

# Effect of heparin on in vivo turnover of clotting factors

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HASSELBACK, R. AND PETER F. HJORT. *Effect of heparin on in vivo turnover of clotting factors.* J. Appl. Physiol. 15(5): 945-948. 1960.—The rapid turnover rate of plasma proteins involved in coagulation has been offered as evidence for a process of continuous subclinical *in vivo* coagulation. By administration of a continuous intravenous drip of heparin to four normal subjects, no significant change was demonstrated in the rate of disappearance of proconvertin and prothrombin from the circulation after blocking the production of these factors by the administration of a large dose of Warfarin sodium. This evidence does not disprove the existence of continuous coagulation, but the rapid turnover rate of coagulation factors would not appear to be sound evidence for such a process.

COAGULATION IS an emergency process which helps to arrest bleeding from damaged vessels. The mechanism and the defects related to this aspect of coagulation have been studied intensively, but is this the only function of coagulation? Many investigators believe that coagulation also has a constant function in the body, playing a role in the integrity of the vascular wall. If this is so, a continuous subclinical coagulation must occur in the normal individual. This theory is attractive, but the evidence is largely indirect.

One type of evidence offered to support this concept is that clotting factors have a more rapid turnover rate than other plasma proteins. For example, proconvertin (factor VII) has been found to have a half-life ( $T/2$ ) of 4.2-10.8 hours (1, 2) and prothrombin (factor II) a  $T/2$  of 10-72 hours (3). If this rapid turnover is caused by continuous coagulation, it should decrease if such coagulation were slowed by heparin.

The purpose of this investigation was to study the effect of heparin on the turnover of proconvertin and prothrombin. The rate of turnover was determined by a method similar to that of Frick (1), based on the rate of disappearance of the factors from plasma after the administration of a large dose of intravenous Warfarin

sodium. We found that a continuous intravenous infusion of heparin did not change the rate of turnover of prothrombin or proconvertin.

## MATERIALS

*Buffered saline.* This contained one part veronal buffer to nine parts saline.

*Calcium chloride.* A 1-M stock solution was diluted with distilled water to the required concentration.

*Heparin.* A-L. (Oslo, Norway), containing 50 mg (5000 IU)/ml, was used.

*Hexamethrine bromide.* Polybrene (Abbott) in an initial concentration of 1 mg/ml was appropriately diluted in buffered saline.

*'Proconvertin reagent.'* This was prepared by the method of Hjort (4). This is an eluate of human serum containing proconvertin and Stuart factor, with very low prothrombin activity. The batch used contained about 30% of Stuart factor when assayed in a one-stage system with Stuart factor-deficient plasma as substrate.

*Saline.* A 0.9-gm% solution of sodium chloride in distilled water was used.

*Thrombin.* Topostasine (Roche, Basel, Switzerland) was used. This was dissolved in distilled water to a concentration of 20 IU/ml and stored at  $-20^{\circ}\text{C}$  in aliquots sufficient for 1 day. When required, this was diluted with buffered saline to a concentration which, with normal plasma, gave a thrombin time of 15-25 seconds in the heparin neutralization system described under figure 1.

*Veronal buffer.* Owren's modification of Michaelis' buffer was used (5).

*Warfarin sodium.* Coumadin sodium (Endo, Richmond Hill, N.Y.) as an intravenous preparation was dissolved in distilled water to a concentration of 25 mg/ml.

## METHODS

All reagents were kept at  $4^{\circ}\text{C}$  while the assays were being carried out, except for the calcium chloride and the calcium-proconvertin reagent mixture, which were kept at  $37^{\circ}\text{C}$ .

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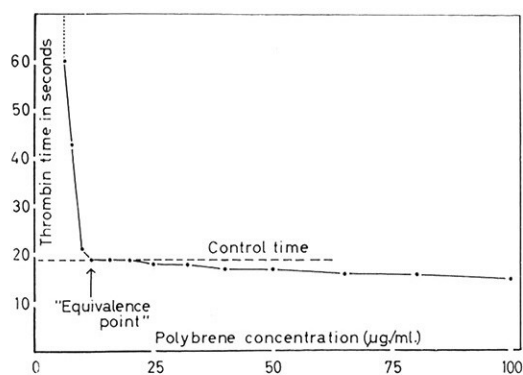


FIG. 1. An example of heparin neutralization procedure. Test system used was as follows: a mixture of 0.1 ml undiluted plasma and 0.1 ml polybrene dilution of buffered saline was preincubated for exactly 2 min. at 37°C, and 0.1 ml thrombin solution added. The appearance of a firm clot was recorded. Control time was the time obtained with nonheparinized plasma and buffered saline.

**Collection of blood.** Venipuncture was performed with a siliconized needle and the initial 2–3 ml of blood discarded. Nine volumes of blood were then collected directly into a Lusteroid tube containing one volume of 3.33 gm% (0.1 M) sodium citrate dihydrate. This was mixed by gentle inversion and centrifuged at 2500 rpm (ca. 1800 rcf) for 30 minutes at 4°C. The plasma was removed and stored in Lusteroid tubes at –20°C until assayed.

**Heparin neutralization procedure.** In order to determine proconvertin and prothrombin in blood containing heparin, it is first necessary to neutralize the heparin completely. This is difficult to do accurately with protamine, since excess amounts of protamine markedly influence coagulation (6). However, satisfactory results were obtained with Polybrene (7).

Progressive dilutions of Polybrene were prepared so that each successive tube contained a concentration 75–80% of that of the next more concentrated tube. With the doses of heparin used, it was found that 18 tubes, with concentrations ranging from 100 to 2.0 µg/ml were adequate. These remained stable throughout the day at 4°C. Increasing concentrations of Polybrene were tested in the clotting system described under figure 1. When heparin was present in excess, no coagulation occurred in 60 seconds. If the heparin in the plasma was effectively neutralized by the Polybrene, a thrombin time equal to that obtained with nonheparinized plasma and buffered saline was found. This was taken as the 'equivalence point' (6, 7). Slight insufficiencies of Polybrene resulted in considerably longer clotting times. An excess of Polybrene produced a further slight shortening of the thrombin time.

Preliminary experiments revealed that a Polybrene excess of four times that indicated at the equivalence point did not alter either the proconvertin or prothrombin assays. It was also possible to assay plasma thromboplastin component (factor IX) in a partial thromboplastin (cephalin) time system after neutraliza-

tion of heparin, but the amount of Polybrene used was much more critical, and the procedure was found to be technically very difficult.

**Proconvertin assay.** The technique of Aas (8) was modified in the dilution phase. Initially, for nonheparinized samples, four parts of plasma were mixed with one part of buffered saline, or, for heparinized plasmas, with one part of Polybrene solution containing four times the Polybrene concentration indicated by the equivalence point. This primary dilution was subsequently diluted 1/20 in diluting fluid II. Assays of serial dilutions of normal plasma in diluting fluid I gave a straight line on double log paper in this system.

**Prothrombin assay.** A modification of the Russell's viper venom-cephalin assay of Hjort *et al.* (9) was used. A 4/5 primary dilution in Polybrene or buffered saline similar to that used in the proconvertin assay was followed by a 1/30 dilution in diluting fluid II. To render the system insensitive to Stuart factor, proconvertin reagent was diluted to 50% with 70 mM calcium chloride. This was used as the 'trigger' reagent (fig. 2). Assays of serial dilutions of pooled normal plasma in diluting fluid I gave a straight line from 5 to 200% on double log paper in this system.

**Whole blood clotting time.** The method of Hjort and Stormorken (6) was used.

#### EXPERIMENTAL PROCEDURE

The subjects were four healthy young adult males. Two experiments were carried out on all subjects, the first lasting 24 hours, the second, 48 hours. In order to avoid any effect of the first experiment on the second, 7 weeks elapsed between the two procedures. Furthermore, subjects 1 and 2 received heparin during the 1st (24-hr.) experiment and subjects 3 and 4 received heparin during the 2nd (48-hr.) experiment. In all experiments blood was collected for a control specimen and initial clotting times were determined at zero time. All subjects then received 150 mg Warfarin sodium intravenously. This dose was twice that found by Frick (1) to produce a maximum rate of fall in proconvertin level. In addition, two of the subjects on each occasion received 150 mg of heparin intravenously. An intravenous infusion of physiological saline was then begun; in those subjects receiving heparin, this contained 300 mg of heparin/l., except subject 1, in whom the concentration was 400 mg/l. The rate of the infusion was regulated to produce a clotting time in excess of 30 minutes, or five times that in the normal; no samples clotted in less time, most in 40–70 minutes, and a few specimens had not clotted in 24 hours. The total heparin dose was approximately 600 and 750 mg in the 24-hour experiments and 1050 mg in the 48-hour experiments. All clotting times from subjects not receiving heparin were less than 6 minutes throughout the experiments. Every 2 hours for the 1st 8 hours and every 4 hours thereafter, blood was collected for test plasma. In the 48-hour experiments, all subjects

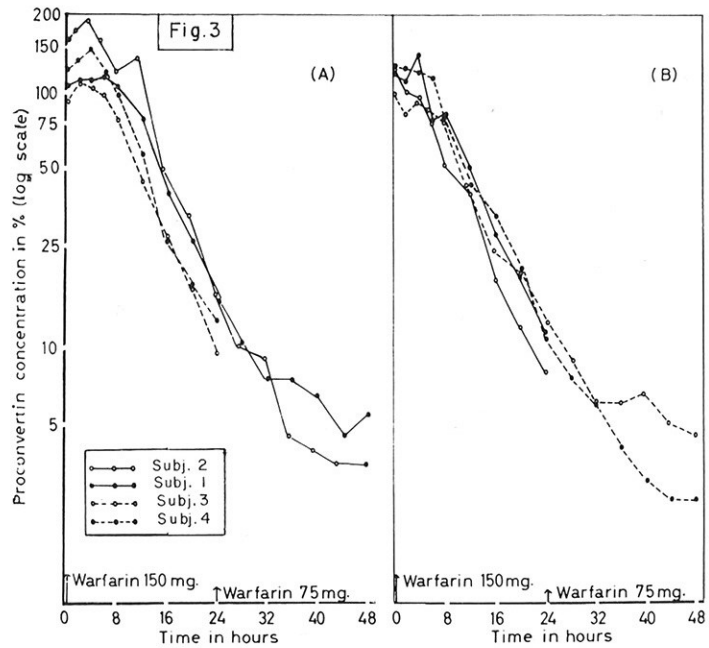
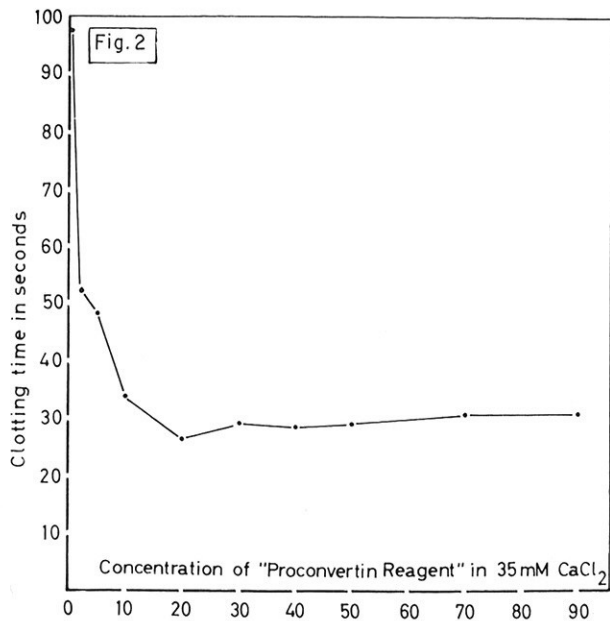


FIG. 2. Russell's viper venom-cephalin prothrombin assay of Stuart factor-deficient plasma, to which various concentrations of proconvertin reagent have been added. Test system used was as follows: a mixture of 0.1 ml of a 1/30 dilution of lyophilized Stuart factor-deficient plasma (kindly supplied by Dr. John B. Graham, Chapel Hill, N. C., and Dr. J. Roos, Utrecht, Holland), 0.1 ml of adsorbed, filtered, oxalated bovine plasma and 0.1 ml

Russell's viper venom-cephalin reagent was preincubated for 3-6 min. at 37°C and 35 mM calcium chloride, containing proconvertin reagent in the concentrations stated, was added. The appearance of a firm clot was recorded.

FIG. 3. Proconvertin levels in four normal subjects following intravenous injection of Warfarin sodium; A, without heparin and B, in the presence of heparin.

received a second intravenous injection of 75 mg of Warfarin sodium after 24 hours.

In a third experiment 6 weeks later, two of the subjects (1 and 4) received intravenous injections of 300 mg Warfarin sodium on each of three successive mornings (total 900 mg each). These subjects remained ambulant and no intravenous infusions were administered as in the previous experiments. Blood was collected for assay twice daily over a 6-day period.

TABLE I. Effect of Heparin on the Half-Life of Proconvertin

Subj.	Without Heparin	With Heparin
1	335	335
2	335	325
3	325	390
4	300	340

Values are T/2 of proconvertin in minutes. T/2 was determined from maximum slope of proconvertin disappearance from plasma after Warfarin sodium.

RESULTS

**Proconvertin.** The results of the assays are shown in figure 3. In all experiments, proconvertin levels remained unchanged during a lag phase varying from 240 to 520 minutes (mean, 344 min.). Thereafter, they dropped with a rate of fall which, on semilog paper, could be graphed as a straight line until some 32 hours had elapsed. The T/2 of the proconvertin in the various experiments is shown in table 1. After approximately 32 hours, when the proconvertin level was less than 10%, the rate of fall decreased.

**Prothrombin.** The assay results are shown in figure 4. Prothrombin levels declined at a much slower rate than did proconvertin levels. It was therefore not possible to measure the lag phase or the T/2. In the two 6-day experiments, the rate of prothrombin fall was the same as that found in the 24- and 48-hour experiments.

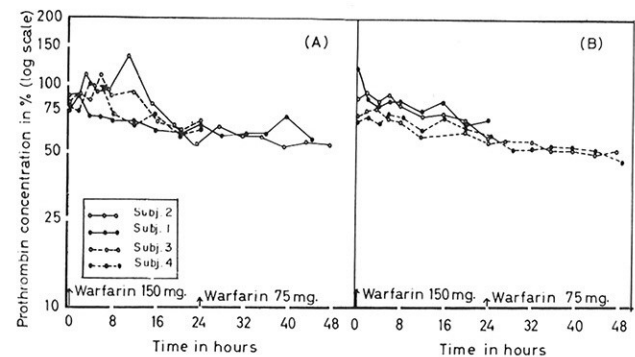


FIG. 4. Prothrombin levels in four normal subjects following intravenous injection of Warfarin sodium; A, without heparin and B, in the presence of heparin.

Any difference between the first two groups of experiments would suggest that prothrombin fell at a slightly more rapid rate in the presence of heparin. However, this difference is not significant.

#### DISCUSSION

† The only function which prothrombin, proconvertin and the other coagulation factors are known to have is in hemostasis. In the normal nonbleeding individual, they exist only for a potential role in the event of vascular damage. Why then should these factors have a very rapid metabolism compared with other plasma proteins? Where are they consumed or destroyed?

‡ It is tempting to postulate a process of continuous coagulation in the normal body, presumably occurring on the vascular wall, as suggested by several authors (10-18). This could be a reasonable explanation for the rapid turnover of those factors which are consumed in coagulation: fibrinogen, prothrombin, proaccelerin and antihemophilic globulin, but this reasoning obviously cannot be applied to those factors such as proconvertin, Stuart factor and PTC, which are not consumed during coagulation, unless one assumes an *in vivo* clotting mechanism quite different from that demonstrable *in vitro*. It is therefore not surprising that these experiments have demonstrated no difference in proconvertin turnover between normal and heparinized subjects.

Prothrombin, however, is consumed in *in vitro* coagulation. Continuous subclinical *in vivo* coagulation could therefore account for the rapid turnover of this protein. Heparin then would be expected to reduce the rate of turnover. These experiments have shown no significant

alteration in the rate of prothrombin disappearance in the presence of heparin.

The conversion of a very small amount of prothrombin to thrombin would be adequate to maintain continuous coagulation. These experiments, therefore, do not disprove the existence of such a process. They do show that the rapid turnover of coagulation proteins cannot be used as evidence for such a process.

A possible explanation for the prothrombin assay results is that an insufficient dose of Warfarin sodium was used in these experiments. If so, the dose Frick found adequate to produce a maximum rate of fall of proconvertin must be inadequate for a comparable prothrombin response. The 6-day experiments in subjects 1 and 4, however, failed to demonstrate a greater rate of fall with a dose four times that used in the first two experiments. This explanation for these results can then be discarded.

The mechanisms by which heparin interferes with coagulation are not fully understood, but it is known to block several steps in the process. In these experiments, heparin may have arrested certain phases of coagulation only, while others continued at a normal rate. Therefore, it cannot be ruled out that heparin did not interfere with that part of coagulation which is essential for the *in vivo* consumption of proconvertin and prothrombin. However, enough heparin was given to prolong the whole blood-clotting time at least five times, and such a dose is known to reduce markedly prothrombin consumption during coagulation *in vitro*. Therefore, the more likely explanation for these results is that the rapid turnover of the coagulation factors is not caused by continuous subclinical *in vivo* coagulation.

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