# Manganous Ion and Activation of Prothrombokinase.* (18380)

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At the time 3-stage analysis of blood coagulation was originally described, it was reported that the activation of prothrombokinase involves an autocatalytic or chain reaction(1). The simplest explanation seems to be that thrombokinase is an enzyme, capable of activating both prothrombin and prothrombokinase(2).

1. Prothrombokinase —Thrombokinase→ Thrombokinase
2. Prothrombin —Thrombokinase→ Thrombin
3. Fibrinogen —Thrombin→ Fibrin

In crude systems, calcium is important for the normal progress of the first 2 reactions. Subsequent results with the 3-stage procedure (3,4) have favored the view that the true activator of the prokinase is kinase, as indicated above, rather than thrombin or lipoid thromboplastin. Further usefulness of the 3-stage procedure is demonstrated by the results with manganous ion.

In unpublished experiments by Hellerman, Milstone and Carnes (mentioned by Hellerman(9), p. 479) it was found that $Ni^{++}$, $Co^{++}$, and $Mn^{++}$ retarded production of thrombin in crude systems [cf. Deetjen(5) and others (6-8)]. At that time it was noted that $Mn^{++}$ which inhibited only at relatively high concentrations, also had an acceleratory effect at low concentrations. Fig. 1 illustrates the results obtained by the 2-stage procedure and confirms the earlier unpublished findings. An outstanding effect was shortening of the latent period. The latent period is concerned with the activation of the prokinase which is contained in the crude prothrombin(2).

The effect of 0.0001 M $Mn^{++}$ on the activation of crude prokinase is brought into bold relief by the 3-stage test of Fig. 2. In the presence of $Mn^{++}$, the peak kinase activity was reached in half the time required for the control. The decline of kinase activity was rapid, as it also was in the control. Subsequently, the activity of the control remained

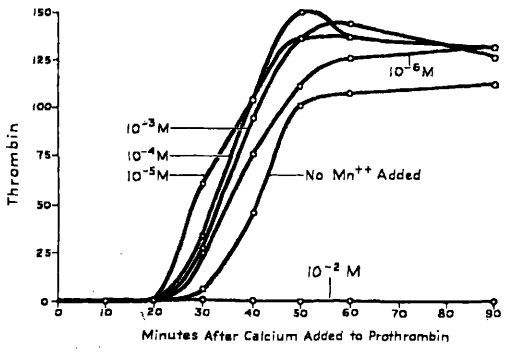


FIG. 1.

Two-stage experiment, showing that latent period in activation of crude prothrombin was shortened by addition of $Mn^{++}$. A stock 0.11 M sol. was prepared from Mallinckrodt analytical reagent, $MnCl_2 \cdot 4H_2O$; and 10-fold dilutions were made in buffer. The figures on the chart give approximate final molarities of added $Mn^{++}$ in respective activation mixtures. Activation mixtures contained 0.1 ml heated crude prothrombin, 0.8 ml buffer, 0.1 M. $MnCl_2$ dilution and 0.1 ml $CaCl_2$ 0.0275 M. $Mn^{++}$ was added 15 sec. before $Ca^{++}$. The tests were started at one-minute intervals and run in parallel, using same batches of reagents. Materials were prepared and tests conducted as previously described(3). The latent period was shortened by all tested concentration of $Mn^{++}$, except $10^{-2}$ M. In the presence of $10^{-2}$ M $Mn^{++}$, appearance of thrombin was completely suppressed during the test period.

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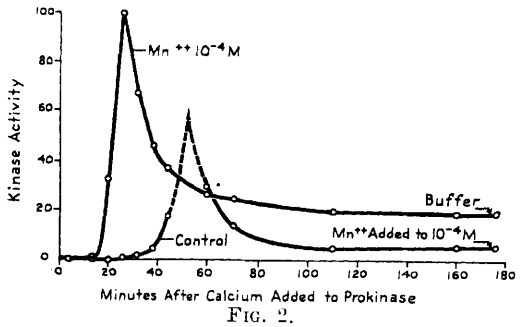


FIG. 2.

Three-stage experiment showing that $Mn^{++}$ accelerated the activation of crude prokinase. Method and materials were essentially those described elsewhere(3). $Mn^{++}$, to make a final concentration of $10^{-4}$ M, was added to prokinase 15 sec. before $Ca^{++}$. At intervals, samples were assayed for kinase activity by determining amount of prothrombin converted to thrombin in 4 min. For example, at 26 min. (first peak on chart), a sample from prokinase mixture (stage 1) was added to prothrombin reagent (stage 2). This stage-2 mixture, sampled at 4 min., gave a clot in 30 sec., when tested on oxalated fibrinogen (stage 3). Test and prokinase control, with buffer instead of $Mn^{++}$, were run one minute apart. 174 min. after the start, $Mn^{++}$ to make a final concentration of $10^{-4}$ M was added to control. This addition reduced the concentration of protein and calcium by 9%. At the same time, a similar 10/11 dilution of the test was made with buffer. These slight dilutions, as well as the uncontrolled 10/11 dilution of $Mn^{++}$ in the test mixture, had no appreciable effect, as shown by the results. It is notable that the late addition of $Mn^{++}$ to the control did not raise its kinase activity, as measured 2 min. after $Mn^{++}$ was added.

below that of the test and was not then raised by addition of $Mn^{++}$ to 0.0001 M. Thus the principal effect of $Mn^{++}$ was exerted before or during the activation of prokinase, and could not be attributed to an enhancement of ripe kinase activity, as *measured by the conversion of prothrombin.* In other words, $Mn^{++}$ did not appreciably accelerate the activation of prothrombin by kinase, under these conditions. This corroborates the impression gained from Fig. 1. There it is seen that, once conversion of prothrombin began, $10^{-3}$ to $10^{-6}$ M $Mn^{++}$ exerted little effect on its rate, with the result that the rising portions of the curves were almost parallel.

Supplementary tests showed that 0.001 M $Mn^{++}$ was more effective than 0.00001 M $Mn^{++}$ in this 3-stage system. Calcium 0.0025 M, was present in all activation tests.

*Discussion.* The contrast between Figs. 1 and 2 emphasizes that the 3-stage test brings out details not readily discernible in 2-stage tests. Consequently, the new test serves not only to localize certain effects, but also to raise new questions. $Mn^{++}$ caused much acceleration of the activation of prokinase and little, if any, of the conversion of prothrombin. Even if the materials were pure, these results would not necessarily militate against the hypothesis that the same enzyme catalyzes both activations. Calcium salts hasten the activation of trypsinogen, but not of chymotrypsinogen, although both conversions are catalyzed by trypsin(10). Since the materials are not pure, it is not known whether $Mn^{++}$ brings about the acceleration by reacting with some impurity in the prokinase; nor is it known whether $Mn^{++}$ would accelerate conversion of prothrombin under other circumstances.

The rapid loss of kinase activity promises to offer difficulties as more attention is given to the activation and assay of prokinase. It is difficult to judge the maximum activity when the peak is sharp, or when it falls between 2 points, as illustrated by the control curve of Fig. 2. Even the true peak probably represents the difference between the total activity developed and that portion of it which has already been lost.

In the present tests, the rise of kinase activity was probably much slower than occurs physiologically; and this may also be true for its fall. Relative to its rise, the decline of kinase activity was so precipitous as to suggest that it could help, *in vivo,* to keep the clotting process within physiologic bounds (11). The *in vitro* effect is possibly related to the losses of kinase or thromboplastic activity reported by others(12-14). It may represent a decrease of kinase, *per se,* or a change in some factor which conditions kinase activity.

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*Summary.* Manganous ion, 0.0001 M, hastened activation of crude prothrombokinase in a 3-stage analysis of blood coagulation. It did not prevent the subsequent steep decline of thrombokinase activity. Rapid quenching of thrombokinase activity might help in the physiologic control of blood clotting.

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## Relation of Hair Proliferation to Damage Induced in the Mouse Skin. (18381)

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Physiological conditions prevailing in the skin at the time of experimental treatment with chemical or physical agents may be expected to influence the nature and degree of the response. A condition that may be readily observed and controlled is the stage of development of the hair. In the developmental cycle of a hair follicle, the anagen stage is the phase of active growth and development (17 days). In a more detailed analysis of the anagen phase, Chase *et al.*(1) have observed 6 clearly defined substages. The subsequent catagen stage is the phase of growth cessation, club hair formation, and setting aside of the dormant hair germ for the next hair generation (2 days). The telogen stage is the resting phase(2). The degree of injury which can be inflicted upon skin appears to depend not only upon the types of treatment but also upon the stage of development of the existing hair follicles. X-radiation with 300 r to 1000 r during anagen stages results in cessation of development in the follicle and the loss of any growing hair. During catagen and telogen stages, such x-ray doses cause no loss of hair, the club hairs remaining apparently undisturbed(3). In addition, x-radiation has also a profound effect upon hair pigment. Pigment loss for the ensuing hair generations is greatest in follicles x-rayed in catagen or telogen(3-5). In contrast to the effects of x-ray on the hair, single or repeated applications by painting of irritants such as $K_2PO_4$, ether, benzene(6), or methylcholanthrene-in-benzene have no effect on hairs in anagen but cause loss of hairs in the telogen stage. Whereas such doses of x-radiation have only a slight effect upon the pigment cells in the epidermis, upon the integrity of sebaceous glands, and upon hyperplasia of the epidermis—a single painting of 0.6% methylcholanthrene-in-benzene results in increased melanization in dendritic cells of the epidermis, loss of sebaceous glands(7-9), and hyperplasia of the epidermis. The painting, however, has no effect upon the growing hairs. After the loss of sebaceous glands by treatment with methylcholanthrene, a complete redifferentiation of the glands from external sheath cells occurs if and when there is active hair development in the follicles(9).

*Methods.* Experiments were carried out to

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