radical anneals by the addition of something at carbon No. 5 and by quenching the unpaired spin. The ESR signal vanishes, but the final photoproduct would be tagged. Alternatively, when the radical vanishes, it could lose either of the protons on carbon No. 6. In this case half the photoproducts would lose the tritium and half the hydrogen, so the final product would again be tagged.

Beukers and Berends (*13*) have reported that paramagnetic metal ions reduce the photodamage of the pyrimidine derivative, orotic acid. Experiments were performed when Co++ and Cu++ ions were bound to the DNA before irradiation in the ratio of one metal ion to ten phosphates. Samples containing the paramagnetic metal ions showed that the concentrations of free radicals were reduced by a factor of about 6 as compared with similar samples irradiated under the same conditions, but containing no metal.

Finally we present the ESR signals from different DNA samples (Fig. 5). The four traces on the upper left are calf thymus DNA; three are moist and one is dry. The three wet samples are from different sources, but the values attained are in excellent agreement. There are significant differences between these and the salmon sperm DNA on the upper right where in comparison the thymine hyperfine lines are less intense with respect to the unidentified central lines. At the bottom are traces of samples of moist DNA from *E. coli* and *Serratia cetals*. The thymine hyperfine structure can be identified in all cases, but in different species its intensity relative to the center line varies. These variations from species to species could originate in the chemical techniques by which the samples are extracted from their parent cells.

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Peptides Attached to Thrombin: Their Influence on Proteolysis

Abstract. *During the activation of prothrombin at least two peptides are split off the molecule. These peptides become attached to thrombin, changing its proteolytic activity. Depending on the peptide adsorbed the endopeptidase activity of thrombin increases or diminishes. Acidification of thrombin by acetylation of amino groups uncovers the influence of peptides on the properties of thrombin.*

The activation of prothrombin is presumed to be an orderly degradation of the protein molecule (*1*). Enzymes with endopeptidase activity, which use prothrombin as substrate, are derived from prothrombin itself. Thrombin (*1*) and autoprothrombin C (*2*) can hydrolyze the prothrombin molecule. Thrombin will be the end product of prothrombin activation whenever this process goes to completion. Thrombin, acting on fibrinogen (as substrate), releases two peptides, thus allowing fibrin to form by polymerization. This is the primary role of thrombin in the clotting mechanism. Thrombin can also utilize synthetic substrates such as tosyl arginine methyl ester (TAMe), showing an activity which is differentiated from the clotting activity; this is called the esterase activity (*3*). Determinations of thrombin activities, performed with equivalent calibrations, show that whenever prothrombin is activated, the first activity that develops is the esterase activity. This is shortly followed by the development of the clotting activity. When activation is completed the esterase activity equals clotting activity (*4*).

These properties of thrombin have been associated with free amino groups, since the acetylation of 50 percent of the free amino groups results in a product without clotting activity but with the same or increased esterase activity (*5*). The affinity for both substrates, namely fibrinogen and TAMe, changes with the acetylation. The acidification of thrombin by acetylation does not allow the interaction of thrombin with fibrinogen to occur, although fibrin is still acted upon by acetylated thrombin (*5*).

The thrombin molecule is known to be associated with two peptides, one containing *N*-terminal glutamic acid (peptide A), and the other containing threonine (peptide B) (*6*). Thrombin as it is formed during activation or after chromatography on amberlite IRC-50 shows glutamic acid as *N*-terminal and after chromatography on phosphate cellulose, shows threonine. This suggests that the peptides may become redistributed on the surface of the molecule as a result of the purification procedures. Only by treating thrombin with a 30-percent solution of urea will the molecule be freed of adsorbed peptides and show the *N*-terminal isoleucine (*6, 7*).

The only form of thrombin that is free of peptides is the one obtained by activation of prothrombin with trypsin (*8*). Consequently, there may be four types of thrombin product: (i) thrombin chromatographed with amberlite, having adsorbed on its surface the peptide with glutamic acid as *N*-terminal, (ii) thrombin chromatographed on phosphate cellulose, having adsorbed on its surface the peptide with threonine as *N*-terminal, (iii) thrombin treated with concentrated urea solutions and free of peptides, and (iv) thrombin obtained by trypsin activation.

To gain some insight into the significance of the peptide structures ad-

Table 1. Changes in esterase activity and the endopeptidase activity induced by acetylation of thrombin.

Esterase (units/ml)	After acetylation		
	Esterase (units/ml)	Endopeptidase activity (expressed as milligrams of tyrosine)	
		500 units thrombin	1000 units thrombin
Amberlite-purified thrombin			
10,000	21,000	0.12	0.19
Cellulose-purified thrombin			
12,000	6,000	0.10	0.18
Trypsin-purified thrombin			
10,000	10,000	0.11	0.20
Urea-treated thrombin			
10,000	11,000	0.10	0.19

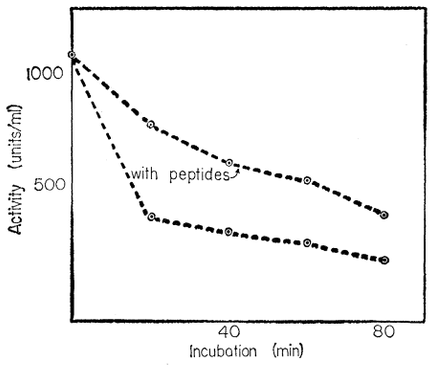

Fig. 1. Inactivation of prothrombin by thrombin. Influence of thrombin peptides. The reaction mixture contained: prothrombin, 1080 units/ml; thrombin, 10 units/ml; and peptides, 20 μg. Dashed lines, prothrombin activity.

sorbed on thrombin and the way they affect the properties of the molecule we have acetylated all four products. Acetylation is carried out with acetic anhydride in a buffer of sodium acetate (*5*). Approximately 10 mg of thrombin preparation was used for each experiment. The thrombin preparations were assayed by the two-stage method with clotting of fibrinogen as the end point (*9*), and with esterase activity being determined by the hydrolysis of TAMe (*3*). The reagents for each of the two determinations were adjusted for equivalent calibrations when a provisional standard of thrombin was analyzed.

Initially, thrombin to which peptide A was adsorbed showed an increase in capacity for hydrolyzing TAMe when about 50 percent of the amino groups were blocked. In a subsequent experiment, a twofold increase in esterase was induced by acetylation. Acetylation of preparations with adsorbed peptide B, however, led to reduction of esterase activity to exactly half the original values (Table 1). Apparently the peptides adsorbed on thrombin can influence its affinity for TAMe. To explore this postulate further, acetylation of peptide-freed thrombin was performed.

Acetylation of 50 percent of the amino groups in thrombin, obtained by trypsin activation or urea treatment, does not alter esterase activity (Table 1). Thrombin dissolved in a urea solution does not adsorb peptides when the urea is diluted or removed by fast dialysis. When the activation of prothrombin is completed, the glutamic acid and threonine, present as *N*-terminal amino acid, are associated with thrombin. Chromatographic fractioning on amberlite IRC-50, phosphate cellulose, DEAE, Sephadex, or precipitation of protein fractions with trichloroacetic acid failed to demonstrate free peptides with these amino acids as *N*-terminal. The conditions for adsorption of peptides on the thrombin molecule apparently have specific requirements that are not met by the simple mixture of peptides and thrombin. These specific requirements are met when peptides and thrombin are being split from the prothrombin molecule.

The endopeptidase activity of thrombin can be demonstrated by using fibrinogen, fibrin, and prothrombin as substrates. However, the endopeptidase activity of acetylated thrombin can be demonstrated only on fibrin (*5*).

In order to determine whether variation of esterase activity, due to acetylation of thrombin, is accompanied by a change in the proteolytic activity, the following experiment was performed. Commercial bovine fibrinogen was clotted with thrombin; the fibrin clot was washed with saline, dissolved in 20-percent solution of urea, and precipitated by dilution with water. This procedure was repeated three times. The fibrin was dried in the oven at 65°C for 12 hours, and a fine powder was made from it. The powder (20 mg) was suspended in acetylated thrombin solution (500 and 1000 units/ml), and the suspension was incubated at 37°C for 2 hours, centrifuged at 10,000 rev/min for 20 minutes and the supernatant was assayed for soluble protein by the tyrosine method (*10*). The tyrosine measured in control tubes containing only acetylated thrombin or only fibrin suspension was deducted from the results. The endopeptidase activity of thrombin, with fibrin as substrate, changes with acetylation in the same way as the esterase activity does (Table 1).

We do not accept the idea that thrombin has two separate activities. Esterase and clotting activity are manifestations of the same endopeptidase activity, demonstrated on different substrates.

The thrombin peptides influence the overall activation reaction by changing the proteolytic activity of thrombin. Thrombin acting on prothrombin forms a derivative that cannot be activated by the thromboplastin-calcium system (*11*). To learn about the influence of thrombin peptides on prothrombin activation, the following experiments were performed. Thrombin was added to a solution that contained prothrombin and thrombin peptides that were purified as described previously (*6*). The peptides inhibited the endopeptidase activity of thrombin (Fig. 1). The same results were obtained when the peptides were added to a prothrombin activation mixture consisting of platelet factor 3, calcium, and prothrombin (Fig. 2). The formation of thrombin as well as the inactivation of prothrombin was inhibited by the peptides. Thus thrombin peptides influence the overall activation reaction by changing the proteolytic activity of thrombin.

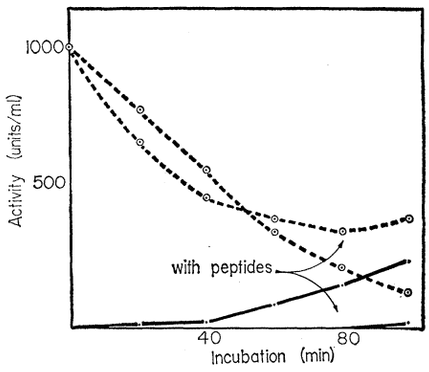

Fig. 2. Prothrombin activation with platelet factor 3 and calcium. Influence of thrombin peptides. The reaction mixture contained: prothrombin, 1000 units/ml; platelet factor 3, 34 units/ml; calcium, 0.02*M*; and peptides, 20 μg. Dashed lines, prothrombin activity; solid lines, thrombin activity.

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