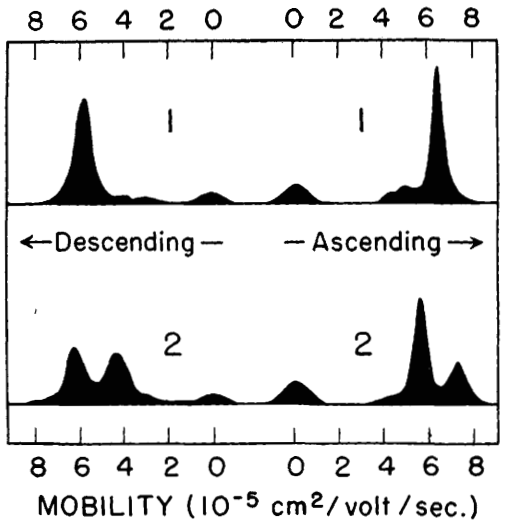

FIG. 1.

The electrophoretic boundary patterns of purified bovine prothrombin and of the same prothrombin after partial inactivation with purified thrombin, in barbital buffer, pH 8.5, ionic strength 0.1, and after 150 min. electrophoresis.

permit a study of the effect of thrombin on the boundary patterns and electrophoretic mobility of prothrombin.

The purified thrombin was prepared by dissolving sufficient purified prothrombin in a 25% solution of sodium citrate to make a 1% solution, adding a small amount of 3-methyl-4, 6, 4'-triaminodiphenyl sulfone and allowing the solution to stand at room temperature for 24 hours. The addition of the sulfone increases the yield of thrombin to a maximum. To separate the formed thrombin from the other substances present it was precipitated by the addition of ammonium sulfate, redissolved in water, and dialyzed free of salt. This method of preparing thrombin will be described in detail and presented for later publication. The important feature of this method is that thrombin is obtained from prothrombin without the use of calcium, Ac-globulin or thromboplastin and is thus free of these substances and other impurities present in even the best Ac-globulin and thromboplastin preparations now available. Thrombin prepared in this way is of higher purity than any previously described, and is of sufficient activity that in the amounts used in our reaction mixtures it could not be detected electrophoretically in the presence of the prothrombin.

Reaction mixtures were prepared from these purified preparations by dissolving in physiological saline sufficient prothrombin to give a concentration of 7,700 units per cc of solution and adding sufficient dry thrombin to bring its concentration to 100 units. The reaction mixture was allowed to stand for 6 hours at room temperature, when samples were removed for measurement of prothrombin activity, by the modified two-stage method[7,8] of analysis and the remainder of the reaction mixture was frozen, dried from the frozen state, dissolved in barbital buffer at pH 8.4 and 0.1 ionic strength and subjected to electrophoresis. The prothrombin activity decreased during the period of incubation to 62% of the original value. Comparison of the electrophoretic patterns of the prothrombin before and after incubation with thrombin (Fig. 1) reveals that the major component of the original sample was reduced in quantity from 87% to 32% in the ascending boundary and from 94% to 53% in the descending boundary. This protein which has disappeared from the major boundary reappeared in a second boundary which represented 64% of the total protein in the ascending boundary and 43% in the descending boundary. The decrease in prothrombin activity as measured by the two-stage method of analysis was in rough agreement with the shift of protein from the main electrophoretic component to the second component. The inactive derivative of prothrombin is characterized by an electrophoretic mobility of 5.60 (ascending) and 4.21 (descending) $\times 10^5$ cm²/volt/sec.

One of the principal difficulties encountered in early work[9] on the purification of prothrombin was the loss of prothrombin activity

7 Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.*, 1949, **19**, 471.

8 Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

9 Ware, A. G., Guest, M. M., and Seegers, W. H., *Am. J. Physiol.*, 1947, **150**, 58.

due to the action of small amounts of thrombin, formed spontaneously from the prothrombin. It was not appreciated at the time that the product formed from the prothrombin was the principal impurity of the preparation. The thrombin produced a derivative of prothrombin which was without typical activity. It is likely that the presence of this inactive prothrombin derivative is responsible for the polydisperse appearance of the prothrombin preparations examined in the ultracentrifuge.[10]

Summary. When purified prothrombin was allowed to react with a small amount of purified thrombin there was loss in prothrombin activity. Comparison of the electrophoretic patterns of the prothrombin before and after alteration with thrombin showed that protein disappeared from the curve representing prothrombin and appeared as a new component with a lower electrophoretic mobility than that of prothrombin.

[10] Seegers, W. H., and Ware, A. G., *Fed. Proc.*, 1948, **7**, 186.

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Acetylcholine and Blood Sugar. (17396)

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Intravenous injection of acetylcholine (100-500 α/kg, i.v.) produces hyperglycemia (25-60%) in rabbits. High doses of acetylcholine (500-1000 α/kg, i.v.) induce convulsions and a marked increase in blood sugar, up to 200%.

The mechanisms of the hyperglycemia provoked by acetylcholine have been investigated in a series of experiments. The observations may be summarized as follows:

1. Atropine (0.25 - 1 mg/kg, i.v.) does not abolish the acetylcholine hyperglycemia

2. The adrenolytic drug SY 28 (α-naphthyl-methyl-ethyl-β-bromo-ethylamine HBr), in doses of 1 to 3 mg/kg, does not abolish the hyperglycemia induced by acetylcholine or epinephrine.

3. The anticholinesterase drug:dimethyl-carbamate of hydroxyphenyl-benzyl-trimethyl-ammonium (Nu-683) (0.1 - 0.3 mg/kg i.v.) sensitizes the animal very markedly to the hyperglycemic actions of acetylcholine.

4. Tetraethylammonium (20 mg/kg i.v.) protects against acetylcholine-hyperglycemia, if doses of acetylcholine which do not induce convulsions are injected.

5. After removal of both adrenal glands and treatment with desoxycorticosterone-acetate, rabbits do not present hyperglycemia after doses of acetylcholine which do not induce convulsions. Higher doses of acetylcholine inducing convulsions still provoke hyperglycemia.

6. Nembutal (Sodium-ethyl(-l-methyl-butyl) barbiturate) anesthesia prevents acetylcholine-hyperglycemia.

7. Control experiments show that no changes in blood sugar occur after injections of the same doses of atropine, tetraethylammonium SY 28 or Nu-683.

8. High doses of Nu-683 (0.5 mg/kg) induce hyperglycemia, which is also prevented by tetraethylammonium.

Conclusions. In rabbits, doses of acetylcholine which do not provoke convulsions induce hyperglycemia.

This increase in blood sugar is prevented by the synaptic blocking agent tetraethylammonium, by nembutal and by removal of both adrenal glands.

The acetylcholine-hyperglycemia thus is induced by adrenal synaptic stimulation, which increases epinephrine output.

The acetycholine and epinephrine hyperglycemia are not abolished by an adrenolytic agent (SY 28). The hyperglycemia induced by high doses of an anticholinesterase drug (Nu-683) is also prevented by tetraethylammonium.

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