Materials and Methods. Female Wistar rats, weighing 200–250 g, were pretreated with 2×10 µg/kg ricin, i.p. Ricin was prepared by us (BALINT[5]) with the help of KOBERT's method and it had a true resemblance to a Merck's preparation, which was stated also to have been produced according to KOBERT's method (E. MERCK Ltd., Darmstadt, W. Germany, Charge No.; Br-23-1776-W. 42721-722721). The preparation was an unfractionated ricin with high enough purity. Its LD_{50} value on rats was 38,15 ± 0,412 µg/kg, i.p. (BALINT[5]).

Between the 2 ricin doses, 4 days elapsed, and the experiment was carried out on the 3rd day after the second ricin dose. The animals belonging to the 2 control groups received treatment as follows: Control group No. 1 was treated with 2×0.25 ml sterile, pyrogen-free normal saline solution, i.p., while the control group No. 2 received 2×80 mg/kg phenobarbital (sodium-salt) i.p. Both control groups received the treatment at the same time when ricin was administered. Each of the groups contained 15 animals.

After the above-mentioned pretreatment, the animals were narcotized i.p. with 100 mg/kg hexobarbital ('Evipan-Natrium', Bayer, W. Germany) and the sleeping-time was measured. As 'sleeping-time' was considered the time interval which elapsed from the injection of hexobarbital till the animals tolerated lying on their back.

The effect of ricin on the hexobarbital sleeping-time on rats

Treatment	Sleeping-time (min) Mean values ± S.D.	*P*
Control (normal saline)	102 ± 12	—
Control (phenobarbital)	57 ± 15	< 0.05
Ricin	68 ± 16	0.05

During the experiments, electron microscopic investigations were carried out also, on another group of animals which received the same treatment. The results of these experiments are reported elsewhere (BALINT[6]) but to show the hypertrophy of the smooth endoplasmic reticulum (Figure 1). The increase in the density of the whole treated cell is one of the most noticeable features.

Results. The experimental results were analyzed statistically with the help of Student's *t*-test and are listed in the Table.

Discussion. The results, listed above, give further suggestions that ricin might have an inductive effect on the drug metabolizing enzyme system in the liver. This experimental result appears unique in the literature, as no material of plant origin is known to have this effect, and no material with so high molecular weight (about 66.000–70.000) as ricin is known to have a similar effect. Further investigations are necessary on this question[8].

Zusammenfassung. Vorversuche mit Rizin, dem toxischen Protein von Rizinussamen (*Ricinus communis*) ergaben einen Verkürzungseffekt der Hexobarbital-Schlafzeit bei Ratten, was für einen induktiven Effekt auf das drogenabhängige metabolische Enzymsystem der Leber spricht.

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Isolation of Thrombin-E and the Evolution of Enzyme Activity from Prothrombin[1]

As a prerequisite for the clotting of blood, thrombin must first be derived from prothrombin. The proteolytic and esterolytic functions of this enzyme are dissociable properties, and during the activation of prothrombin, esterase activity appears first. This is followed by the emergence of proteolytic activity or the capacity to induce the coagulation of fibrinogen. This latter property then disappears leaving only esterase activity. These facts were presented in several papers from this laboratory beginning in 1957[2–4]. At that time, the technology of protein chemistry was not adequately developed to enable an interpretation of the activation sequence in terms of protein structure.

What structure does the protein have when the enzyme can hydrolyze *p*-toluenesulfonyl-L-arginine methyl ester (TAMe), and how is it changed when this property is retained while the capacity to clot fibrinogen evolves and subsequently disappears? This report, based on the use of technology previously described[5–9], provides information on the question formulated above. Thrombin has the capacity to hydrolyze TAMe and to 'clot fibrinogen'. Itis a two chain structure in which the A chain is connected to the B chain by a disulfide bond[10]. Our new experiments serve to demonstrate that the B1 portion[8] of the B chain is removed by autolysis and the remaining structure, called thrombin-E, possesses only the quality to hydrolyze TAMe. We present a simple procedure for the isolation of thrombin-E and the B1 chain. We have found that the

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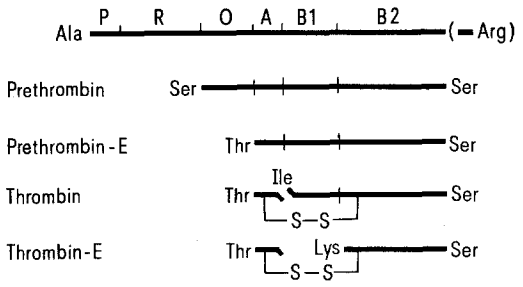

Fig. 1. Purified prothrombin viewed as a single chain after reduction and alkylation. In the model, the molecular weight (69,800), with carbohydrate included, is proportional to the length assigned to each fragment. Each bond broken by autoprothrombin C or thrombin is an arginyl. The NH_2-terminal amino acid for R fragment is threonine. Thrombin, as well as autoprothrombin C, can remove PR fragment. From prethrombin to prethrombin-E requires autoprothrombin C. From thrombin to thrombin-E requires thrombin. Prethrombin-E has esterase activity. By breaking its Arg-Ile bond with autoprothrombin C, the B1 portion of the B chain is free and the structure is that of classical thrombin. By autolysis, the B1 chain is broken from the B chain, and the active histidine site which it contains can no longer function. Thrombin-E has only esterase activity. In the prothrombin activation sequence, there is one pathway as follows: Prothrombin → Prethrombin + PR fragment → Prethrombin-E + 0 fragment → Thrombin → Thrombin-E + B1 chain.

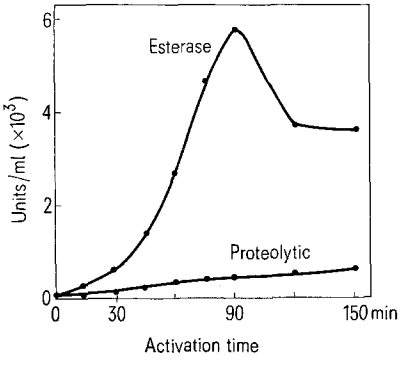

Fig. 2. Activation of purified bovine prethrombin (2.3 mg/ml) with purified autoprothrombin C (0.14 mg/ml) in 0.05 *M* *Tris*-HCl in 0.9% NaCl at pH 7.8 and at 30 °C. No calcium added.

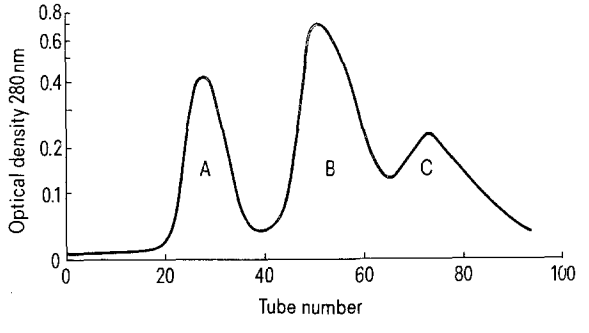

Fig. 3. Thrombin after autolysis on Sephadex G-100 column (2.5 × 93 cm) in 0.1 *M* ammonium bicarbonate, pH 8.0. Flow rate 19 ml/h. Fraction volume 6 ml/tube. Fraction A was B1 chian. Fraction B was thrombin-E and fraction C unidentified thrombin autolysis products.

first esterase activity is associated with prethrombin-E, which is a single chain polypeptide (Figure 1). Its molecular weight and amino acid composition are identical with that of thrombin, and it is converted to thrombin by breaking an Arg-Ile bond with autoprothrombin C (Factor Xa)[11].

Our experiments are consistent with the conclusion that all 3 enzymes, namely, prethrombin-E, thrombin, and thrombin-E, have an active serine site, but the B1 chain, with its active histidine, is the structure which accounts for functional variability. In prethrombin-E, B1 chain is bound within a straight polypeptide chain, in thrombin it is free to function as the NH_2-terminal end of the B chain, and in thrombin-E it is absent.

We found special conditions for activating purified prethrombin which serve to demonstrate the esterase activity of prethrombin-E. Only autoprothrombin C (Factor Xa) was used (Figure 2). Esterase activity developed, but only a small amount of thrombin clotting activity was generated. Throughout the activation, the two-stage prethrombin assay yielded the full thrombin titre possessed by the purified prethrombin at the outset. By using polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, we could identify prethrombin-E, a small amount of thrombin, and the 0 fragment, from the non-thrombin NH_2-terminal portion of prethrombin, in the activation mixture. We have isolated the 0 fragment from the reaction mixture and are in the process of determining its primary amino acid structure. The prethrombin-E has also been isolated[11], but as an inactive protein. We are currently attempting to isolate prethrombin-E in its active form. Difficult separation problems exist because thrombin and prethrombin-E have similar properties. Their net charge and molecular weights are similar.

Instead of converting prethrombin to prethrombin-E, the usual procedure is to convert it completely to thrombin. The classical thrombin which then arises from prethrombin has esterase and 'clotting' activi-

Table I. Proteolysis of purified thrombin at 4 °C, pH 8.0

Days	Proteolytic [a]	Specific [b]	Esterase [a]	Specific [b]
0	280,000	9,300	280,000	9,300
5	170,000	5,700	280,000	9,300
10	142,000	4,700	290,000	9,700
15	101,000	3,400	305,000	10,200
20	56,000	1,900	340,000	11,400
25	23,000	770	365,000	12,100 [c]

[a] Activity in U/ml. [b] Specific activity in U/mg. [c] When 12,100 is corrected for loss of B1 chain, the value is 14,500.

Table II. Inactivation of enzymes with purified antithrombin, TLCK and DFP

Enzyme	Antithrombin	TLCK	DFP
Prethrombin-E	Inhibited	Inhibited	Inhibited
Thrombin	Inhibited	Inhibited [a]	Inhibited
Thrombin-E	Inhibited	Active	Inhibited

[a] Esterase and clotting activity inactivated.

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Table III. Amino acid composition of thrombin-E and related preparations[a]

	Prethrombin	0 Fragment	Prethrombin-E	Thrombin	Thrombin-E	B1 chain
Lysine	26	2	23	24	17–18	5
Histidine	7	0	7	7	5	3
Arginine	29	7	22	22	15	5
Aspartic acid	44	16	29	29	23–24	7
Threonine	19	5	13	13	11	3
Serine	23	8	14	12	12	5
Glutamic acid	49	14	35	34	29	8
Proline	25	8–9	16	17	12	5
Glycine	35	11	22	25	19–20	5
Alanine	24	9	15	15	12	5
Half-cystine	12	4	8	8	6	2
Valine	24	5	17	21	17	6
Methionine	5	0	5	5	4	1
Isoleucine	15	1	22	15	11–12	4
Leucine	36	8	24	28	22	12
Tyrosine	16	4	11	11	8–9	2
Phenylalanine	16	3	13	12	9–10	2
Tryptophan	12	2	10	10	5	2
Total residues	417	107–108	306	308	237–242	82
MW amino acids	47,506	11,744–11,873	35,549	35,552	27,647–28,292	9,272

[a] Beckman amino acid analyzer. Hydrolysis for 22, 48 and 72 h. For threonine, serine, half-cystine, methionine, and tyrosine, an extrapolation to zero was made for loss during hydrolysis. Tryptophan, method of DUGGAN and UDENFRIEND[18].

ty[12]. Material with the highest specific activity obtained to date was obtained by isolation of the thrombin from an activation mixture consisting of purified prethrombin + purified autoprothrombin C + purified Ac-globulin (Factor V), phospholipid + calcium ions. The purified product forms crystals, and consists of an A chain bound to the B chain by one disulfide bond[13,14].

For the purpose of preparing thrombin-E, which has only esterase activity, we isolated thrombin from a purified prethrombin activation mixture. This was set aside for autolysis (Table I) at 4 °C, at pH 8.0, in 0.2 *M* ammonium bicarbonate. The clotting activity gradually declined, while the esterase activity remained and even increased. During the autolysis, a precipitate formed and was isolated by centrifugation. It proved to be the B1 fragment of the B chain of thrombin.

After 1 month of autolysis, the mixture was passed through a G-100 Sephadex column. This is a satisfactory method for isolating B1 chain as well as thrombin-E. In the first fraction, there was no enzyme activity. It was an aggregate of pure B1 chain. Thrombin-E and some heterogeneous material appeared in separate fractions (Figure 3). The purified thrombin-E was identified as a single component by several criteria, including disc gel electrophoresis. With the ultracentrifuge, we found $S^0_{20,w} = 2.69 - 0.008\,X$, where X is protein concentration in mg/ml. In 0.1 *M* KCl, pH 7.2, at 59,780 rpm, 20 °C, 65° angle, the schlieren pattern consisted of a single peak. An aliquot of the thrombin-E was reduced and alkylated for the purpose of isolating the A chain, identical with that in thrombin, and the B2 chain (previously called β-chain[8]).

Each one of the enzymes was subjected to different kinds of inhibitors; namely, 1. diisopropylfluorophosphate (DFP) which binds to an active serine site residue of serine proteinases, 2. tosyl-L-lysine chloromethyl ketone (TLCK) which binds to an active histidine residue[15], and 3. purified bovine antithrombin III which combines with thrombin in a mutual depletion system[16]. The concentrations at pH 7 were DFP 5.5×10^{-4} *M*, TLCK 2.6×10^{-3} *M*, and antithrombin purified by the method of MONKHOUSE[17] was used in 'excess'. With one exception, the enzymes were inactivated by each one of the inhibitors (Table II). The exception was the refractoriness of thrombin-E to TLCK, which is accounted for by the fact that it has no B1 chain in which the essential active center histidine is found.

The amino acid analyses (Table III), activity measurements, and facts about the B1 chain of thrombin are all consistent with the interpretation that esterase activity is the first to arise from prothrombin and is associated with a single chain structure. Upon cleavage of an Arg-Ile bond, thrombin forms. When the B1 chain of thrombin is removed by autolysis, the two chain structure retains only esterase activity.

Résumé. La préthrombine-E a seulement une activité estérasique et, après la perte d'un lien Arg-Ile, elle provoque la formation dlune thrombine protéolytique composée de 2 chaines (A et B) liées dans une maille bisulfide. A la suite d'un procédé de décomposition autolytique, la chaîne B se détache et on obtient la thrombine estérasique formée par 2 chaînes A et B_β. On a introduit de nouvelles méthodes pour l'isolement de la thrombine-E. L'antithrombine et le DFP neutralisent effectivement les 3 enzymes.

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