Prothrombin Activation Cycle.* (23831)

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It was remarkable to observe that prothrombin loses its activity when mixed with a small amount of thrombin(1). This loss in activity consists of becoming refractory to activation in the presence of lung thromboplastin, calcium ions and Ac-globulin (2-stage analytical reagents). When the proportion of thrombin to prothrombin is increased the prothrombin not only loses its activity but also partly regains it again, and then finally becomes thrombin. All this takes about 2 days (2). Apparently the same events occur in 25% sodium citrate solution, but far more rapidly(3). The sequence may be described by the following: Prothrombin (sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin-derivative I (not sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin-derivative II (sensitive to calcium + Ac-globulin + lung thromboplastin) → Thrombin and possibly other reaction products.

The prothrombin activation cycle described above is completed when thrombin is added to prothrombin. Perhaps the cycle is also negotiated when prothrombin activates rapidly in the presence of procoagulants, but then this transpires so fast that no means devised thus far is suitable for following the changes. Any phenomena that are natural in accelerated autocatalytic activation of prothrombin might be expected to occur in the molecule left by itself, if no side reactions interfere. For this reason we supposed that prothrombin alone might lose its activity and subsequently regain it. In this brief presentation we describe conditions that fulfill this requirement.

Methods. Prothrombin. This was prepared from bovine plasma by methods developed in this laboratory(4). The last traces of Ac-globulin were not removed. The specific activity was usually near 25,000 U/mg tyrosine. *Prothrombin and thrombin activity.* These assays were performed as previously described(5,6).

Results. A spontaneous activation cycle was first noticed when a lyophilized product of prothrombin was dissolved in physiological saline, buffered with imidazole at pH 7.35 to give a solution containing 11,600 U/ml. This was kept in a stoppered test tube, refrigerated (4°C), and the activity of prothrombin was measured every 2 to 3 days. The prothrombin activity decreased to 5000 U/ml in 4 days but gradually returned to the original level and higher in 15 days. By 29 days it declined to 1000 U/ml and further analyses were impossible because the prothrombin solution was exhausted.

Further studies were then started. A prothrombin product was dissolved in M/15 phosphate buffer at pH 6.0 and 7.2 and a third sample was dissolved in veronal buffer of 0.1 ionic strength at pH 8.6. Each of these solutions contained 5150 U/ml of prothrombin. These were stored at 4°C and prothrombin activity was measured every 2-3 days. The prothrombin activation cycle (Fig. 1) shows itself at each of these hydrogen ion concentrations. The loss and gain in prothrombin activity was without detectable thrombin in the solutions. In experiments previously described(1,2,3) thrombin was added and presumably accelerates the alterations that prothrombin alone can undergo. In these experiments the appearance of thrombin was seen late. After 28 days there were about 100 U/ml of thrombin in the solution of prothrombin at pH 8.6 and thrombin activity increased to 300 U/ml 2 days later. Only a very small amount of thrombin was measured in solutions at pH 6.0 and 7.2 by this time, but a month later there were 140

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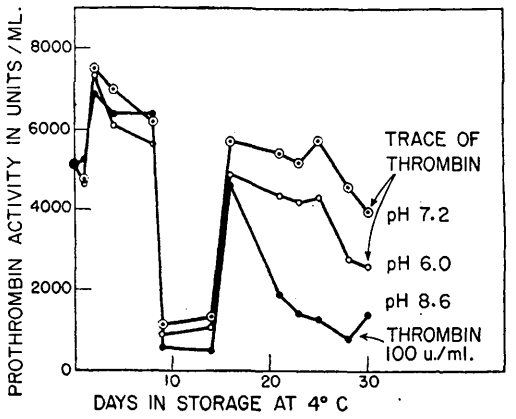

FIG. 1. Changes in reactivity of purified prothrombin stored at 4°C, in M/15 phosphate buffer or veronal buffer 0.1 ionic strength and pH 8.5. Assay by 2-stage analysis.

U/ml and 1050 U/ml of thrombin respectively.

All prothrombin preparations so far used in these experiments were dried from the frozen state. To see whether this process of drying had anything to do with the activation cycle the lyophilizing process was omitted and a prothrombin product in water solution was stored and observed. Two products of prothrombin were followed through the first phases of the cycle illustrated by means of Fig. 1. The original activity of each of these 2 prothrombin solutions was 15,000 U/ml. After 14 or 20 days of storage the prothrombin activity went up to 18,000 U/ml then gradually came down to 10,000 U/ml and finally reached 5000 U/ml in one and 2000 U/ml in the other case. At this point which was 49 days after storage at 4°C thrombin developed in both solutions.

In further experiments prothrombin was studied with respect to salt. Prothrombin (not lyophilized) was dissolved respectively in distilled water, 0.9% NaCl solution and saturated NaCl solution all at pH 7.0. Again the events observed were essentially like that seen in other experiments, however, the prothrombin in the saturated NaCl solution lost its activity more rapidly and did not regain reactivity very extensively. By about 35 days all produced thrombin ranging from 400 U/ml (water and 0.9 NaCl) to less than 1 U/ml (saturated NaCl). Similar results were obtained with a prothrombin product which was first dried from the frozen state.

Discussion. Prothrombin preparations can go through an activation cycle spontaneously, and without detectable thrombin until the last stages. This is most likely a property of the prothrombin molecule itself and does not require the addition of procoagulants or even the activator which is thrombin. Since this is natural to prothrombin, and occurs more rapidly in the presence of thrombin we think this very likely is a cycle that is partly or fully completed with prothrombin activation in our ordinary physiology. Material that has only gone through the inactivation phase could be a constituent of normal plasma. Since no one has observed these changes in rapid prothrombin activation the view is based on inferences based on various observations about slow activation. Moreover, small quantities of brain thromboplastin which is all lipid, or platelet factor 3, which is a lipoprotein, can be mixed with prothrombin and "inactivation" occurs. This is really the beginning of the activation cycle which is not completed because other substances are needed under the circumstances. When such a cycle is started with platelet factor 3 the prothrombin activity can be regenerated with mitochondria(7). So the change is not "irreversible" after activation with platelet factor 3. Whether the regenerated prothrombin activity is the same as the original prothrombin is not known, but we are inclined to favor the view that this part of the cycle is a continuous run-off process involving intramolecular space relationships. These might create configurations refractory to the environment created by the procoagulants and then with further unfolding and/or folding of prothrombin the procoagulants would again favor thrombin formation—the latter actually being the activator.

Summary. The prothrombin activation cycle consists of a sequence described by the following: Prothrombin (sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin derivative I (not sensitive to calcium + Ac-globulin + lung thromboplastin) → Prothrombin derivative II (sensitive to calcium + Ac-globulin + lung thrombo-

plastin) → Thrombin. Solutions of prothrombin standing at 4°C go through most of this cycle, the prothrombin molecule alone possessing the peculiar properties that enables it to do this.

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Plasma Ketosteroid in Adrenal-Tumor Bearing Mice.*†‡ (23832)

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Several investigators have shown that transplantable adrenal tumors in mice may produce steroid hormone. This has been demonstrated by anatomical changes in the hosts, such as the development of atrophic adrenal glands, mammary and uterine changes, or chemical studies either of urinary steroid or *in vitro* response of tumors to ACTH(1-5).

This report concerns the measurement of plasma ketosteroid in CDF_1 hybrid and Balb-c mice bearing a hormonally active, transplantable, adrenal tumor which arose spontaneously in a Balb-c mouse in the laboratory of Dr. H. B. Andervont of the National Cancer Institue.

Materials and methods. Determination of plasma 17-ketosteroids. Plasma ketosteroids were determined by a modification of the procedure of Gardner(6). Each determination represented a pool of plasma obtained from 4 mice. Mice were lightly anesthetized with ether and blood from the severed femoral vein was aspirated into a heparinized syringe. *Tumor inoculation and animal maintenance.* Two months old CDF_1 were injected subcutaneously with 0.1 ml of a 50% tumor-saline emulsion. Tumor-bearing animals were sacrificed on twenty-fifth day after inoculation for plasma steroid determination; control mice which had been maintained under similar conditions were sacrificed simultaneously. Mice were maintained at 24°C and fed Purina Chow.

Results. Tumor growth. After about 5 weeks of subcutaneous growth, the tumor was hemorrhagic and the animal died. Metastases were never observed in unoperated animals. The hosts developed atrophic adrenals; enlarged uteri were observed in females. Gross and microscopic details of tumor growth are being studied by Dr. T. B. Dunn of Nat. Cancer Inst.

The effect of tumor growth in male mice is illustrated in Fig. 1. After 25 days of tumor growth, the ketosteroid level is elevated with respect to the plasma of control mice. Gonadectomy-adrenalectomy decreased the plasma ketosteroid level of control mice to trace values but did not affect the elevated level found in the plasma of tumor-bearing animals. The tumor effected an increase in plasma ketosteroid roughly similar to that caused by administration of 1 mg of ACTH for 5 successive days. An unexpected finding was the presence of widespread gross metastases in such organs as lung, liver, and spleen in 11 of

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