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Experiments on Decalcifying Anticoagulants.

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Reagents used.[1] 1. Prothrombin-free fibrinogen (dog). 2. Howell prothrombin. 3. (Calf) brain cephalin (active in 1: 2,000,000 dilution). 4. M/10 $CaCl_2$. 5. M/1 (etc.) potassium oxalate and trisodium citrate. Incubations and tests conducted at 37-38°C.

Controls. An initial series of tests established the lower limits of the calcium optimum in the activation of prothrombin-cephalin mixtures. Incubation of coagulant mixtures for at least 10 minutes prior to addition of fibrinogen ensured thrombin of maximal potency: *e. g.,* 1 cc. of fibrinogen was clotted solidly in a matter of seconds by the following: prothrombin, 0.5 cc. + 0.1% cephalin, 0.1 cc. + M/10 $CaCl_2$, 0.1 cc. In order to validate the significance of the clotting time (C.T.) delay in the accompanying experiments, routine controls were run with and without simple dilution (distilled water or 0.9% NaCl). The control thrombins retained their potency with but slight diminution of activity (? metathrombin formation) over several hours.

Oxalation (or citration) during thrombin formation and aging. A 30-50-fold excess (chemical equivalents) of oxalate (or citrate): added calcium (of the thrombic mixture) entirely prevented the formation of thrombin if the anticoagulant was added during the first 4-5 minutes of incubation. During the next hour the already activated thrombin gave good clots although somewhat delayed by the presence of oxalate (or citrate). Older thrombins yielded progressively slower and poorer clots in contact with the anticoagulant.

Effect of increasing concentrations of anticoagulant on thrombin of fixed (maximal) potency. Fresh (maximally active) thrombin gave correspondingly longer clotting times when added to a series of test fibrinogens containing increasing amounts of oxalate or citrate. There was a minimum below which the C. T. was identical with controls, and a maximum above which the C. T. delay was not significantly extended. Indeed, with very great excess, especially in the citrate experiments (verified repeatedly), a return of C. T. to

[1] Ferguson, J. H., *Am. J. Physiol.,* 1936 (in press).

definitely shorter values was noted. A possible correlation with clinical haemostasis by citrate injections is mooted.

Progressive inactivation of thrombin on incubation with oxalate or citrate. After maximal potency had developed (*e. g.,* 10-15 min. incubation) the thrombin was divided into two parts, mixed respectively with (a) 50-100 equivalents of oxalate or citrate, and (b) an equal volume of simple diluent, and the incubation continued. Tests made at varying intervals ranging from a few seconds to 2 hours invariably demonstrated (1) an *immediate* retarding effect of the anticoagulant, varying in degree according to the concentration of oxalate (or citrate) used and comparable to the findings of the foregoing experiments; and (2) a *progressive* inactivation, resembling the phenomenon described by Loucks and Scott.[2] Whereas controls still clotted in a minute or two (even after 2 hours), the oxalated (or citrated) thrombin could be weakened to give clots only after many minutes or hours or (in a few instances) not at all. The inhibitory phenomenon was also manifest in the slower completion of clotting after its commencement, and certain of the delayed clots were flocculent and incomplete.

Conclusion. It has long been established that thrombin, once formed, is active in the presence of excess of oxalate, etc., this being the classical experiment in support of the view that ionized calcium is not necessary for the clotting process proper ("second phase" of Hammarsten). It is less widely appreciated that clotting under these conditions is definitely slower than normal. Forty years ago Hammarsten[3] failed to inactivate thrombin by oxalation, and Kastl[4] and Eagle[5] have recently confirmed. The divergent findings of Loucks and Scott[2] are supported by the present experiments. Since the data appear to be unequivocal, and since their importance in the elucidation of the calcium factor in coagulation is obvious, it is imperative that further investigations be made with a view to explaining the discrepancies in the literature and elucidating further the mechanism of action of the so-called "decalcifying" anticoagulants.

2 Loucks, M. M., and Scott, F. H., *Am. J. Physiol.*, 1930, **91**, 27.

3 Hammarsten, O., *Hoppe Seylers Ztschr. f. physiol. Chem.*, 1896, **22**, 333; 1899, **28**, 98.

4 Kastl, O., *Biochem. Ztschr.*, 1934, **274**, 452.

5 Eagle, H., *J. Gen. Physiol.*, 1935, **18**, 531, 547.