Fibrino-peptide

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Investigations on the *N*-terminal groups of fibrinogen and fibrin, as described in previous papers (Bailey, Bettelheim, Lorand & Middlebrook, 1951; Lorand, 1951*a*; Lorand & Middlebrook, 1952), revealed that *N*-terminal residues of glycine are set free in the fibrinogen molecule as a result of the action of thrombin. Whatever the underlying mechanism of clotting, it was shown that the fundamental enzymic step in the transformation of fibrinogen to fibrin is closely linked up with the liberation of these end groups. This was the first direct evidence that thrombin acts as a protease. Assay of the *N*-terminal residues showed, furthermore, that the glutamic acid end groups of the fibrinogen molecule were absent in fibrin. Such a change implied that their 'disappearance' during clotting was due, if they were not recombined in some way, to a splitting-off of part of the fibrinogen molecule.

Current ideas on the enzymic action of thrombin are governed by the conclusions of Jaques (1938), who stated that all fibrinogen nitrogen was converted into fibrin nitrogen. These findings (Astrup, 1950) do not favour the concept that the fibrinogen-thrombin reaction produces another substance besides fibrin. It became clear, however (Lorand, 1951*a*, *b*), that Jaques's approach in studying the *N*-partition of the clotting system cannot be regarded as adequate because of the possibility of a second reaction product being occluded within the clot and so being estimated as fibrin. If such a substance is produced in the clotting system, it will not be detected unless looked for in a special way. A short account of the work presented here in detail has already been published (Lorand, 1951*b*).

MATERIALS

Fibrinogen. This was purified from commercial Bovine fraction I (Armour) by precipitation with 0·25 vol. saturated $(NH_4)_2SO_4$ (Laki, 1951*a*). The clottability of the final product exceeded 90%, and care was taken that only freshly dialysed solutions were used in the experiments.

Thrombin. This was obtained by the courtesy of Prof. Seegers and was used in aqueous, freshly dialysed solutions (2 mg./ml.).

Other reagents were of analytical purity, and glass-distilled water was used throughout the experiments.

EXPERIMENTAL AND RESULTS

The release of non-protein N *from the fibrinogen-thrombin system*

The fibrin gel could be converted into a flocculent precipitate by dissolving it in 1% (w/v) monochloroacetic acid and then precipitating the proteins with 7% (w/v) trichloroacetic acid. This allows the release of non-protein material in the supernatant which was assayed for nitrogen by the micro-Kjeldahl method. Monochloroacetic acid, like urea (Lorand, 1950*a*, 1951*a*), dissolves the clot rapidly to produce a clear solution. It neither interferes with the nitrogen estimation nor does it inhibit the deproteinizing action of trichloroacetic acid.

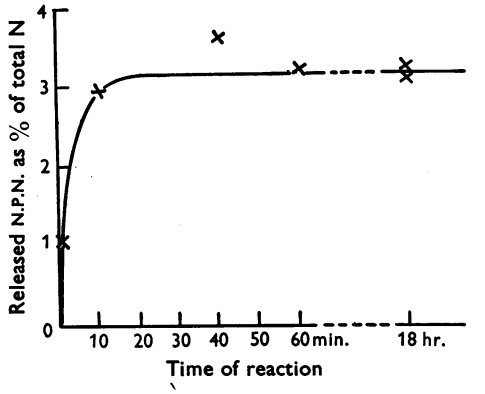

Fig. 1. Increase of N.P.N. (% of total N) with time of action of thrombin on fibrinogen.

In typical experiments the following procedure was adopted. To 8 ml. portions of fibrinogen (0·378 mg. N/ml.) dialysed against 0·2 M-KCl, 2 ml. 0·05 M phosphate buffer, pH 6·8, and 0·2 ml. thrombin were added. Clotting occurred within 30 sec., and the gels were dispersed at different times with 9·8 ml. of 2% (w/v) monochloroacetic acid. After dissolution of the clots in 2 min., 10 ml. of 20% (w/v) trichloroacetic acid were added. Half an hour after the precipitation of the proteins the mixtures were filtered and N was estimated in duplicate on suitable portions of the filtrates. Kjeldahl digestions were carried out for 8 hr. using the catalyst mixture recommended by Chibnall, Rees & Williams (1943), and the method of estimating N was that of Ma & Zuazaga (1942). A solution of fibrinogen,

to which mono- and tri-chloroacetic acid was added prior to thrombin, was used as control. The results are shown in Fig. 1.

The asymptotic level of the time-curve, maintained for more than 18 hr., indicates that the increase of non-protein nitrogen (N.P.N.) in the supernatant is not due to fibrinolysis, but is a characteristic feature of the enzymic action of thrombin on fibrinogen. The reaction does not stop with the onset of gelation, but goes on to completion when about 3% of the total N appears as N.P.N. This implies that a pure fibrinogen preparation cannot contain more than about 97% clottable N if fibrin could be recovered by an adequate method. (For an attempt along such lines see Lorand, 1951*a*.)

Peptide liberated by the action of thrombin on fibrinogen

Filtrates of deproteinized solutions of fibrinogen and fibrin were shaken with ether in order to remove the chloroacetic acids. Portions (1 ml.) of each of the ether-extracted solutions were dried *in vacuo* and hydrolysed with 0·2 ml. 5·7 N-HCl in sealed tubes for 24 hr. at 110°. After the acid had been evaporated, both hydrolysates were subjected side by side on the same paper strip to one-dimensional ascending chromatography. Phenol-NH_3 was used as mobile phase, and the chromatograms were developed with a freshly made solution of 0·1% (w/v) ninhydrin in ether, heated for 5 min. at 100°.

The chromatogram corresponding to the hydrolysate of the protein-free supernatant of fibrinogen (repeated on three different preparations) showed a complete absence of amino-acids; while that representing the protein-free supernatant of fibrin produced several spots of varying intensity.

In later experiments, in order to avoid the distortion of the chromatograms by the presence of salts, the original fibrinogen solution was dialysed against a solution of 0·05% (w/v) ammonium carbonate. Clotting could be conducted adequately in this medium, and the ammonium carbonate was volatilized during subsequent steps of the experiment. The results obtained by this modified process were substantially the same as described above, and the quality of the chromatograms was improved.

The R_F values of the amino-acids in the hydrolysate from the protein-free supernatant of fibrin were found, with phenol-ammonia as the mobile phase on a one-dimensional chromatogram, to be as follows:

R_F values	0·1	0·29	0·4	0·5	0·65	0·87	0·92
Relative intensity of colour	Strong	Strong	Weak	Strong	Weak	Weak	Weak

Chromatograms of unhydrolysed samples of supernatants did not show spots corresponding to free amino-acids.

Isolation of peptide from the fibrinogen-thrombin system

The fibrinogen was thoroughly dialysed against a solution of 0·05% (w/v) $(NH_4)_2CO_3$ at 0° until all other salts were replaced. Thrombin was mixed with the freshly dialysed fibrinogen solution at room temperature, so that the clotting time was 1–2 min. 2 hr. after gelation an equal volume of 2% (w/v) monochloroacetic acid was added, whereupon the clot dissolved rapidly. When a clear solution was obtained, the proteins were precipitated by addition of half a volume of 20% (w/v) trichloroacetic acid. The precipitate was allowed to settle and the mixture was then filtered. The filtrate was concentrated by vacuum distillation at about 13° into a smaller volume. This solution was thoroughly extracted with peroxide-free ether in order to remove the chloroacetic acids. The ether-extracted solution was then evaporated *in vacuo* and the separated material washed with ether.

The substance obtained was readily soluble in water; the solution gave no precipitate on adding trichloroacetic acid, showing that no protein was present. A paper chromatogram of the substance demonstrated the absence of free amino-acids. The acid hydrolysate of the material, however, gave rise to a number of amino-acid spots on the chromatogram, similar to those described in the previous section.

The peptide can be precipitated from its solution in water by 75% (v/v) acetone, and by this method an apparently pure sample can be obtained. Investigations for establishing the composition and molecular structure of the peptide are being carried out and will be reported on later. Preliminary results (Lorand & Middlebrook, 1951) indicate that the free amino end of the peptide chain consists of a glutamic acid residue, in good agreement with the previously reported finding that the *N*-terminal glutamic acid groups of the fibrinogen molecule are absent in fibrin (Lorand, 1951*a*; Lorand & Middlebrook, 1952). From a personal communication it is learnt that Dr K. Bailey and Mr F. R. Bettelheim (1951), in parallel experiments at Cambridge, have also isolated a homogeneous peptide from the clot liquor. They have prepared this peptide both in the free form and also as a water-soluble dinitrophenyl compound; its terminal amino groups account for the difference found in the content of glutamic acid *N*-terminal groups between fibrinogen and fibrin.

According to private communications, Laki (1951*b*) noticed recently the appearance of a substance in the protein-free supernatant of the iodinated fibrinogen and thrombin system. The supernatant was found to be biologically active in stimulating the frog heart (Laki, 1951*c*), and it is known now to give the biuret reaction. It is believed,

therefore, that the substance when isolated may turn out to be identical with the peptide reported previously by the present author (Lorand, 1951*a*, *b*).

DISCUSSION

Previous authors (Bailey *et al.* 1951; Lorand, 1951*a*; Lorand & Middlebrook, 1952) had reported that peptide bonds involving the amino group of glycine are split by thrombin within the fibrinogen molecule when it is converted to fibrin, and it had also been shown that the glutamic acid *N*-terminal residues of fibrinogen cannot be found in fibrin. The present paper reports further that the fibrinogen-fibrin transformation is accompanied by a simultaneous liberation of N.P.N., and evidence is presented to show that a peptide is set free at the same time. As suggested in the preliminary note (Lorand, 1951*b*), this peptide should be called 'fibrino-peptide', for it is believed that it is produced by splitting off a portion of the fibrinogen molecule during the formation of fibrin. The possibility remains that the free glutamic acid *N*-terminal residues always present in fibrinogen preparations are due to a tenaciously adsorbed peptide which is released only if the underlying molecule is converted to fibrin; but in view of the uniform results obtained with various preparations, this cannot be a likely explanation. The facts that (*a*) the peptide is not released from the fibrinogen preparation without the action of thrombin, (*b*) thrombin has been shown to be a specific protease, (*c*) the *N*-terminal residues of glutamic acid of the original fibrinogen are found as end groups of the released peptide, and (*d*) the liberation of N.P.N. in the fibrinogen-thrombin system proceeds parallel with clotting without the occurrence of fibrinolysis, are all strong arguments in favour of 'fibrino-peptide' being derived by fission of the fibrinogen molecule during its transformation into fibrin. Further evidence to establish the relationship between the release of fibrino-peptide and the clotting mechanism is being sought by studying the molecular structure of the peptide.

Since it appears that fibrin is not the sole reaction product of the enzymic action of thrombin on fibrinogen, the process can accordingly be represented as follows:

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} + \text{fibrino-peptide}.$$

The reactions occurring when pepsinogen is activated by pepsin (Herriott, 1941), or when ovalbumin is converted to plakalbumin by a bacterial enzyme (Linderstrøm-Lang & Ottesen, 1949), must be very similar to the fibrinogen-fibrin transformation. In all three cases a new protein species is created merely by splitting off a peptide or a few amino-acid fragments from the main body of the original substance.

$$\text{Protein A} \xrightarrow{\text{Enzyme}} \text{Protein B} + \text{low-molecular substances (peptides)}.$$

The resulting product (protein B) displays quite different behaviour from that of the primary protein, though differing so little from it in molecular constitution. The concept of such a relatively minor change of the fibrinogen molecule during clotting would explain the findings that there is no appreciable difference between the viscosity of fibrinogen and fibrin in urea (Lorand, 1948; Lorand, 1950*a*, *b*; Mihalyi, 1950*a*), and that both proteins have identical sedimentation constants in urea solution (Shulman, Ehrlich & Ferry, 1951). It should also be noticed that differences in the electrophoretic mobilities and isoelectric points of fibrinogen and fibrin (Mihalyi, 1950*b*) can most readily be interpreted in terms of a proteolytic change involving fission of a peptide from the fibrinogen.

The proposed mechanism of the enzymic step in the clotting of fibrinogen (Lorand, 1951*a*, *b*), as outlined above, is supported by the recent observations of Laskowski, Rakowitz & Scheraga (1952). These authors showed that the reaction

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin} + \text{fibrino-peptide}$$

is a reversible one, and they demonstrated that the equilibrium could be shifted to the left by adding fibrino-peptide to the system.

'Non-specific' clotting agents of the proteolytic type (papain, and certain snake venoms) are known to produce clots which, like fibrin, are soluble in urea (Janszky, 1949; Lorand, 1951*a*); but it is not known yet if these enzymes cause the same molecular alteration of fibrinogen as thrombin does, namely the liberation of glycine *N*-terminal groups with the splitting off of fibrino-peptide. The type of the reaction may be similar, but different enzymes may show individual variations in their actions at the molecular level. It is conceivable that in the course of the continuous degradation of fibrinogen by these agents a transitory stage is reached when the composition of the molecule happens to be similar to that of true fibrin, so that the particles then associate to a clot which, as the non-specific enzyme activity proceeds, is eventually digested. The action of thrombin, however, seems to be limited by its high specificity simply to producing fibrin.

SUMMARY

1. It is shown that the clotting activity of thrombin, known to be a specific protease, is accompanied by the liberation of non-protein nitrogen.

2. A peptide appears in the fibrinogen-thrombin system.

3. It is suggested that this hitherto unknown substance should be called 'fibrino-peptide', since it is believed to be derived by splitting off part of the fibrinogen molecule.

4. A method for isolating apparently pure fibrino-peptide is given.

5. The release of fibrino-peptide is correlated with previous results, and the clotting of fibrinogen is discussed in the light of the new finding.

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The Branched-Chain Fatty Acids of Mutton Fat

1. THE ISOLATION OF (+)-14-METHYLHEXADECANOIC ACID

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Recent investigations in this laboratory have revealed the presence of branched-chain fatty acids as minor constituents of butterfat (Hansen & Shorland, 1950, 1951, 1952; Hansen, Shorland & Cooke, 1951*a*) and of beef fat (Hansen, Shorland & Cooke, 1952). The branched-chain acids isolated from butterfat comprised two C_{17} and one C_{18} saturated mono-methyl acids, and a saturated multi-methyl acid. From beef fat was derived a C_{17} methyl-substituted acid corresponding with one of the C_{17} acids isolated from butterfat.

Prior to the above findings, branched-chain acids had been located in the lipids of a number of bacilli (Anderson, 1927, 1941; Anderson & Chargaff, 1929; Velick, 1944*a*, *b*; Hofmann & Lucas, 1950) and in wool grease (Weitkamp, 1945), while the short-chain acid *iso*valeric was found by Chevreul in 1817 (cf. Hilditch, 1947) in the head and jaw oil of the dolphin.

The purpose of this paper is to describe in detail the isolation of (+)-14-methylhexadecanoic acid from the external tissue fat of ewes. The occurrence of this acid in ewe fat was briefly reported last year (Hansen, Shorland & Cooke, 1951*b*). Although (+)-14-methylhexadecanoic acid has been shown by Weitkamp (1945) to be present in wool grease, where it occurs as a sterol ester, and although Velick & English (1945) synthesized this acid and confirmed its structure, it has not hitherto been reported as a constituent of natural fats.

Since this manuscript has been prepared Dr G. Weitzel and Dr K. Thomas have, in a private com-