On the Purification and Conversion of Human Prothrombin

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In 1893, Alexander Schmidt first stated that in plasma, thrombin has an inactive precursor, prothrombin (22). He believed that prothrombin was converted to thrombin by the action of zymoplastic substances.

In 1934, Quick published the first method for the quantitative determination of prothrombin (20). Today his original "prothrombin" cannot be regarded as an uniform substance, because it is now recognised as a complex composed of several components (factors V, VII, Stuart-Prower and prothrombin itself). During the last two decades, many new results and hypotheses have been published on the purification, structure, and the mode of conversion of prothrombin. For example, Quick states that prothrombin is derived from a precursor which he calls, prothrombinogen (21). Seegers et al. devised a method for the purification of bovine prothrombin (24). They insist that prothrombin occupies a central position in the clotting system. Under certain conditions it can be converted to auto-prothrombin I (25) and auto-prothrombin II (26). Probably these two proteins are identical with factor VII (and/or Stuart-Prower factor) and factor IX. Oncley et al. extended the ethanol fractionation method of Cohn (19). They found prothrombin in fraction III-2. Duckert et al. purified human prothrombin on a $BaSO_4$ column (7). Seegers determined the molecular weight of his prothrombin preparation and found it to be 63 000 (24). Laki et al. analysed the amino-acid composition in the same product (16). Lasch and Roka (17) and Alkjaersig (1) described the conversion of factor VII to prothrombin by means of liver mitochondria.

The aim of this work was to purify human prothrombin by chemical and immunological methods, to compare prothrombin preparations obtained by different methods and to study the conversion of prothrombin to thrombin by the action of blood thromboplastin.

Materials and Methods

Veronal-acetate buffer, pH 7.35 (Michaelis).

a) Stock solution: $CH_3 \cdot COONa\ 3\ H_2O$, 9.714 g. Na-veronal, 14.714 g. Dissolve the mixture and bring the total volume to 500 ml with distilled water.

b) Buffer solution: Stock solution 250 ml., 4.25% NaCl 200 ml., N/10 HCl 217 ml., Distilled water 683 ml.

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Fibrinogen solution: Human oxalated plasma fractionated according to Cohn method No. 6. Precipitate I (Cohn fraction I) is dissolved with equal volumes of 0.9% NaCl and buffer solution, making the final amount equal to half the original volume of plasma used. 9 parts of fibrinogen are mixed with 1 part of M/10 Na-oxalate, and then adsorbed on $BaSO_4$ for 20 mins. at room tempt., (5 g $BaSO_4$ per 100 ml oxalated solution). After adsorption, it is centrifuged and the supernatant deep-frozen at — 25° C.

$BaSO_4$: prepared for X-ray examination by Merck, Germany.

Thrombin: bovine-thrombin, "Roche" 43 N.I.H. units/mg protein.

Thromboplastin generation test: according to Duckert et al. (8).

Determination of fibrinogen: according to Clauss (6), prothrombin: according to Koller et al. (15), factor V: according to Kappeler (14), factor VII-complex: according to Koller et al. (15), factor VII: according to Zollinger (27), factor VIII: according to Geiger et al. (12), factor IX: according to Geiger et al. (12), PPA: according to Fisch (10), Stuart-Prower: according to Bachmann et al. (2), Tyrosin: according to Folin-Ciocalteu (11).

Purification of Human Prothrombin

1) *The Procedure*

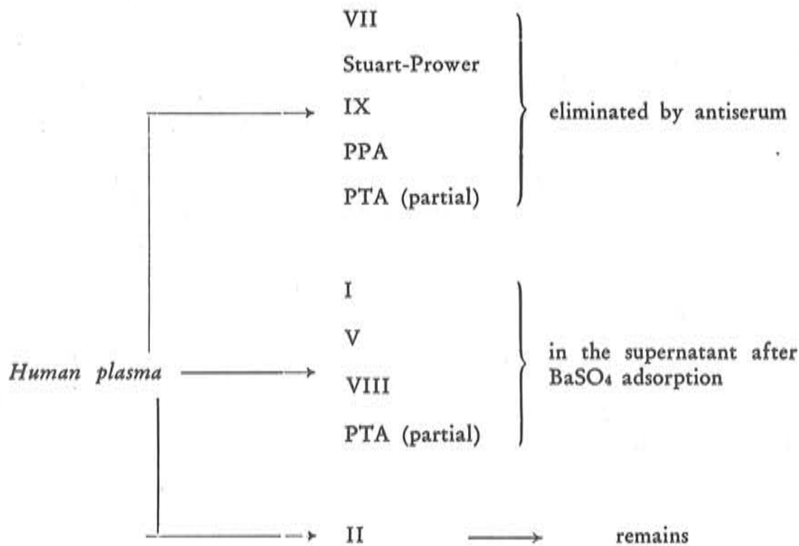
The proteins involved in blood coagulation can be divided into two groups according to their physico-chemical properties. We differentiate the "Prothrombin group" (prothrombin, factors VII, IX, PPA and Stuart-Prower) from the "Fibrinogen group" (fibrinogen, factors V and VIII), PTA (plasma thromboplastin antecedent) being the only factor occupying an intermediate position. The proteins of the prothrombin group are so closely related to each other that, in our opinion, it is quite impossible to separate them by chemical means, with only one exception, i.e. chromatography. Duckert et al. (7) obtained good results by chromatography on a $BaSO_4$ column. The following methods gave us very unsatisfactory results in the separation of prothrombin from the other factors of the "Prothrombin group":

- Adsorption on various adsorbents such as $BaSO_4$, $BaCO_3$, $Al(OH)_3$ etc. and elution with different eluents.
- Ethanol fractionation according to Cohn (method No. 6).
- Precipitation at low pH.
- Filtration through asbestos filters (this method is possible to some extent with ox plasma but not human plasma).

We wish to emphasize that, in spite of the close relationship of the factors belonging to the "Prothrombin group", each of them plays a different and definite role in blood coagulation. Our findings do not give any evidence that prothrombin can be converted to other factors of the "Prothrombin group". This observation will be pointed out in the discussion of this paper.

During normal blood coagulation, 95—99% of prothrombin is converted to thrombin. Then, the thrombin is completely inactivated within a short time. Factors VII and Stuart-Prower remain unaltered during coagulation. It is found that Factor IX and PPA are present in plasma in the inactive precursor state, whereas, in serum both are found to be in the active form. This means that only prothrombin is eliminated from the factors of the "Prothrombin group" during the process of coagulation. If we assume that the four other remaining factors which are known today, have the same immunological properties in plasma and serum, it should be possible to produce antibodies by inoculating serum into animals and to precipitate those factors out of plasma. The only factor remaining will be prothrombin. It can then be separated from the factors of the "Fibrinogen group" (fibrinogen, factors V and VIII) by adsorption on $BaSO_4$. Table 1 demonstrates the hypothesis.

Tab. 1: Prothrombin purification scheme.



I = fibrinogen
II = prothrombin
V = proaccelerin
VII = proconvertin
VIII = AHG
IX = PTC, Christmas F.
PPA = prephase accelerator
PTA = plasma thromboplastin antecedent.

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2) *Results*

a) *The antiserum:* Pooled human serum is oxalated (9 parts of pooled serum added to 1 part of M/10 Na-oxalate) and adsorbed on $BaSO_4$ (25 mg $BaSO_4$/ml pooled oxalated serum) for 20 minutes at room temperature. It is then centrifuged at 0° C, and the supernatant discarded. The deposit is washed with (pre-cooled) 0.9% NaCl at 0° C. It is again centrifuged at 0° C and the supernatant discarded. Elution is carried out at 0° C on the deposit with (pre-cooled) Na-citrate (pH 7.8) for 60 mins. The volume of Na-citrate used is 1/24 the volume of pooled serum started with. After that, it is centrifuged at 0° C and the supernatant is dialysed first against (pre-cooled) distilled water for 1 hour and then against (pre-cooled) 0.9% NaCl for 24 hours at the same temperature. Table 2 gives the values of pooled serum and serum concentrate, (the mean value of different products).

Tab. 2: Comparison of activities of clotting factors in pooled serum and serum concentrate.

	amount	II	VII	IX	PPA	Stuart	Protein
PS	1 ml	3U*)	100U	100U	100U	100U	70 mg
SC	1 ml	25U	3100U	1700U	1000 —2000U	1200U	5.6 mg

*) U = units (1.0 ml human oxalated plasma containing 100 units of each clotting factor).

By means of paper electrophoresis, Fisch demonstrated that such a serum concentrate contains the proteins involved in coagulation as well as other proteins (10). With the exception of fibrinogen, the absolute amount of coagulation factors in plasma is not yet known. This is the reason why we cannot calculate the ratio of total protein to coagulation-active protein. Our findings obtained from chromatography on $BaSO_4$ column indicate that the concentration of each factor of the Prothrombin group is 7—10 mg% or even less. Graph 1 shows an electrophoretic pattern of serum concentrate. For further details see Fisch (10).

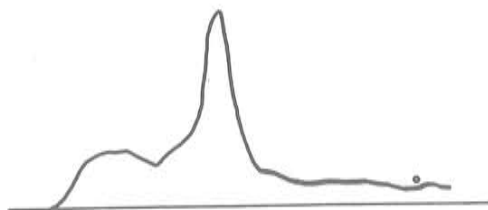

Fig. 1: Paperelectrophoresis of serum concentrate.

b) *Immunisation:* We used healthy rabbits, weighing between 3000—3500 g. Serum concentrate was injected either only subcutaneously or alternatively by intravenous, intraperitoneal or subcutaneous inocculation. Either method gives similar results. The total protein injected into each rabbit was 500—1000 mg. 2200—4400 ml of pooled serum are required to produce the necessary amount of serum concentrate.

c) *Preparation of antiserum:* Blood is collected from the marginal ear vein of the rabbit. The hair over the vein is dry-shaved and sterilised with alcohol. If dilation of the vein is required, it is gently rubbed with xylol or better still with chloroform. If xylol is used, the ear is washed with alcohol and lightly covered with vaseline after blood collection. An incision is made into the vein by means of a sharp scalpel. 30 ml of blood from each rabbit are allowed to drop into three 10 ml test tubes. One can safely collect this amount of blood on three consecutive days without causing them any harm. After the blood has clotted at room temperature it is incubated at 37° C in the water-bath for 3 hours. The blood is centrifuged and the serum removed.

It is necessary to eliminate the rabbit's own serum factors (VII, IX, PPA, Stuart-Prower and partially PTA). After the addition of Na-Oxalate, (9 parts of serum to 1 part of M/10 Na-oxalate) the serum is twice absorbed on $BaSO_4$ (50 mg $BaSO_4$/ml oxalated serum). The clear centrifugate is free from the serum factors with the exception of PTA. The antibodies are not adsorbed on $BaSO_4$. The serum treated in this way can be safely deepfrozen at — 25° C without any loss of antibody activity during storage.

With the aim of obtaining pure antibody fractions, we tried to fractionate the $BaSO_4$ treated rabbit serum according to the Rivanol-method devised by Horejesi and Smetana (13). These authors treat human serum with 0.4% aqueous rivanol solution (2-aethoxy-6,9-diamino-acridinlactate). They add 3 parts of rivanol solution to 1 part human serum in order to precipitate out the albumins, α and β-globulins. The rivanol is later removed by adsorption on charcoal powder. The γ-globulin fraction obtained is 97—99% pure. This method is not applicable if rabbit serum is used, because during the process some of the antibodies will be precipitated. Only by mixing equal proportions of rabbit serum and 0.4% rivanol solution the antibody titre is not altered, and to a certain extent the antiserum is purified.

d) *Titration of the antiserum:* To 1 ml of human oxalated plasma different amounts of 1/10 diluted, pre-treated rabbit serum are added, and the total volume brought up to 2 ml with buffer solution:

1.0 ml oxalated human plasma
0.1—1.0 ml 1/10 rabbit serum
0.9—0.0 ml veronal-acetate buffer

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The mixture is incubated for 15 mins. at 37° C. The clotting factors in the incubation mixture are then determined. Graph 2 gives a titration curve.

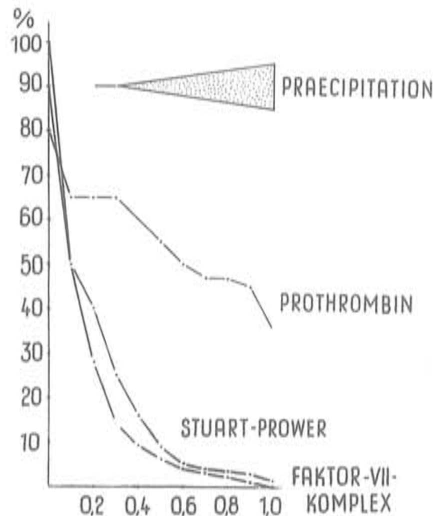

Fig. 2: Antiserum titration curve. Abscissa: Amount of rabbit serum added (1/10 dil.). Ordinate: % of clotting factors.

This test enables one to determine the amount of antibodies formed against each serum factor and also to calculate the quantity of antiserum required to inactivate 1 ml of oxalated human plasma. From the titration curve, it is striking to note that even the prothrombin level falls to a certain degree. This can be explained by the fact that the antigen injected contains traces of prothrombin. Furthermore, if we assume that the amount of antibodies formed is proportional to the amount of antigen injected, the lowering of the prothrombin level can be understood.

e) *Comparison of chemical and immunological specificity:* By testing the clotting factors of the rabbit with the usual one-stage methods or by using the rabbit serum or $BaSO_4$-treated plasma in the normal thromboplastin generation test, it is found that the proteins from different species of mammals react together. Based on this wellknown fact, ox plasma is used (whole or partial) as substrate in the factor VII, Stuart-Prower and prothrombin determinations. The active centers of the clotting factors of man and rabbit must therefore be identical or very similar to each other. On the other hand, if we inject human coagulation factors into the rabbit, it produces antibodies against the foreign proteins. This means that, immunologically the coagulation factors of both species have different molecular structures. This specificity can even be more definite,

as proven in cases of haemophilic patients with acquired circulating anti-coagulants (5).

The following findings, already described by Lewis and Didisheim (18), were repeated and confirmed by us. They demonstrate clearly the difference between chemical and immunological specificity.

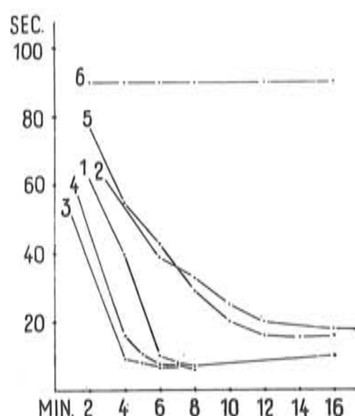

Fig. 3: Thromboplastin generation tests. Curves 1—6 refer to table 3.

Tab.: 3: Thromboplastin generation tests with:

No.	$BaSO_4$-plasma 1/10 dil.	Serum 1/10 dil.	Thromboplastin-formation
1	Human	Human	Possible
2	Human	NOT Immunised rabbit	Possible
3	NOT immunised rabbit	Human	Possible
4	NOT immunised rabbit	NOT immunised rabbit	Possible
5	Human	Immunised rabbit	Possible
6	Immunised rabbit	Human	Impossible

Incubation mixture:

0.3 ml $BaSO_4$-plasma 1/10 dil.
0.3 ml serum 1/10 dil.
0.3 ml chloroform-brain extract
0.3 ml M/40 $CaCl_2$.

In the incubation mixture No. 6, although all the factors necessary for the formation of blood thromboplastin are present, no thromboplastin is formed. This is so, because the factors in the human serum (factors IX, PPA and Stuart-Prower) are inactivated by the antibodies present in the $BaSO_4$-treated rabbit

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plasma. On the other hand, in the incubation mixtures No. 1—5 thromboplastin formation is possible.

f) *"Pseudocoagulation":* A striking incident was observed when we added oxalated rabbit serum containing antibodies to human oxalated plasma. This mixture, in spite of enough anticoagulant present, began to coagulate after 60 mins'. incubation at room temperature. After 24 hours, a solid clot was formed. We did the following experiments in order to determine why the above incubation mixture coagulated.

Tab. 4: "Pseudocoagulation". All tests done at room temperature.

Human oxalated plasma	1.0	1.0	1.0	1.0	1.0	1.0	1.0 (ml)
1 Human oxalated serum	1.0						
2 Rabbit oxalated serum (NOT immunised)		1.0					
3 Rabbit oxalated serum (Immunised)			1.0				
4 Rabbit oxalated serum (NOT immunised) $BaSO_4$ adsorption)				1.0			
5 Rabbit oxalated serum (Immunised, one $BaSO_4$ adsorption)					1.0		
6 Rabbit serum (NOT immunised)						1.0	
7 Rabbit serum (Immunised)							1.0
Clot after 1/2 hr.	—	—	—	—	—	—	—
Clot after 1 hr.	—	+	+	+	—	++	++
Clot after 2 hrs.	—	+	+	+	+	++	++
Clot after 15 hrs.	(+)	+++	+++	+++	+++	+++	+++
Prothrombin (at once and after 15 hrs.)	55%	45%	0%	40%	0%	50%	0%
Thrombin	—	—	—	—	—	—	—
Fibrinogen mg%	160	25	35	35	30	25	20

These findings show that clot formation occurs when either serum of normal or immunised rabbits is used in the incubation mixture. Furthermore, they indicate that there is no prothrombin consumption in mixtures 2, 4 and 6. The consumption in mixtures 3, 5 and 7 is due to the inactivation by excess of antibodies and not due to conversion of prothrombin to thrombin. If one tests the incubation mixture immediately after the addition of the antibody-containing rabbit serum, one will find neither prothrombin nor thrombin and the fibrinogen is present in the original concentration. The interpretation of these results gives us this inference: rabbit serum probably contains a substance (proteolytic enzyme?) different from thrombin which is able to convert human fibrinogen to fibrin. Up to now we have no further information regarding this conversion.

g) *Fractionation of human oxalated plasma:* Before producing an antibody-antigen reaction, we tried to find a suitable way to fractionate human plasma in order to remove unnecessary proteins. We chose the ethanol fractionation method by Cohn (method No. 6) (9). We found that this method has the further advantage of relatively enriching prothrombin. Table 5 shows the distributions of fibrinogen, prothrombin, factor VII and Stuart-Prower factor in Cohn fractions I, II + III and supernatant II + III (mean value of 8 fractionations).

Precipitate II + III is dissolved with veronal-acetate buffer (half the original starting volume) and Ringer solution, (half the original starting volume). Ringer solution proves to be advantageous for the antigen-antibody reaction.

Tab. 5: Comparison of clotting factor activities in Cohn fractions.

	Human oxalated plasma	Cohn I	Cohn II+III	Supernatant II+III
Fibrinogen	300 mg%	260 mg%	40 mg%	—
Prothrombin*)	100%	5.5%	68%	17%
Factor-VII-complex**)	100%	4%	49%	50%
Stuart-Prower*)	100%	3%	31%	59%

*) Slight prothrombin and Stuart-Prower denaturation during fractionation.

**) Besides a probable denaturation of factor VII there is a conversion of a precursor (which we cannot determine) to the active state of factor VII (= which we measure). For this reason, the sum of the three fractions is greater than 100%.

h) *Reaction-product:* It consists of

1) Cohn's fraction II + III from human oxalated plasma.
2) the purified antibody-containing rabbit serum.

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With the aid of the titration curve in section d) one can determine the amount of rabbit serum required for full inactivation. Cohn's fraction and the rabbit serum are mixed in correct proportions, and left at 4° C for 24 hours. The antigen-antibody reaction takes place during this period. This temperature ensures minimum denaturation of prothrombin; the traces of fibrinogen which were not eliminated during the ethanol fractionation, are converted to fibrin by the process called "pseudocoagulation". Afterwards, the reaction-product is centrifuged at 3000 r.p.m. for 15 mins. at 0° C.

The supernatant now contains, besides a large amount of other proteins, also the following proteins involved in coagulation:

out of the human oxalated plasma:	factors II
	V
	VIII
	PTA (partial)
	? Hageman
out of the rabbit serum:	factors V (little)
	PTA (partial)
	? Hageman

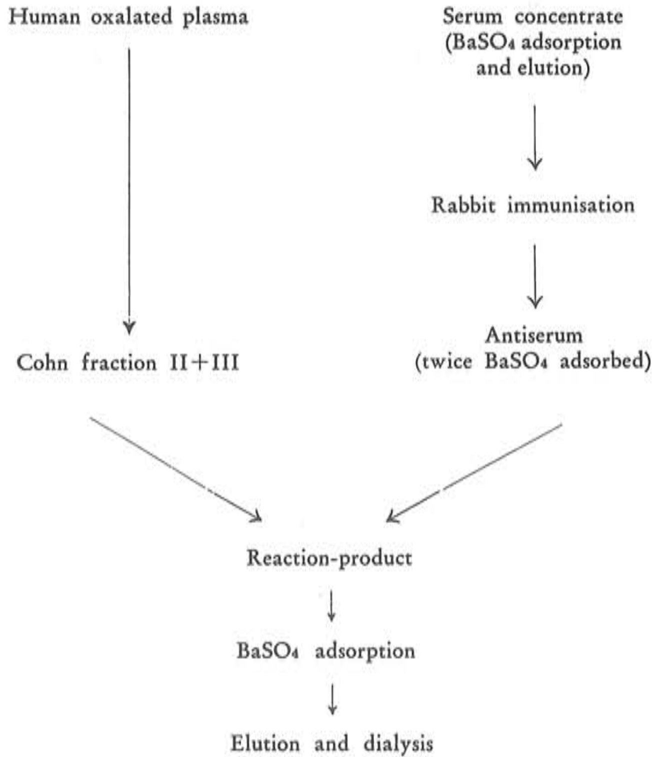
The final step is to separate prothrombin from the above factors. $BaSO_4$-adsorption is again used. Out of the rabbit serum which had been previously twice adsorbed with $BaSO_4$ (see section c) only traces of protein should now be adsorbable. Theoretically, the Cohn fraction should contain only prothrombin which is the sole adsorbable protein, because all the other proteins should have been precipitated by the antibodies which have been produced by the injection of $BaSO_4$-eluate into animals. The comparison between the activity of the prothrombin preparation and the total protein shows that such a consideration is not quite correct. Physiologically this preparation is pure, but we are still quite far from being able to produce a chemically pure one. One can not expect from such a complexity of antigens and antibodies that by chance all antigens are inactivated and precipitated quantitatively.

The reaction mixture is adsorbed an $BaSO_4$ for 30 mins. at room temperature (25 mg $BaSO_4$/ml mixture). The next steps are carried out (except where mentioned) at 0° C and all reagents used are pre-cooled. After centrifugation the deposit is twice washed with 0.9% NaCl. The centrifugate is discarded and the deposit is washed once with 0.006 M Na-Citrate. This is spun, and prothrombin is eluted from the deposit with 1/6 to 1/10 the original volume 0.14 M Na-Citrate (pH 7.8) for 60 mins. The eluate is now dialysed against distilled water at 4° C until its specific conductance is $0.38 \pm 0{,}07 \times 10^{-3}$ Ohm^{-1} cm^{-1} measured

at 10° C (this corresponds to a period of 24—48 hours of dialysis with frequent changing of distilled water). During dialysis some proteins which are insoluble in water precipitate and can be removed by centrifugation.

Table 6 summarises the stages involved in the isolation of prothrombin and its corresponding results.

Tab. 6: Purification of Prothrombin.



	II	V	VII-complex	Stuart	VIII	IX	PPA
Plasma	10 000U*)	10 000	10 000	10 000	10 000	10 000	10 000
Cohn II+III	6 800	—	4 900	3 100	—	—	—
Antiserum	0	—	0	0	—	0	0
Reactionproduct	3 950	—	0—30	0	—	—	—
Eluate	2 250	0	0—30	0	0	0—30	0

*) U = units.

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The isolated prothrombin exhibits from the physiological point of view, a good degree of purity, (the other known clotting factors range between 0%—1%) but chemically it is unsatisfactory, because it contains 75%—90% of impurities.

In order to purify it further, we tried isoelectric-precipitation with cysteine-HCl (0.1 M, pH 1.50 "Roche") which gave a better result than by using N/10 HCl (?protection from oxydation, protection of SH-groups in the prothrombin molecule, protection from formation of aggregates). With cysteine-HCl, it is possible to precipitate out the prothrombin between pH 4.70 and 4.90, at 0° C. After centrifugation, the precipitate is dissolved in distilled water and adjusted to pH 7.0 with N/10 NaOH. Table 7 compares the prothrombin preparations before and after isoelectric precipitation.

Tab. 7: Comparison of prothrombin concentrations before and after isoelectric precipitation.

	Prothrombin U/ml	Protein/ml
Before	100	0.78 mg
After	340	1.09 mg

Calculated on its protein content, the prothrombin is 90 times purer before isoelectric precipitation, and 210 times purer after, than in plasma (normal value: 70 mg/ml plasma).

The Conversion of Prothrombin to Thrombin

For the conversion of prothrombin (proenzyme), to thrombin (enzyme) there are two different physiological ways known today:

a) the intrinsic system (blood thromboplastin)

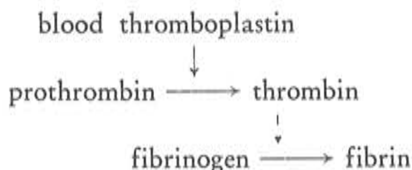
b) extrinsic system (tissue thromboplastin + factors V, VII and Stuart-Prower).

How this conversion is brought about is still not clear. Neither has it been decided whether the activation is catalytic or stoichiometric, nor how thrombin looks like as compared to its proenzyme.

1) *Conversion in the intrinsic system*

a) *Production of blood thromboplastin.* Biggs et al. (4) were the first to show that it is possible to produce a powerful thromboplastin in an incubation

mixture prepared with $Al(OH)_3$-adsorbed plasma, serum, isolated thrombocytes and $CaCl_2$. This blood thromboplastin works according to the following scheme:



Duckert et al. devised a modification of this test by using $BaSO_4$ for the adsorption of plasma and, instead of thrombocytes they use an equivalent of platelet-factor 3 which is a chloroform brain extract (method of Bell and Alton [3]). In both the original and the modified tests, the thromboplastic activity reaches its maximum after an incubation period of 4—6 mins. and is then followed by a rather rapid inactivation. The reason for this inactivation is still unknown. If one cools the incubation mixture rapidly to 0° C when its thromboplastic activity is at its highest, and then centrifuges (1000 r. p. m. at 0° C) to bring down the traces of fibrin, furthermore if the supernatant is re-centrifuged (20 000 r. p. m. corresponding to 25 000 g, at 0° C), the deposit dissolved in veronal-acetate buffer (1/8 original starting volume) and M/80 $CaCl_2$ added (1/8 original starting volume), the resulting product will show identical thromboplastic activity as that of the incubation mixture. Table 8 is a guide for isolating blood thromboplastin:

Tab. 8: Assay method for production of blood thromboplastin.

1) 8.0 ml $BaSO_4$-plasma 1/10 dil.
   8.0 ml serum 1/10 dil.
   8.0 ml chloroform-brain extract 1/100 dil.
   8.0 ml $CaCl_2$ M/40
   } at 37° C

2) e.g.

Incubation time in mins.	3	4	5	6	7
Clotting time in secs.	15	10	9	10	11

3) As soon as miximum activity is reached, cool rapidly to 0° C and centrifuge at same temperature.

4) Sediment (trace!) is dissolved in 4 ml veronal-acetate buffer.

The final Ca^{++} concentration is M/160 in both the incubation mixtures of the normal test and in the isolated thromboplastin. The thromboplastic activity is dependent on the amount of Ca ions present. If one dissolves the deposit only in veronal-acetate buffer or in 0.9% NaCl, poor thromboplastic activity is

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obtained. If Ca^{++} are added to make a final concentration of M/160, the thromboplastin becomes fully active. On the other hand, if one treats the fully active thromboplastin with ion-exchange resin (Dowex 50, Na cycle) thromboplastin loses its activity. If Ca^{++} is re-added thromboplastin becomes active again.

b) *Conversion of prothrombin to thrombin by blood thromboplastin.* From the following incubation mixture at different intervals 0.1 ml is added to 1.0 ml fibrinogen solution (300 mg fibrinogen/100 ml):

	1.0 ml	prothrombin solution (= 100 units)
at 37° C	0.1—0.4 ml	blood thromboplastin (dissolved only in buffer)
	0.4—0.1 ml	buffer
	0.5 ml	M/80 $CaCl_2$
	2.0 ml	

Graph 4 shows the amount of thrombin formed by the addition of different amounts of thromboplastin. The units of thrombin are calculated by means of a calibration curve.

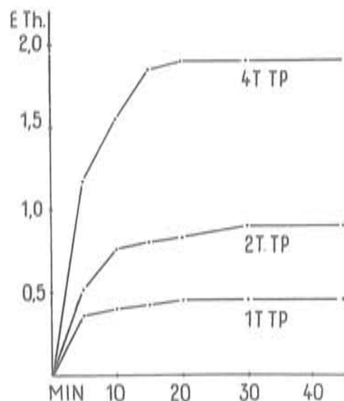

Fig. 4

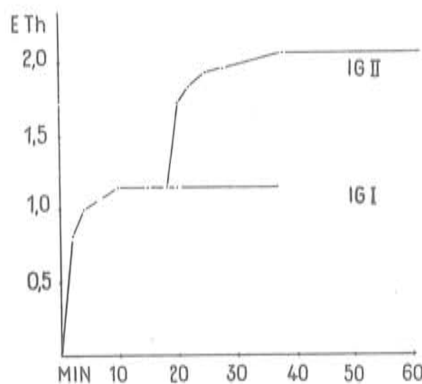

Fig. 5

Fig. 4: Conversion of prothrombin to thrombin by blood thromboplastin. Abscissa: Incubation time in minutes. Ordinate: Amount of thrombin units (N.I.H. units) / 0.1 ml. T.TP = part thromboplastin (1 part = 0.1 ml of thromboplastin as prepared and described above).

Fig. 5: Conversion of prothrombin to thrombin by blood thromboplastin. Abscissa: Incubation time in minutes. Ordinate: Units of thrombin (N.I.H. units). — IG I = Incubation mixture I (same type of assaying as in fig. 4 middle curve, using 2 parts of TP); IG II = Incubation mixture II (at 18 mins. incubation time. 1.0 ml of IG I added to 1 part TP).

There are three possible ways to interpret these conversion experiments:

a) After a certain time has elapsed, the thromboplastin in the incubation mixture is inactivated. The amount of thrombin formed remains constant and can be determined over a long period.

b) At the beginning thrombin production prevails. After incubating for 15—25 mins. thrombin production and thrombin inactivation are in equilibrium. In cases a) and b) thromboplastin would act as an enzyme.

c) Prothrombin and thromboplastin react together in a stoichiometric way: e. g.
Prothrombin (= Thrombin-X) + Thromboplastin = Thrombin + Thromboplastin-X.

The following assays show how we tried to elucidate those problems:

The isolated thromboplastin is stable for 60 mins. at 0° C. After 120 mins. there is a loss of 5—10%. If one does a conversion assay with prothrombin (see graph 4) and thromboplastin is added while the thrombin amount formed is constant, the production of thrombin starts again. This amount of thrombin produced is proportional to the amount of thromboplastin added (see graph 5).

On the other hand, if prothrombin is added at the same point we only find a dilution effect.

Stability assays show that prothrombin and thrombin activities are unaltered over a period of 60 mins. under these conditions. Therefore this indicates that interpretation b) must be incorrect.

Thromboplastin is rather unstable. By testing its stability at 37° C in an incubation mixture as described above, but without the addition of prothrombin, this following curve is obtained:

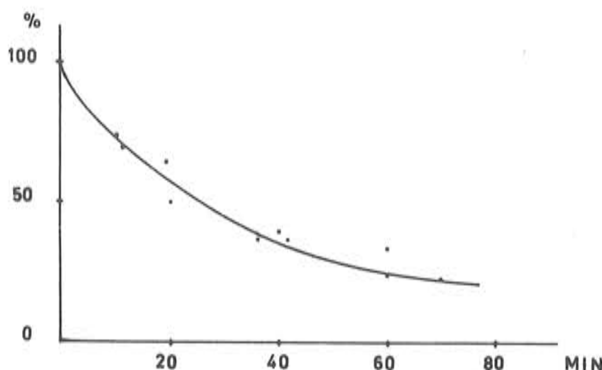

Fig. 6: Thromboplastin stability at 37 ° C. Abscissa: Incubation time in minutes. Ordinate: Thromboplastic activity in %. — Method: 0.1 ml thromboplastin, 0.4 ml buffer, 0.5 ml M/80 $CaCl_2$ Aliquots added to 0.1 ml Dowex-50 treated human plasma at different intervals. Clotting times in seconds are converted to %-activity by a calibration curve.

If one compares this stability curve with that of the conversion of prothrombin to thrombin, it will be seen that at the point where no more thrombin is formed, the loss of thromboplastin activity is only about 50%. Assuming

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that the thromboplastin undergoes inactivation in the stability assay at the same rate as in the conversion assay, the reaction between prothrombin and thromboplastin cannot be enzymatic. The production of thrombin stops because an equilibrium is reached. To prove the above statement, one should really be able to show that the loss of thromboplastic activity in the conversion assay and in the stability are the same. Unfortunately, we were not able to determine thromboplastin separately in a system containing thromboplastin and thrombin. But it is a well known fact that, in an incubation mixture of a thromboplastin generation test, the rate of thromboplastic inactivation becomes faster the more prothrombin is present. This prothrombin is converted to thrombin, and it seems that there is a relationship between the amount of thrombin formed in the incubation mixture and the rate of thromboplastic inactivation. Most probably the thrombin itself is not responsible for this inactivation. Graph 5 shows that in spite of the presence of thrombin the second addition of thromboplastin is able to produce more thrombin. But one can imagine that prothrombin is split in thrombin and another protein or polypeptide, the latter inhibiting the thromboplastin molecule. This would mean that thromboplastin acts on prothrombin enzymatically, but it is inhibited stoichiometrically by another reaction-product.

Further investigation must be carried out in order to reveal the exact kinetics between blood thromboplastin and prothrombin.

2) *Conversion in the extrinsic system:*

In the following incubation mixture, no thrombin is formed:

0.1 ml prothrombin solution (= 10 units)	at 37° C
0.1 ml fibrinogen solution	
0.1 ml brain thromboplastin	
0.1 ml M/40 $CaCl_2$	

Brain thromboplastin together with Ca^{++} is not able to convert prothrombin to thrombin. In our opinion, it is incorrect to call the other factors determined in Quick's "prothrombin time" (factors V, VII and Stuart-Prower) "accessory factors". They are essential for the conversion of prothrombin to thrombin by brain thromboplastin.

3) *Conversion in the "citrate assay":*

Seegers (23) showed that prothrombin can be converted to thrombin autocatalytically under certain conditions. Bovine prothrombin is incubated

together with some thrombin in a 25% Na-citrate solution at room temperature. After 24 hrs., all the prothrombin is converted to "citrate thrombin".

Through the courtesy of the Behring Werke*) we obtained bovine prothrombin prepared according to Seegers' method (24). This enabled us to do this important assay with different prothrombin preparations:

1. with the above-mentioned bovine prothrombin ⟶ thrombin formation
2. with human prothrombin purified by chromatography (Duckert [7]) ⟶ no thrombin formation
3. with human prothrombin purified immunologically (as described in this paper) ⟶ no thrombin formation.

By testing the bovine prothrombin prepared by Seegers' method with the one stage methods we found it to contain besides prothrombin, significant amounts of factors VII, IX, Stuart-Prower and small amounts of factors V and VIII. In the thromboplastin generation test this prothrombin is able to replace normal serum, whereas our prothrombins when used in place of serum gives no thromboplastin formation. If brain thromboplastin, Ca^{++} and this bovine prothrombin are mixed together thrombin is rapidly formed.

We believe that an autocatalytic conversion of prothrombin to thrombin does not exist. As the prothrombin prepared by Seegers' method contains all the factors (some of them in very small amounts) required for thromboplastin formation, it is possible that the thrombin formation in a 25% Na-citrate solution takes place in a similar manner as in the intrinsic system. However, such conditions are quite extraordinary as regard to ionic strength, pH and Ca^{++} concentration.

Discussion

At the beginning of this paper we have insisted on the fact, that the physicochemical properties of the factors belonging to the "Prothrombin-group" are very similar to each other. For this reason, the separation of these proteins is quite difficult. It was an attractive task to purify prothrombin by applying chemical and immunological methods together. A preparative immunological method offers the advantage of high specificity. We are aware of the fact that the results are not yet satisfactory in every respect. It seems to us that we have attained at least the main goal of separating prothrombin from the other clotting factors.

*) We are indebted to Prof. Schultze of the Behring Werke, Marburg, Germany for providing us with samples of bovine prothrombin.

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There is yet another problem to be discussed here, namely the physiological relation between prothrombin and its chemically related factors. During our investigations we have never observed a conversion of prothrombin to factors VII or IX. We had the oportunity to investigate the plasmas of 4 cases of hypoprothrombinaemia.*) We found them to have prothrombin levels of 3—7%, while all the other factors were within the normal range. On the other hand, patients with a deficiency of factor VII or IX or Stuart-Prower have a normal prothrombin level. If one stores serum at 4° C for a long period the concentration of active factor VII is increased up to three folds the concentration determined at the beginning, whereas the residual serum prothrombin is unaltered. Immunologically all the discussed factors are identical in plasma as in serum. Based on these facts we believe that the factors of the "Prothrombin group" are different chemical entities, and not derivatives of prothrombin. They are all produced in the liver but differ from prothrombin by their functions in the clotting system and their turnover rate.

Summary

A combined chemical and immunological method for the purification of human prothrombin is described. After isoelectric precipitation a purified prothrombin is obtained. Its activity per mg of protein is 210 folds higher than that of plasma. This prothrombin and the prothrombin purified on a $BaSO_4$ column are not converted into thrombin in a 25% sodium-citrate solution. No thrombin is formed by the addition of calcium ions and brain thromboplastin. For its conversion, a complete clotting system (extrinsic or intrinsic) is necessary. Our experiments present no evidence for the hypothesis that Factors VII, IX, PPA and Stuart-Prower are prothrombin derivatives.

The conversion of prothrombin to thrombin by means of isolated blood thromboplastin is studied. The results still do not permit one to decide whether the reaction is stoichiometric or enzymatic.

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Résumé

Une méthode de purification de la prothrombin humaine est décrite. La fraction II + de Cohn, obtenue à partir de plasma oxalaté, est traitée

*) We wish to thank Profs. Quick (USA) and Van Creveld (Holland), for sending us plasmas of their patients B. B. and T. B., G. R. and W. R. respectively.

par un sérum de lapin préablement immunisé par une préparation concentrée des facteurs de coagulation sériques (facteur VII, IX, PPA et Stuart-Prower). Après réaction, seule la prothrombine est adsorbée sur le $BaSO_4$. Après élution et précipitation isoeléctrique la prothrombine est enrichie 210 fois par rapport au plasma. Elle ne contient pas ou seulement des traces d'autres facteurs de coagulation.

Elle n'est pas convertie en thrombine par le citrate de sodium à 25%, ni par la thromboplastine de cerveau et le Ca^{++} seuls. La conversion prothrombine thrombine nécessite un système complet.

Les facteurs VII, IX, PPA et Stuart-Prower ne sont pas des dérivés de la prothrombine.

La conversion prothrombine thrombine par l'action de la thromboplastine sanguine est étudiée. Les résultats ne permettent pas encore de décider si cette réaction est enzymatique ou non.

Zusammenfassung

Es wird eine Methode beschrieben, die es gestattet, mit Hilfe eines kombinierten chemischen und immunologischen Verfahrens menschliches Prothrombin zu isolieren. Die Präparate weisen gerinnungsphysiologisch einen guten Reinheitsgrad auf, sind jedoch immer noch mit anderen Eiweißkörpern verunreinigt (Anreicherung 210mal).

Wir untersuchten die Umwandlung des Prothrombins in Thrombin durch Blutthromboplastin (zu dessen Isolierung ebenfalls eine Methode angegeben wird) und Hirnthrombokinase. Die Frage, ob Blutthromboplastin stöchiometrisch oder enzymatisch wirkt, wird diskutiert. Die Lösung dieses Problems gelingt allerdings noch nicht.

Die Faktoren VII, IX, PPA und Stuart-Prower können nicht als Derivate des Prothrombins betrachtet werden. Sie unterscheiden sich in ihrem Stoffwechsel, und ihre physiologische Funktion ist unabhängig vom Prothrombin.

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