creased incidence of convulsions on B_{12} supplement, this group showed the highest body weight of any group (after 15 weeks of age). Whether or not the unsupplemented basal diet contained the optimal amount of B_{12} for our C_3H mice cannot be determined from the data available to us.

As seen in the Tables the incidence of "spontaneous" convulsive seizures in the first group of experimentals is higher than that obtained from the Exp. 2 regardless of diet. This difference may be attributed to the differences in caging. The first experimental group of mice was caged singly while the latter group was kept two per cage. It has been reported(1) that when C_3H male mice are housed 2 per cage, the incidence of "spontaneous" seizures was reduced and with twenty to a large cage the incidence was negligibly small. A factor of a "social" nature appears to be implicated.

Summary. 1. The incidence of "spontaneous" convulsions and of convulsive deaths in C_3H male mice is greatly reduced by caloric restriction or by supplement of vit. B_{12} in the diet fed *ad libitum*. Substitution of hydrogenated vegetable fat for lard decreased the age of onset of convulsions and increased the incidence of convulsive deaths. 2. Studies with auditory and electric stimulation appear to confirm the existence of a difference between the effect of HVF and of lard.

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Influence of Thrombin on Rate of Prothrombin Conversion. (23169)

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There appears to be general agreement among investigators of blood clotting that thrombin accelerates its own formation from prothrombin. However, the specific mode of this action of thrombin is, at present, not completely resolved. The present work is concerned with an attempt to show that traces of thrombin will accelerate the formation of a prothrombin-conversion factor. Inasmuch as it has been found that plasma accelerator-globulin*(1,2) and probably also other clotting factors(2) may be associated with platelets, a phospholipid preparation from beef brain corresponding to the phosphatidyl ethanolamine fraction of Folch(3) has been used as a substitute for platelets.

Materials. Antihemophilic Factor (AHF) and Plasma Accelerator-Globulin (PAcG): The method used for preparation of these 2 factors was a combination of the procedures described by Bidwell(4) and by Lewis and Ware(5). $BaSO_4$-treated oxalated bovine plasma was diluted 1:20 with distilled water and the pH adjusted to 5.5. Precipitate from 200 ml plasma was dissolved in 90 ml of 0.9% NaCl (pH 7.0) and the solution ad-

* For a different terminology of factors referred to in this paper reference is made to Appendix, The Coagulation of Blood. Methods of Study. Edited by L. M. Tocantins, Grune and Stratton, N. Y., 1955.

justed to a concentration of 10% sodium citrate by the dropwise addition of a 40% sodium citrate solution. Precipitate and supernatant solution were then worked up separately. Antihemophilic factor (AHF); precipitate was taken up in 75 ml of 0.9% NaCl (pH 7.0), heated for 10 min at 52°C and precipitate discarded. Supernatant fluid was taken to 0.3 saturation of $(NH_4)_2SO_4$ with solid $(NH_4)_2SO_4$. Precipitate was dissolved in a small amount of 0.9% NaCl (pH 7.0) and dialyzed in the cold against 0.9% NaCl (pH 7.0). The solution was then lyophilized. Plasma accelerator-globulin (PAcG); supernatant solution (described above) was brought to 25% sodium citrate concentration. Precipitate was dissolved in 70 ml of cold 0.9% NaCl (pH 7.0) and precipitated between 0.33 to 0.5 saturation of $(NH_4)_2SO_4$ by the addition of a saturated $(NH_4)_2SO_4$ solution. Precipitate was dissolved in a small amount of 0.9% NaCl (pH 7.0) and dialyzed in the cold against 0.9% NaCl (pH 7.0). This material was frozen in small aliquots and used as a stock solution. *Prothrombin*: Prepared according to the procedure described by Surgenor *et al.*(6). The prothrombin preparation, assayed according to the method of Ware and Seegers(7), was found to contain 100 units per mg of protein. *Serum eluate preparation (SE)*: Previously frozen rabbit serum was thawed, allowed to stand at room temperature for 24 hours and 0.1 M sodium oxalate added in the ratio of 1 volume to 9 volumes of serum. After addition of $BaSO_4$ (100 mg/ml) the mixture was stirred for 20 min. The $BaSO_4$ was collected by centrifugation and washed with an equal volume of 0.9% sodium chloride-sodium oxalate mixture (9 parts of 0.9% NaCl to 1 part of 0.1 M sodium oxalate) and then with 0.9% NaCl. The $BaSO_4$ was eluted twice with 0.1 M sodium citrate equal to 1/10 the original volume of serum. The eluates were combined, brought to 50% saturation with solid ammonium sulfate and kept at 4°C overnight. The precipitate was filtered off, collected in a small amount of distilled water and dialyzed in the cold against distilled water until completely free of ammonium sulfate. After centrifugation the precipitate was extracted with 0.1% ammonium carbonate, centrifuged and the supernatant solution lyophilized. The proconvertin activity of this preparation, determined according to the method of Owren and Aas(8), was found to be 400 units per mg. One unit of proconvertin was arbitrarily defined as that amount of preparation which will give a clotting time equal to that of 0.1 ml of a 1:50 dilution of normal rabbit plasma as assayed by the above method. *Thrombin*: Parke, Davis Topical Thrombin (1% solution in oxalated 0.9% NaCl) was stirred with $BaSO_4$ (50 mg/ml) for 20 min at room temperature. The $BaSO_4$ was centrifuged down, the decanted supernatant solution adjusted to pH 7.2 with 2 N NaOH and taken to 38% saturation with solid ammonium sulfate. After centrifugation the supernatant solution was adjusted to pH 5.2 with 2 N HCl and taken to 70% saturation with solid ammonium sulfate. The precipitate obtained after centrifugation was dissolved in a small amount of water and dialyzed against cold distilled water until free of ammonium sulfate. The dialysate was centrifuged and the supernatant solution stored in small aliquots in the frozen state. Thrombin activity was equal to 250 u/ml with a protein concentration of 3.56 mg/ml. *Fibrinogen*: Fibrinogen was prepared according to the procedure described by Ware *et al.*(9). The stock solution was diluted 1:20 with 0.9% NaCl adjusted to pH 7.0. Final concentration of fibrinogen was 0.16%. *Phospholipid*: Folch's (3) method was used for the preparation of a cephalin fraction corresponding to phosphatidyl ethanolamine.

Methods. The rate of prothrombin conversion was determined in two ways: A "1-stage" procedure in which the reactants involved in prothrombin conversion were mixed simultaneously, and a "2-stage" procedure in which certain reactants supposedly involved in the formation of a prothrombin-conversion factor were preincubated prior to addition of the prothrombin preparation. The testing procedure consisted of preparing a reaction mixture of different factors and measuring amount of thrombin formed at various time intervals of incubation at 26°C by adding a measured amount of the reaction mixture to a fibrinogen solution at 37°C. Activity of the

reaction mixtures used in this investigation was determined by degree of conversion of prothrombin to thrombin. *One-stage reaction*: The reaction mixture consisted of the following: prothrombin 0.2 ml (5 mg/ml), serum-eluate 0.2 ml (0.2 mg/ml), phospholipid 0.05 ml (1 mg/ml), plasma accelerator-globulin 0.25 ml (2 mg/ml), antihemophilic factor 0.5 ml (0.4 mg/ml), thrombin 0.02 ml (5 NIH units), and 0.125 M $CaCl_2$ 0.1 ml. The final volume was adjusted to 2.70 ml by the addition of 0.9% NaCl (pH 7.0); the final reaction mixture had a pH of 7.0. At various time intervals of incubation at 26°C 0.1 ml of the reaction mixture was added to 0.4 ml of fibrinogen at 37°C. When the clotting times (as obtained by the tilt method) were below 15 sec, the 0.1 ml portions of the reaction mixture were suitably diluted with 0.9% saline (pH 7.0) before addition to the fibrinogen solution. The recorded clotting times were interpreted in terms of National Institutes of Health (NIH) Thrombin units from a curve prepared with a Standard NIH Thrombin preparation.† *Two-stage reaction*: This reaction was carried out with the same preparations in identical proportions as described for the 1-stage reaction. However a preliminary reaction was allowed to take place by first incubating serum-eluate, phospholipid, plasma accelerator-globulin, antihemophilic factor, and $CaCl_2$ in the absence or presence of thrombin for 10 min at 26°C before adding the prothrombin. After addition of prothrombin the generation of thrombin at 26°C was followed in the same manner as described above for the 1-stage reaction.

Results. The results reported here are average values of at least 8 separate experiments. Variation in any given series of experiments never exceeded 10%. Though the results reported are for a period of only 15 minutes, experimental measurements were recorded for a period of 60 minutes. It was found that the percent conversion reached a maximum within 15 minutes and remained constant throughout the 60 minute period. A

† Kindly supplied by Biologics Control Laboratory, N.I.H., Bethesda, Md.

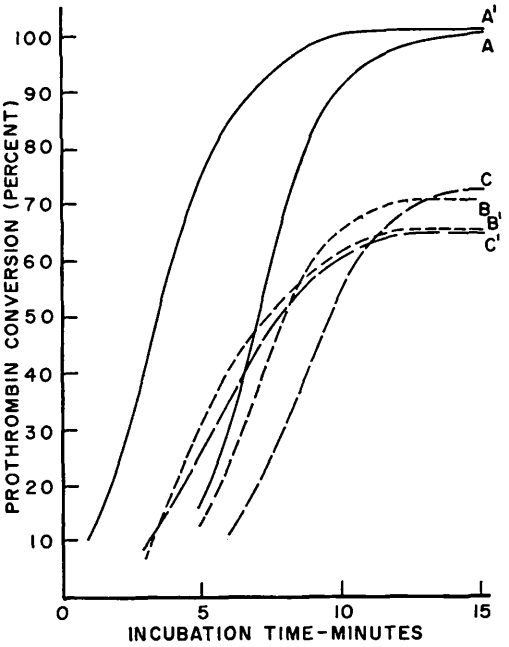

FIG. 1. Influence of thrombin on rate of prothrombin conversion (one-stage). A = Complete reaction mixture without thrombin. A′ = Complete reaction mixture with thrombin. B = Reaction mixture minus AHF without thrombin. B′ = Reaction mixture minus AHF with thrombin. C = Reaction mixture minus PAcG without thrombin. C′ = Reaction mixture minus PAcG with thrombin.

reaction mixture containing serum-eluate, phospholipid, $CaCl_2$ and prothrombin yielded only traces of thrombin. However, if PAcG is added to the above reaction mixture (Fig. 1 curve B) an average of 71% of the prothrombin present is converted to thrombin. If in place of PAcG, AHF is added to the reaction mixture (Fig. 1 curve C), again only about 77% of the prothrombin is converted to thrombin. It is not until AHF and PAcG are present together in the reaction mixture containing serum-eluate, phospholipid, $CaCl_2$ and prothrombin (Fig. 1 curve A), that complete conversion of prothrombin to thrombin takes place.

It will be noticed in Fig. 1 that in each of the reactions there is a definite lag period during which there is no detectable conversion of prothrombin to thrombin. However, after a definite period of time has elapsed and thrombin formation has started, conversion of prothrombin takes place quite rapidly. It can also be seen (Fig. 1) that addition of

thrombin (5 NIH units) to either one of the reaction mixtures decreases this lag period. In the complete reaction mixture, the lag period is almost entirely eliminated. It was found that addition of thrombin did not reduce the lag period or enhance conversion of prothrombin in the reaction mixture containing only serum-eluate, phospholipid, $CaCl_2$ and prothrombin. It would seem, therefore, that thrombin affects the activity of PAcG and AHF. Fig. 1 also shows that addition of thrombin caused a faster conversion of prothrombin to thrombin but did not increase amount of thrombin formed.

Since the activities of the PAcG and AHF preparations seemed to be alike in this test reaction, and since the effect of thrombin on each of these preparations appeared to be similar, varying concentrations of both factors were used in the reaction mixture. If both preparations were one and the same factor, doubling the concentration of one factor in the absence of the other should have increased degree of prothrombin conversion. It was found that degree as well as rate of conversion of prothrombin to thrombin remained the same when the amount of AHF was doubled. Similarly, twice the amount of PAcG in a reaction mixture containing no AHF did not increase extent or rate of conversion of prothrombin. This would indicate that AHF and PAcG preparations employed in these studies are different agents and that both factors must be present in the reaction mixture to attain complete conversion of prothrombin to thrombin.

If rate of prothrombin conversion obtained in a 1-stage procedure using a complete reaction mixture minus thrombin is compared with rate of prothrombin conversion in a 2-stage procedure using the same agents (Fig. 2 curves A and B), it may be seen that preincubation of serum-eluate, AHF, PAcG, phospholipid and $CaCl_2$ shortened the lag phase. These results seem to indicate that incubation of phospholipid, AHF, PAcG, serum-eluate and calcium ions yields a prothrombin-conversion factor. In the absence of phospholipid or of serum-eluate there was found to be no formation of a prothrombin-conversion

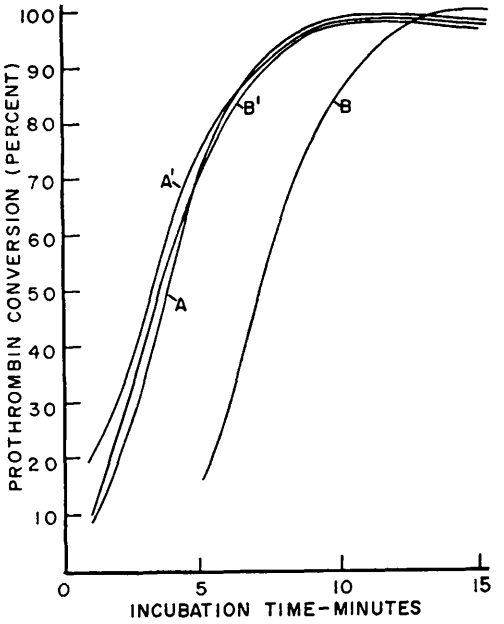

FIG. 2. Influence of thrombin on the formation of prothrombin conversion factor (two-stage). A = 2-stage, complete reaction mixture without thrombin. A' = 2-stage, complete reaction mixture with thrombin. B = 1-stage, complete reaction mixture without thrombin. B' = 1-stage, complete reaction mixture with thrombin.

factor. The activity of the serum-eluate preparation may be due to the presence of plasma thromboplastin component* or of proconvertin* or of both.

It is interesting to note that the lag period (Fig. 2) was greatly shortened in the 1-stage reaction containing all the reactants including thrombin and in the 2-stage reaction containing a complete reaction mixture with or without thrombin. It might seem from inspection of the curves A and A' of Fig. 2 that the 2-stage reaction mixture in the presence of thrombin produced the prothrombin-conversion factor at a faster rate than did the 2-stage reaction mixture without thrombin. However, the difference was too small to allow any definite conclusions.

Discussion. The results on rate of prothrombin conversion in a 1-stage procedure (Fig. 1) seem to indicate that action of PAcG as well as that of AHF is enhanced by traces of thrombin. These observations support the finding of Seegers and his associates(10) demonstrating the importance of traces of throm-

bin on rate and completeness of prothrombin conversion under various experimental conditions in a 1-stage reaction procedure. They expressed the opinion that thrombin converted PAcG in the reaction mixture to the more active serum accelerator-globulin. According to Lanchantin and Ware(11) thrombin has a specific proteolytic effect on plasma accelerator-globulin which is associated with a significant increase in accelerator-globulin activity. The above results are also in agreement with the assumption of Quick and his associates(12) that thrombin activates thromboplastinogen (AHF). As can be seen from Fig. 1, traces of thrombin shortened the lag period of prothrombin conversion. It appears that once a certain amount of thrombin (either added as such or formed during the reaction) is present, the conversion of prothrombin to thrombin proceeds at a comparatively rapid rate.

From the data presented here, it appears that thrombin will accelerate formation of the prothrombin-conversion factor. Fig. 1 and 2 illustrate that the slopes of the curves are the same in the absence or presence of thrombin. This indicates that rate of prothrombin conversion is the same in all cases. The effect of thrombin was to shorten the lag period of the start of thrombin formation. This lag period seems to be a function of rate of formation of the prothrombin-conversion factor. In the presence of thrombin the rate of formation of the prothrombin-conversion factor becomes accelerated. Once sufficient prothrombin-conversion factor is formed, the conversion of prothrombin to thrombin takes place quite rapidly. Inasmuch as traces of thrombin will apparently accelerate formation of a prothrombin-conversion factor, a proper evaluation of a thromboplastin generation test should take into account the possible presence of trace amounts of thrombin derived from prothrombin in the serum employed.

Summary. Both antihemophilic factor and plasma accelerator-globulin are necessary to obtain complete conversion of prothrombin in the presence of a $BaSO_4$-eluate preparation from serum, phospholipid-thromboplastin and $CaCl_2$. In the presence of traces of thrombin conversion of prothrombin to thrombin becomes greatly accelerated. Results indicate that thrombin greatly accelerates formation of a prothrombin-conversion factor. This influence of thrombin appears to be mediated through its activating effect on plasma accelerator-globulin and, perhaps, also on the antihemophilic factor.

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