The Reduction of Platelet Cofactor II (Autoprothrombin II) Activity with Anticoagulants

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Platelet cofactor II (Autoprothrombin II, Plasma thromboplastin component, Christmas factor, Factor IX etc.) is a substance found in plasma and serum. It is adsorbed on barium carbonate, and in the clotting of blood perhaps functions with platelet factor 3, Ac-globulin and calcium ions to convert prothrombin to biothrombin (1). This is represented by the following equation:

$$\text{Prothrombin} \xrightarrow{\begin{array}{l}Ca^{++}\\ \text{Platelet factor 3}\\ \text{Ac-globulin}\\ \text{Platelet cofactor II}\end{array}} \text{Thrombin}$$

With the administration of dicumarol to patients, the plasma platelet cofactor II concentration is reduced (2, 3) and may actually decrease to zero (4). Reduction to these low levels occurs without any evident bleeding tendency and is observed when the prothrombin concentration is in the therapeutic range. With the exception of Coumadin sodium (Warfarin [5]) and phenylindanedione (6) it is not known whether other orally administered anticoagulants also alter the plasma platelet cofactor II concentration. If they do, another question arises: how rapidly and to what extent does the reduction occur and how soon is there a return to normal levels after the drug is withdrawn. The work described in this paper was largely motivated by these questions.

Materials and methods

Purified Prothrombin. This was prepared from bovine plasma by the method of Seegers and associates (7, 9, 10). Small amounts of Ac-globulin were in each preparation used in the study. Prothrombin activity was measured by two-stage method (8).

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Platelets. The platelets were of bovine origin and were prepared by differential centrifugation by the method of Schneider and coworkers (11).

Calcium and Imidazole Buffer. $CaCl_2$ was dissolved in imidazole buffer as follows: 2.396 Gm. $CaCl_2 \cdot 2H_2O$ were dissolved in 100 ml of solution containing 25 ml of physiological saline and 75 ml of imidazole buffer of pH 7.2.

Plasma and Serum Samples. Blood was collected from patients to receive or receiving the respective anticoagulant. A two-syringe technique was used, and the blood was immediately mixed with 3.2% sodium citrate solution in the proportion of 9 parts of blood to 1 part citrate. The plasma was separated within a short time after collection and stored at —35° C until analyzed. 5 ml of the plasma were defibrinated by addition of 4 ml of physiological saline and 1 ml of purified thrombin (100 U/ml) and the fibrin wound out. This defibrination involves a two-fold dilution of the plasma. The serum samples were, therefore, similarly diluted by addition of an equal volume of saline. The plasma and serum samples were ether treated briefly to remove antithrombin by shaking the sample at room temperature for one-half minute with an equal volume of ethyl ether. The plasma or serum sample was separated from the ether phase in a separatory funnel. This procedure was repeated twice. Most of the ether was removed by passing air through the sample.

Fibrinogen. Purified bovine fibrinogen was prepared according to the method of Ware, Guest and Seegers (12).

Platelet Cofactor I and Platelet Cofactor II Assay. The cofactor assays were carried out as described before (4). The following reaction mixture was set up.

Purified prothrombin (4500 U/ml)	1.0 ml
Defibrinated Plasma or serum	0.5 ml
Calcium, imidazole buffer	0.5 ml
Platelet suspension (Packed platelets 1 part plus 4 parts saline)	0.5 ml
Physiological saline	0.5 ml
Total volume	3.0 ml

The reactions were followed as they occurred in a water bath at 28° C. At intervals, samples were taken out and the thrombin units in the reaction mixture were determined according to the method of Seegers and Smith (13).

Results

General Perspective. In the conversion of purified prothrombin to thrombin, conditions can be arranged so that the thrombin yield is a reflection of the quantity of platelet cofactor II in the reaction mixture, the actual reaction being as described by the equation above. In addition to platelet cofactor II, plasma contains another substance that can function as a platelet cofactor. This substance is platelet cofactor I (also called antihemophilic factor, antihemophilic globulin, factor VIII, etc.) and the equation representing its function in the conversion of prothrombin to thrombin is given as follows:

$$\text{Prothrombin} \xrightarrow[\text{Platelet factor 3, Ac-globulin, Platelet cofactor I}]{Ca^{++}} \text{Thrombin}$$

If both factors, namely, platelet cofactor II and platelet cofactor I yield thrombin by nearly the same mechanism, we must pay attention to both and try to make a differentiation. Two possibilities are open. Platelet cofactor II is adsorbed on barium carbonate while platelet cofactor I is not. Platelet cofactor I is not found in serum because it combines with an inhibitor during the clotting of blood. Platelet cofactor II, on the other hand, increases in concentration during the clotting of blood.

In our work, we elected to measure the combined activity of platelet cofactor II and platelet cofactor I in plasma. Then we also measured the platelet cofactor II activity of serum. Our results are expressed in terms of thrombin yield from a standard prothrombin substrate. These measurements are not subject to the influence of the factor variously called autoprothrombin I, factor VII, stable factor, etc.

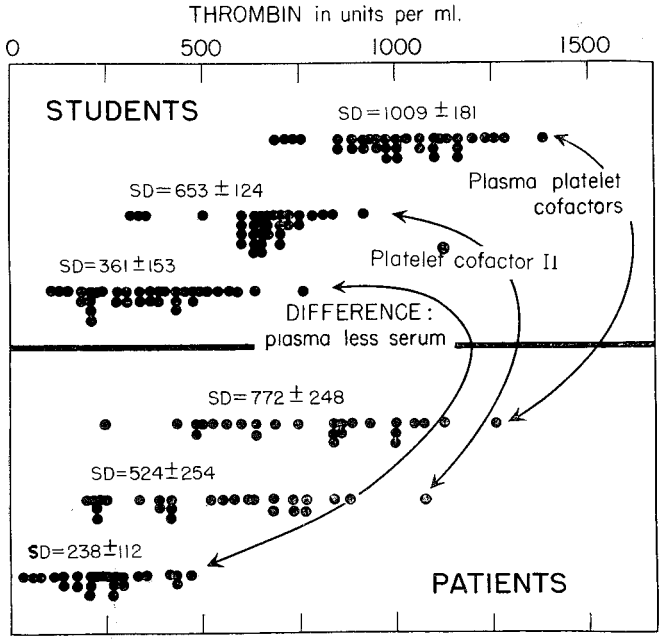

Fig. 1: The yield of thrombin from a prothrombin substrate of 1500 units per ml. The mean and standard deviations are given and each point represents a determination per person. Analysis for students include total plasma platelet cofactor activity and the serum platelet cofactor activity designated as platelet cofactor II. The difference between these two was calculated for each individual. Samples from patients were taken before anticoagulants were given.

Analysis of Plasma and Serum of Students and of Patients before Use of Anticoagulants. The methods used in this work have not previously been applied extensively to the study of blood, and we wanted to find out what the values for healthy individuals are. Thirty-five medical students in the Wayne State

University College of Medicine kindly volunteered to give blood. The total plasma platelet cofactor activity (Platelet cofactor II plus platelet cofactor I) was found to be 1009 ± 181 expressed as units of thrombin yield (fig. 1). The yield with serum was 653 ± 124 and indicates the platelet cofactor II concentration of serum. The difference found between these two values was 356 ± 153.

We have not translated the respective thrombin yields 1009 ± 181 and 653 ± 124 into units of total platelet cofactor activity or platelet cofactor II activity, nor has there been a correction made for a certain amount of background material (i.e., cofactor activity) in the prothrombin substrate. This means that the numbers used for expressing the results are considered in somewhat the same manner as clotting times. For example, a clotting time of 15 seconds corresponds to one unit of thrombin and 17 seconds corresponds to 0.85 units and 13 seconds corresponds to 1.20 units. The difference is two seconds in each instance but the units of thrombin involved are not the same. The difference between 1009 ± 181 and 653 ± 124 gives numbers that cannot be compared directly with the difference between 653 ± 124 and 361 ± 153. Low yields of thrombin are more easily obtained in the assay procedure than high yields of thrombin. Despite these limitations imposed by a difficult circumstance not surmounted in the face of difficult technical manoeuvers, our results have quantitative significance when due allowances are made. Our discussion focuses on substantial differences and by-passes the marginal considerations that can probably some time be taken up when more exact and less cumbersome methods are devised.

Before the patients were given any anticoagulants, their plasma and serum was analyzed. The average total cofactor activity in the plasma from 26 patients yielded 772 ± 248 units of thrombin (fig. 1). This value is lower than for students and there was greater variability. In fact, some of the plasma samples showed such low activity that we were unable to use additional data obtained during the therapeutic period for deciding exactly what the effect of the therapy was. Perhaps if we had anticipated this fact, we would have included only those persons in our study who hand substantially a high total platelet cofactor activity.

Under the conditions of our analysis, the cofactor activity found in serum never yielded as much thrombin as obtained from plasma. Evidently, the loss of platelet cofactor I activity during clotting could not be offset by the platelet cofactor II already present plus some increase in platelet cofactor II activity during clotting. This general observation applies not only to the student blood samples, but also to the patient material before and after drugs were taken. Undoubtedly, these drugs do not lower platelet cofactor I concentration very much, and most likely not at all. This generalization will be implied in the subsequent discussion without further mention.

Studies With Sintrom (nitrophenyl-acetyl-ethyl-4-oxycumarin). Our study included extensive observations of 14 patients. An attempt was made to gain information during the three periods when the following occurred a) the initial dose was taken, b) the maintenance dose was taken, and c) no drug was taken. With Sintrom the platelet cofactor activity decreased in concentration at the same time or even a little later than the prothrombin concentration. When the latter was in the therapeutic range, the platelet cofactor II was near zero. When the patient stopped taking the drug, the platelet cofactor activity increased within a day or so after the prothrombin concentration increased (fig. 2). In general, the above description applies to all our cases except one, where the increase in platelet cofactor activity did not follow shortly after the increase in prothrombin concentration.

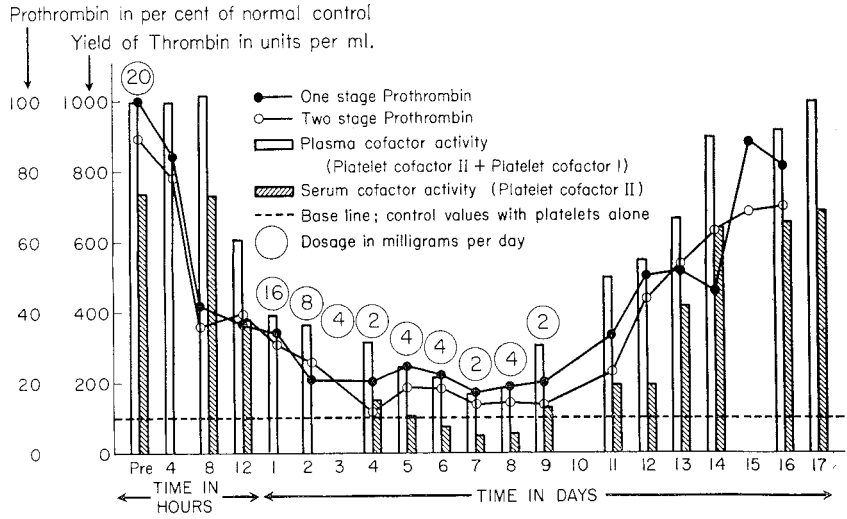

Fig. 2: Sintrom as anticoagulant. The prothrombin substrate was 1500 units per ml. The yield of thrombin is indicated without correction for background of platelet cofactor activity indicated by broken horizontal line.

The changes which occur in total platelet cofactor activity and which we regard as principally, if not entirely, changes in platelet cofactor II are somewhat sluggish. Figure 3 illustrates this fact better than figure 2. The fluctuations in prothrombin concentration are more responsive to the giving and withdrawal of the drug than alterations in platelet cofactor II.

Phenylindanedione (2-phenylindane-1:3-dione). Three patients were observed to provide the detailed information such as is illustrated by figure 4. All three patients gave the same impression indicated by figure 4. The platelet cofactor II concentration decreased almost coincidentally with prothrombin. The former was near zero when the prothrombin concentration was at the desired low level;

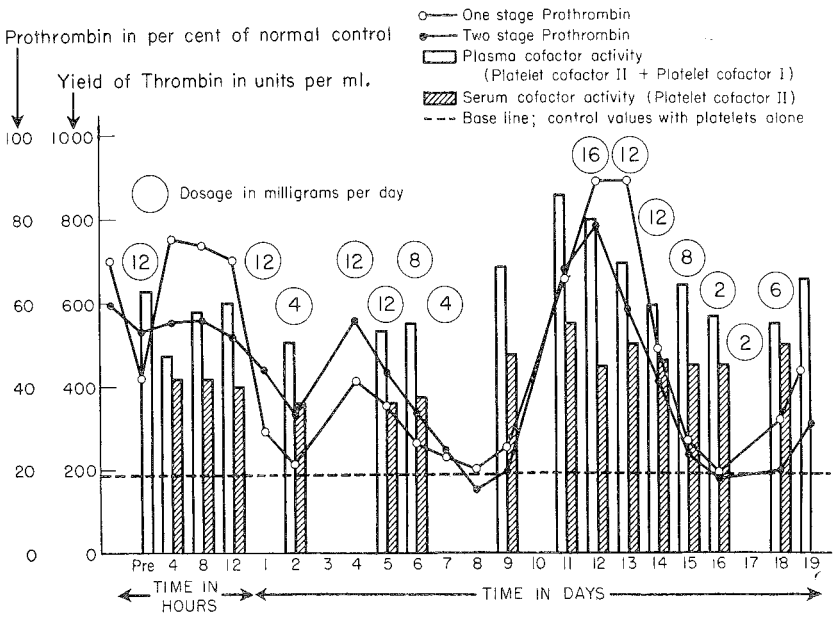

Fig. 3: Sintrom as anticoagulant when taken intermittently.

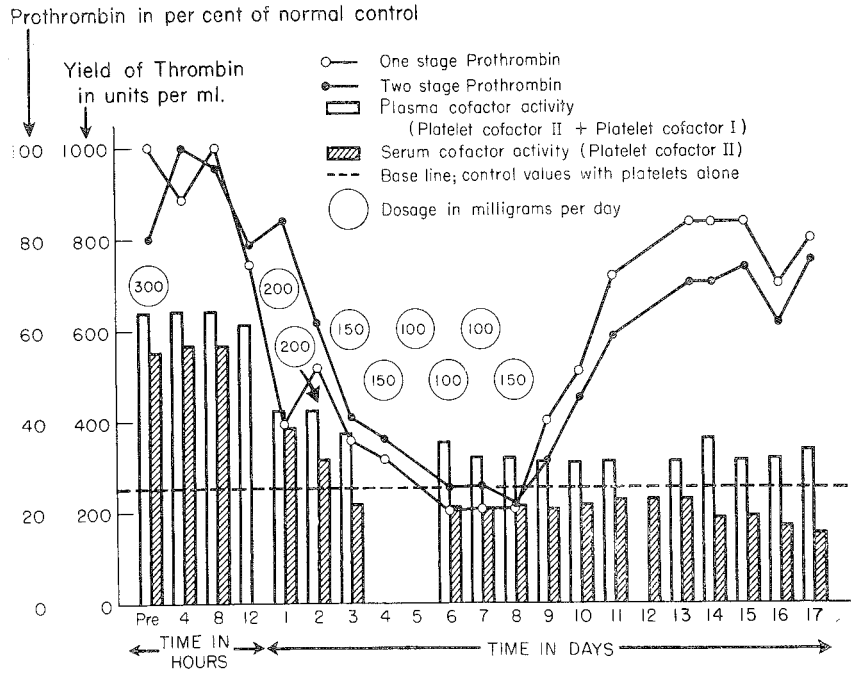

Fig. 4: Phenylindanedione as anticoagulant without return of platelet cofactor II activity to normal.

however, when the prothrombin concentration increased following withdrawal of the drug, the platelet cofactor II did not increase in concentration. In the case of the illustration of figure 4, there were observations for 10 days after the drug was not taken. With another patient, 12 days of observation were recorded and still there was no cofactor II activity. Our unexpected findings found us unprepared to find out how long it takes for the platelet cofactor II concentration to return to normal; because, we transported plasma between Chicago and Detroit, and in addition, the analytical work was cumbersome and was sometimes completed quite a number of days after the blood was collected.

Preliminary Study of Other Drugs. The response of patients taking Sintrom differs from that of those taking phenylindanedione. Possibly other drugs would have individual characteristics too. We made enough observations to support this preconceived view, but our work did not advance far enough to state definitely the specific characteristics of each drug worked with. Accordingly, our impressions are recorded below.

With Dicumarol (3,3'-methylenebis-[4-hydroxycoumarin]), the observations included two patients. In one, platelet cofactor II activity decreased somewhat ahead of prothrombin and went to zero. The return was not followed in one patient but in the other the return paralleled that of prothrombin.

With Dipaxin (2-[diphenylacetyl]-indane-1:3-dione) three patients were studied in detail. One of these had a very high platelet cofactor II concentration and this was only slightly reduced on the thirteenth day of therapy. The other two patients had a very low platelet cofactor II concentration prior to the administration of the drug. In each case, during the three weeks of observation, no significant changes were noted when the drug was first taken, while it was being taken, and after withdrawal. The prothrombin concentration, however, followed the anticipated changes, first decreasing in concentration, then remaining at a low level, and lastly, returning to normal.

With Marcumar ([3-1'-phenylpropyl]-4-hydroxycoumarin]) one patient with a moderately low platelet cofactor II concentration was followed through to a low prothrombin concentration and subsequent return to the pretherapy level without any significant change in platelet cofactor II concentration. This patient incidentally had a very large amount of platelet cofactor I activity in his plasma.

With Tromexan (bis-3-[4-hydroxy-courmarinyl]-ethylacetate) three patients were studied. Two with high platelet cofactor II concentration were followed while the prothrombin concentration decreased. The platelet cofactor II concentration did not drop. A third patient was observed for 28 days to include complete decrease and return of prothrombin activity without any change in the platelet cofactor II of the serum; however, the concentration of the latter was very low at the outset.

With Cyclocumarol (3:4-[2'-methoxy-4'-phenyl]-dihydropyrano-coumarin) one patient was followed through induction and recovery without change in platelet cofactor II concentration. The latter was very low at the beginning.

With Coumadin sodium (Warfarin) (3-α-phenyl-β-acetylethyl-4-hydroxy-coumarin) one patient was studied for 28 days. When the prothrombin concentration was in the therapeutic range, no platelet cofactor II activity was found. Later when the prothrombin concentration was again high, some platelet cofactor II was found though this patient also possessed only a small concentration of this factor initially.

Discussion

The quantity of information related to Sintrom and phenylindanedione enables us to observe with practical assurance that their use is not followed by the same responses. With Sintrom, the platelet cofactor II concentration approximately follows in parallel with prothrombin throughout; but with phenylindanedione the return of platelet cofactor II to normal is greatly retarded. The preliminary work on other drugs indicates that they too have individual characteristics and most likely some of these do not appreciably alter the platelet cofactor II concentration even though the prothrombin activity decreases. Definite conclusions must await the study of further cases. It is difficult to judge how many cases might constitute an adequate study. Even with our 14 cases who took Sintrom, one patient's platelet cofactor II did not return to normal, and thus was an exception to the average experience.

We have no comprehensive information on the significance of platelet cofactor II in thrombosis. But when it is known which drugs change platelet cofactor II concentration and which ones do not, there will exist readiness to inquire about this aspect of anticoagulants on an experimental basis. At the moment, it is not possible to say whether a drug that lowers the platelet cofactor II concentration is better than one that does not, or whether there is an advantage if the platelet cofactor II concentration returns only slowly to normal.

The platelet cofactor II concentration can be brought to zero without noticing a bleeding tendency. Moreover, some of our patients had a very low platelet cofactor II level even without anticoagulants. They were certainly not recognized as hemophiliacs or with bleeding tendencies of any kind. Consider, however, that this laboratory has analyzed (14) blood from plasma thromboplastin component (PTC) patients and found that they contain more platelet cofactor II than some of these patients and perhaps fully as much as a few of the healthy medical students. One cannot suppose that there was a mistake in the diagnosis of the PTC cases, because one of these was Mr. Christmas which

is the patient in whom Christmas disease was discovered (15), and another was Mr. Kincaid in whom plasma thromboplastin component „deficiency" was discovered (16). Most likely, this type of hemophilia involves more metabolic abnormalities than a simple decrease in platelet cofactor II concentration.

Summary

The total platelet cofactor activity in plasma and the platelet cofactor II activity in serum was measured. The normal variations are high. Some individuals have less serum platelet cofactor II than found in certain cases of PTC "deficiency"; and when the serum platelet cofactor II concentration was reduced to zero with the use of anticoagulants, there was no bleeding tendency observed. With the use of Sintrom and phenylindanedione, the total platelet cofactor activity of plasma was reduced and the platelet cofactor II activity of serum was reduced to near zero. With the latter drug platelet cofactor II of serum was still low 10 days after the drug was withdrawn. With Sintrom given intermittently the fluctuations in prothrombin concentration are noticeably more rapid than the changes in platelet cofactor II. In a preliminary study of other drugs, there were indications that platelet cofactor II concentration is not altered with some anticoagulants even though the prothrombin concentration is reduced. Apparently, the platelet cofactor II changes which occur are characteristic for each anticoagulant. The platelet cofactor I levels of the plasma are not appreciably, and most likely not at all altered by the drugs studied.

Résumé

Des variations importantes de l'activité globale des cofacteurs plaquettaires du plasma (Facteur VIII et IX) et du cofacteur plaquettaire II du sérum (Facteur IX) ont été trouvées chez des individus normaux. Certaines personnes ont moins de cofacteur plaquettaire II dans le sérum que certains malades ayant une déficience de la PTC; en diminuant la concentration de ce facteur jusqu' à zéro en administrant des anticoagulants, il n'y avait aucune tendance hémorragipare.

L'activité globale du cofacteur plaquettaire II du plasma fut abaissée et l'activité du cofacteur plaquettaire du sérum était pratiquement absente après l'administration de Sintrom ou de phenylindanedione. Ce dernier produit provoque une diminution du taux du cofacteur plaquettaire II du sérum jusqu'au dixième jour après la fin du traitement. Le Sintrom, administré à intervalles réguliers, provoque des fluctuations du taux de la prothrombine considérablement plus importantes que les changements de l'activité du cofacteur plaquettaire II. Nous avons signalé dans une étude préliminaire que certains médicaments provoquent une diminution du taux de la prothrombine, mais ne provo-

quent aucun changement de la concentration du cofacteur plaquettaire II. Il semble que la diminution de l'activité du cofacteur plaquettaire II est caractéristique pour l'anticoagulant étudié. Le taux du cofacteur plaquettaire I du plasma est peu ou pas diminué par l'administration des substances étudiées.

Zusammenfassung

Es wurde die gesamte Plättchen-Cofaktor-Aktivität (Faktor VIII + Faktor IX) im Plasma und die Plättchen-Cofaktor-II-Aktivität (Faktor IX) im Serum bestimmt. Die Streuung der Normalwerte war groß. Einzelne Individuen haben weniger Serum-Plättchen-Cofaktor II als gewisse Fälle von PTC-„Mangel" (Hämophilie B); wenn der Serum-Plättchen- Cofaktor II (Faktor IX) durch Antikoagulantien auf Null reduziert wurde, so konnte keine Blutungsneigung beobachtet werden. Durch Sintrom und Phenylindandion wurde die gesamte Plättchen-Cofaktor-Aktivität (Faktor VIII + Faktor IX) im Plasma vermindert und die Plättchen-Cofaktor-II-Aktivität (Faktor IX) im Serum auf nahezu Null gesenkt. Bei Verwendung des zweitgenannten Medikamentes blieb der Plättchen-Cofaktor II im Serum noch 10 Tage nach Absetzen des Medikamentes niedrig. Bei intermittierender Verabreichung von Sintrom schwankt die Prothrombin-Konzentration schneller als die des Plättchen-Cofaktor II. Vorläufige Untersuchungen mit anderen Antikoagulantien ergeben Hinweise dafür, daß die Plättchen-Cofaktor-II-Konzentration sich bei Anwendung einzelner Antikoagulantien nicht ändert, obwohl die Prothrombin-Konzentration vermindert wird. Die Veränderungen des Plättchen-Cofaktor II sind anscheinend für jedes Antikoagulans charakteristisch. Die Konzentration von Plättchen-Cofaktor I (Faktor VIII) wird durch die untersuchten Antikoagulantien nur unmerklich und wahrscheinlich überhaupt nicht verändert.

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