FIBRINOGEN COAGULATION AND FIBRINOPEPTIDES IN LOWER VERTEBRATES

by

C. S. Hann, B. Sc.,

Institute of Medical and Veterinary Science

and

Department of Biochemistry, University of Adelaide.

A thesis submitted in part fulfilment of the requirements for the Degree of Master of Science.

February, 1967.

CONTENTS

--------oOo--------

SUMMARY

Chapter I of this thesis describes the preparation of highly coagulable and stable fibrinogens of one reptilian and four avian species, using methods involving precipitation with ethanol and glycine. N-terminal amino acid analysis before and after coagulation of the fibrinogens of two bird species suggested that mammalian thrombin cleaved two of three polypeptide chains with the release of two fibrinopeptides bearing no free α-amino groups. The mammalian-type three strand model of an avian fibrinogen was confirmed by electrophoretic and chromatographic separation of the individual polypeptide chains after oxidative sulphitolysis. After coagulation, one of these chains showed a much reduced anodic mobility consistent with the loss of a large net negative charge.

Chapter II describes the isolation and properties of fibrinopeptide material from the clot supernatant of all species after coagulation with mammalian thrombins. In the case of the lizard, two fibrinopeptides were recovered in approximately equal yield (i. e. mammalian like), but electrophoresis revealed a wide spectrum of avian fibrinopeptides, a finding inconsistent with that deduced from the original N-terminal analyses. The possibility that this arose from the heterologous coagulation system used (viz. mammalian thrombin on bird fibrinogen) was examined and excluded. Extensive

heterogeneity of the fibrinogen molecular species was also excluded.

Chapters III and IV are concerned with N-terminals, total amino acid compositions and partial amino acid sequences of certain lizard and bird fibrinopeptides. The lizard peptides appeared to be of the mammalian 'A' and 'B' classes. The main fibrinopeptide of the bird species was akin to a mammalian B-type, but no A-type fibrinopeptide appeared in substantial yield. The implications of this peculiar finding, and the origin of the range of 'minor' fibrinopeptides appearing is discussed.

I declare that, except as stated herein, this thesis contains no material which has been accepted for the award of any other degree or diploma in any University, and that, to the best of my knowledge, the thesis contains no copy or paraphrase of material previously published or written by another person except where due reference is made in the text of the thesis.

Signed: .

(C. S. HANN, B. Sc.)

ACKNOWLEDGEMENTS

I wish to express my thanks to my supervisors, Dr. E. Hackett of this Institute and Dr. G. Rogers of the Biochemistry Department, for their enthusiastic encouragement throughout the course of this investigation. I am indebted also to Dr. J. Welles of the Biochemistry Department for several amino acid analyses; to Mr. W. Nolan for assistance with the photography; to Mr. J. McCarthy for generously allowing me to collect blood of the bird species; and to Miss S. Whitington and Mrs. J. Guiney for typing the manuscript.

GENERAL INTRODUCTION

GENERAL INTRODUCTION

Essentially, the mammalian blood coagulation system may be divided into three phases:

(i) the generation of intrinsic plasma thromboplastin (prothrombin activator or prothrombin converter) triggered by damage to vascular epithelium and involving plasma or cellular and tissue components, and ionic calcium;

(ii) the production of thrombin by the thromboplastic conversion of its zymogen (prothrombin);

(iii) the enzymatic alteration, by thrombin, of soluble plasma fibrinogen to fibrin, which forms the insoluble meshwork of the clot.

The elaboration of staunching mechanisms adequate to cope with the risks associated with high-pressure circulatory systems must have been at a premium in the evolutionary development of warm blooded vertebrates. Blood clotting in mammals is exceedingly complex, probably because any mechanism of haematosis needs to have imposed upon it sufficient restraints to prevent untimely intravascular coagulation. A composite scheme for human blood coagulation is given in Figure 1.

It has so far been impossible to isolate and study the early stages in the clotting sequence at a biochemical level. Elucidation of the mechanism has depended very much on the recognition of

BLOOD COAGULATION MECHANISM

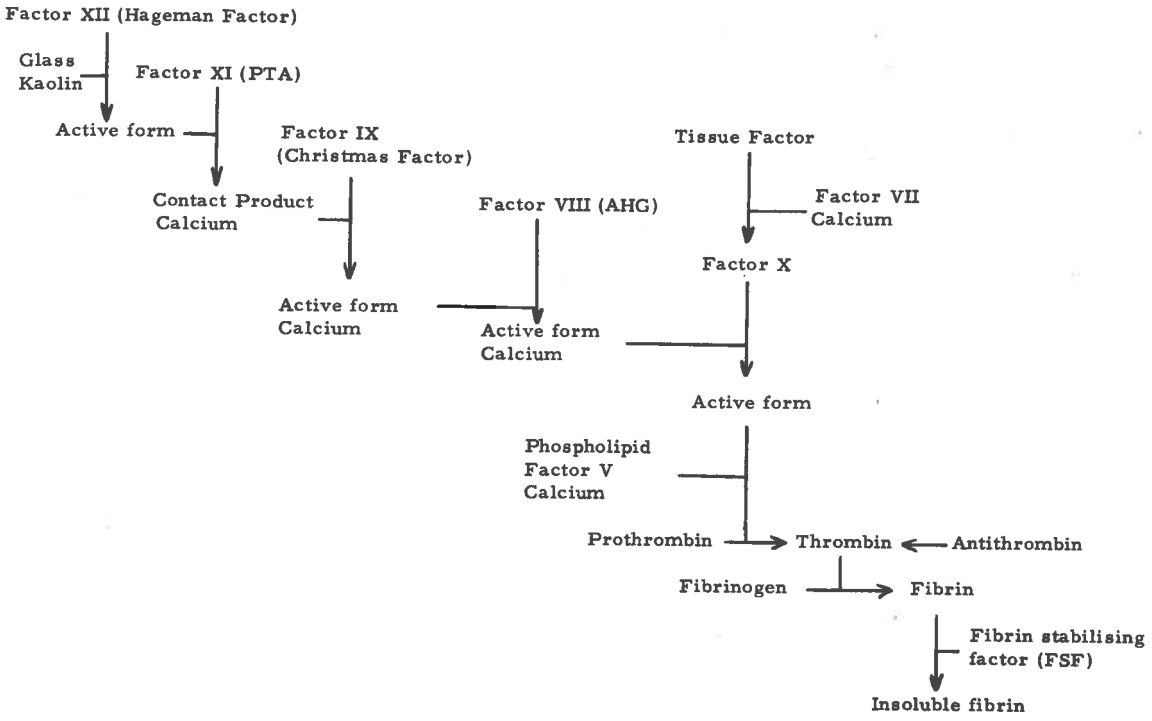


FIGURE 1

deficiency syndromes manifested in clinical or genetic abnormalities and on such empirical findings as the presence or absence of a given factor in serum, its lability on heating or storage or whether it can be removed by inorganic adsorbents such as aluminium hydroxide, calcium phosphate or bentonite. The discovery of Factors VIII (anti-haemophilic globulin), X (Stuart-Prower factor) and XII (Hageman, or "contact" factor) are classical examples, their presence and roles in normal plasma being quite overlooked until the recognition of specific disease states in haemorrhagic patients.

More recently, particular attention has been directed to the role of the platelet in coagulation and thrombus formation; platelet aggregation, triggered by contact with the collagen of the vascular wall and perhaps mediated by ADP now seems to be of fundamental importance in initiating thromboplastin generation and the ultimate formation of the coagulum.

The conversion of prothrombin to thrombin which subsequently clots fibrinogen is far better understood than the preliminary sequence of reactions through which thromboplastin is generated. These two steps have been demonstrated in all classes of vertebrates from fish, amphibia (Hackett and Le Page, 1961 a, b), reptiles (Fantl, 1961) and birds (Soulier, Wartelle and Ménaché, 1959) to mammals, although some varying degrees of species specificity with

respect to the rate of reaction exist (Quick, 1938; Osbahr, Gladner, Laki and Irreverre, 1964).

Bovine prothrombin is a carbohydrate-containing protein of molecular weight of 68,000 and isoelectric point of 4.2 (Lamy and Waugh, 1953). Inherent instability of the molecule is suggested by the autolytic breakdown which highly purified prothrombin undergoes in 25 per cent sodium citrate solution (Seegers, 1955) ultimately producing thrombin activity, and by changes in the C-terminal residues during chromatography on DEAE cellulose (Thomas and Seegers, 1962). The process is a complex one, involving dissociation into several electrophoretically distinct entities, and then reassociation with appearance of thrombin activity. Citrate-thrombin, of molecular weight near 31,000 has glutamic acid as an N-terminal in contrast to the alanine of the original prothrombin. Thrombin, whether generated by intrinsic thromboplastin or "prothrombin converter" or by addition of tissue extracts, has properties similar to citrate-thrombin (Seegers et al., 1959) and there is reason to believe that the two products are fundamentally similar. It is now apparent that during the activation of prothrombin, derivatives from the (auto) proteolysis have autocatalytic activity. Autoprothrombin-C is one such enzyme product (Marciniak, Cole and Seegers, 1962). After DEAE cellulose chromatography of

prothrombin, autoprothrombin-C activity disappears.

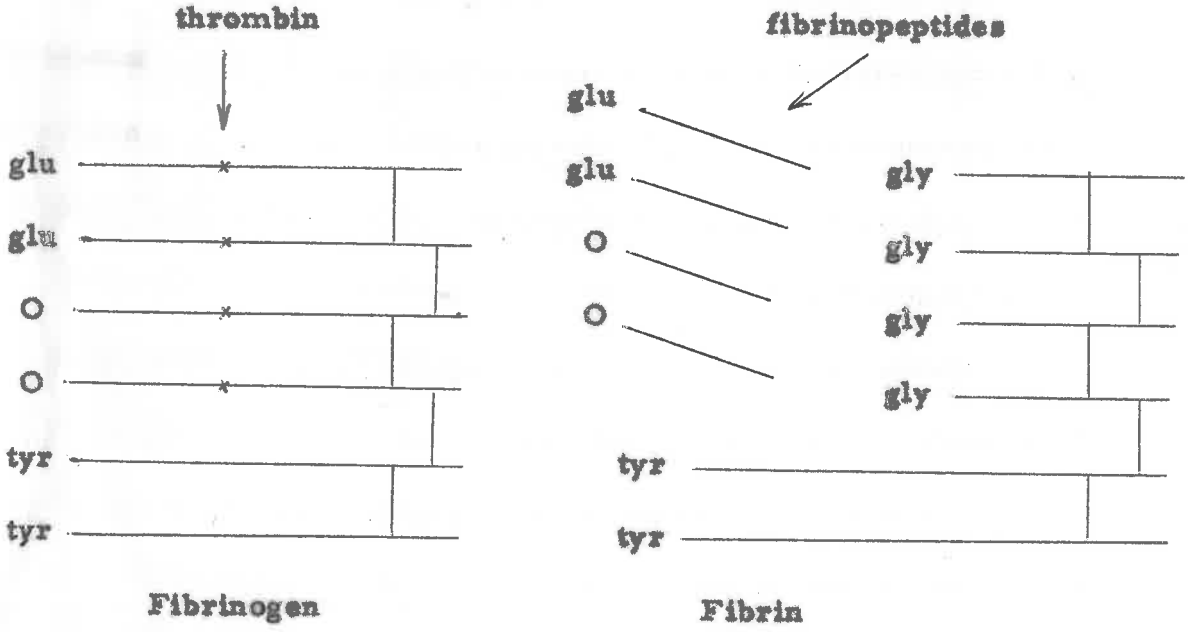
The very last step in blood clotting - the coagulation of fibrinogen by thrombin - is the one best understood biochemically. Although thrombin had long been recognised as an enzyme, its mode of action remains speculative. As early as 1909 it was classed as protease (Mellanby, 1909) but in succeeding decades a variety of other plausible mechanisms was proposed. Thrombin is able to clot fibrinogen in the absence of any co-factors. Its remarkably potent and specific activity was, for instance, variously ascribed to that of a polymerase (Mommaerts, 1946 a, b), a thiol oxidising agent (Lyons, 1945), and a denaturase. Difficulty in obtaining thrombin free from other plasma proteins greatly retarded investigations of its mode of action. Elaborate plasma fraction techniques (Cohn et al., 1946) made increasingly purified samples of fibrinogen available, and with some modifications of these methods (Morrison, Edsall and Miller, 1948) products coagulable to the order of 98 per cent were obtained.

It was soon evident that if thrombin were a proteolytic enzyme its action on fibrinogen must be very restricted. Comparison of the sedimentation patterns of fibrinogen and fibrin in the ultra-centrifuge when both were dispersed in 6 M urea showed that they were not significantly different (Laki, 1953). A similar conclusion

had been drawn from light-scattering studies (Steiner and Laki, 1951). Nevertheless, there was a small but consistent loss of some 3 per cent protein nitrogen during coagulation suggestive of a limited hydrolysis. Final proof that thrombin was a protease with a high degree of specificity was afforded by the discovery that glycine appeared constantly as a new N-terminal amino acid residue after the coagulation of bovine fibrinogen (Bailey, Bettelheim, Lorand and Middlebrook, 1951). Using 1-fluoro-2:4-dinitrobenzene (Sanger, 1945) glutamic acid was shown to occur in bovine fibrinogen (Lorand and Middlebrook, 1952) and this was replaced by glycine in fibrin (Bettelheim and Bailey, 1952; Lorand, 1952). Glutamic acid appeared in the clot supernatant as peptide material. Tyrosine was present as another N-terminal in both fibrinogen and fibrin.

Preliminary quantitative estimations of the N-terminal residues of ox fibrinogen (based on a molecular weight of about 350, 000) indicated four protein chains, two bearing glutamyl and two having tyrosyl end-groups, while in the corresponding fibrin six N-terminal residues were found: two tyrosines and four glycines. This apparent paradox was resolved when two peptides (later designated fibrinopeptides A and B and originally portions of the parent fibrinogen molecule) were recovered from the clot

supernatant. The A peptide bore N-terminal glutamic acid, while the B peptide had no demonstrable end group (Bettelheim and Bailey, 1952). From further quantitative estimations of the N-terminals of ox fibrinogen (Blombäck and Yamashina, 1958; Blombäck, 1958 a) it was evident that two molecules of each of the fibrinopeptides A and B were cleaved from fibrinogen during coagulation with thrombin.



It is apparent (see diagram) that the native fibrinogen molecule of M. W. in the vicinity of 350, 000 is dimer. The release of the fibrinopeptides, both of which bear net negative charge at neutral pH, in some way prepares the remainder of the molecule for polymerization (Mihalyi, 1954). Electron microscopy has revealed that fibrinogen molecules which appear as three globules joined by

rod-like connections undergo both end-to-end and side-to-side aggregation after being acted upon by thrombin (Hall and Slayer, 1959). This strongly suggests that after release of the fibrinopeptides electrostatic repulsion is reduced and polymerization can take place. Fibrin formation is pH dependent (Scheraga, 1958).

Since the C-terminal amino acid of both ox peptides is arginine (Folk, Gladner and Levin, 1959; Blombäck, Sjöquist and Wallen, 1959) it is evident that thrombin cleaves an arginyl-glycine linkage. Two main fibrinopeptides have been obtained by thrombin coagulation of the purified fibrinogens of a number of mammals including human (Blombäck, Blombäck, Edman and Hessell, 1962), rabbit, sheep, goat, pig and reindeer (Blombäck and Doolittle, 1963 a, b) and the entire amino acid sequences have been deduced. Their structures are given in Appendix II.

Thrombin behaves, then, as an endopeptidase with trypsin-like specificity, though its action is much more limited. Like trypsin, thrombin shows esterase activity toward certain synthetic substrates, p-tosyl arginine methyl ester and lysine ethyl ester being readily hydrolysed. Of the several arginyl sequences in fibrinogen susceptible to trypsin (Bailey and Bettelheim, 1955) only those four near the amino terminus of the molecule are available to thrombin. Regularities of amino acid sequences

adjacent to the particular arginyl-glycine bond hydrolysed are apparent when the fibrinopeptides A and B from different species are examined. It may be that the limited specificity shown by thrombin is determined at least in part by the primary structure of fibrinogen. Of particular interest is the occurrence of a phenylalanyl-leucyl sequence six residues removed from the bond attacked by thrombin (Appendix II) in the fibrinopeptides A of a number of species. It is known that in ox and rabbit the A-peptide is released more rapidly than the B-peptide (Blombäck and Vestermark, 1958), implying either that marked evolutionary conservation of primary structure has been essential for removal of the A-peptide or that the B-peptide chain has remained susceptible to thrombin in a variety of forms. Comparison of the primary structures of the fibrinopeptides of a large number of species should indicate which amino acid residues are essential for function for these should be strongly conserved by selection. Other residues, acting only as "spacers" and serving to keep vital amino acids in specific position might be more readily replaceable.

No information has become available yet on the amino acid sequences on the fibrin side of the bonds attacked by thrombin, but it is possible that these, too, may partly determine thrombin specificity.

A biochemical classification of organisms paralleling traditional taxonomy has recently become an intriguing possibility. Greater degrees of correspondence of amino acid sequences of proteins are found in species which are closely related. Investigations of this kind have been carried out on the haemoglobins of thirteen primates (Hill and Buettner-Janusch, 1964; Buettner-Janusch and Hill, 1965) the cytochromes-C (Margoliash, Needleman and Stewart, 1963; Smith and Margoliash, 1964) and the fibrinopeptides of mammals of the order Artiodactyla (Doolittle and Blombäck, 1964).

The general question of amino acid replacements, especially those of so-called "neutral" substitutions (such as one acidic residue for another) is one which demands the accumulation of more data. Chemically neutral replacements are presumably not selectively neutral or they would not be maintained in the stock for more than a few generations. Further observations may throw light on this, and also on whether these substitutions are in accordance with a single base change of the proposed triplet code (Eck, 1963).

From the foregoing it can be seen that analytical biochemistry has recently made useful inroads into the field of blood coagulation. Comparative blood coagulation studies in vertebrates in the past have often been restricted to searches for "factors" with roles analagous

to those described in the human. Biochemical investigation may show that some of these "factors" described in the human system do not exist as distinct entities. For instance, from the work of Seeger's group it would appear that "deficiency" of Factor X (Stuart-Prower factor) is in reality due to a functionally abnormal molecule. Highly purified normal prothrombin, but not prothrombin chromatographed on DEAE-cellulose on which it undergoes some structural alterations, can correct the clotting abnormality of Factor X "deficient" plasma (Marciniak, Cole and Seegers, 1962). A similar situation may apply to antihaemophilic globulin (Factor VIII). It has not yet been possible to chemically isolate Factor VIII activity from the fibrinogen fraction. Antihaemophilic-globulin activity might well reside within the normal fibrinogen molecule itself, with the clotting defect being due to an altered molecular species. There is not yet substantial evidence for this, but the complete mapping of tryptic peptides from normal fibrinogen and fibrinogen of Factor VIII 'deficiency' patients could resolve the issue.

In this thesis the action of thrombin on the fibrinogens of a number of lower vertebrates has been investigated. The species reported upon are the Australian stumpy-tail lizard (Trachydosaurus rugosus rugosus), three birds from the order Anseriformes (the Muscovy duck Cairina moschata, the Peking duck Anas platyrhincos,

the domestic Goose Anser anser) and the domestic Turkey (Meleagris gallopavo). The characteristics of the fibrinogen molecule and the nature and structure of some of the fibrinopeptides released were examined.

CHAPTER I

THE PREPARATION AND PROPERTIES OF THE FIBRINOGENS OF SOME LOWER VERTEBRATES.

CHAPTER I

INTRODUCTION

Fibrinogen is the species of plasma protein coagulated by thrombin; the molecular weights of human and bovine material are approximately 340, 000 (Caspary and Kekwick, 1954), but it is now apparent that this figure pertains to a dimeric form.

Numerous methods have been used in the purification of mammalian fibrinogens. Simple dilution of plasma causes flocculation of a protein fraction rich in fibrinogen (Mellanby, 1909), but protein precipitants are generally employed. Sodium chloride at half-saturation was first used by Hammarsten (1875), a technique subtly modified by a number of later authors (Fiorkin, 1930; Eagle, 1935; Chargaff and Bendich, 1943). Stability of the final product is of prime importance, particularly its resistance to denaturation and freedom from coagulating factors (especially prothrombin) and lytic enzymes which might lead to degradation. Repeated sodium chloride precipitation has given good results for horse fibrinogen, but is unsuitable for the preparation of ox or chicken fibrinogen (Astrup and Darling, 1942). Conversely, the dilution method of Mellanby (1909) is satisfactory for chicken, but ox fibrinogen is reportedly unstable.

Ammonium sulphate precipitation at 25% saturation after removal of prothrombin with magnesium hydroxide (Smith, Warner and Brinkhouse, 1934) was reviewed by Astrup and Darling (1942) and such preparations were considered, in general, to be the best with respect to both purity and stability. Co-precipitating globulins contaminating preparations of ox fibrinogen were removed by precipitating them from phosphate buffer at pH 4. 1 (Laki, 1951) and a product coagulable to 95% was obtained.

Ox fibrinogen was easily prepared by the Seegers group by slowly thawing frozen plasma, whereupon, at or near $0^{\circ}C.$, the fibrinogen remained insoluble and was obtained in high purity after washing with chilled isotonic saline (Ware, Guest and Seegers, 1947).

Large scale plasma fractionation using ethanol was devised by Cohn (e. g. Method 6, Cohn et al., 1946) and approximately 80% of the total fibrinogen content of human plasma was precipitated in "fraction I" at an ethanol concentration of 8%. Fibrinogen solubility was found to be sharply dependent on ethanol concentration and pH; contaminants of Cohn fraction I were removable by subtle adjustments of these variables (Morrison, Edsall and Miller, 1948). Providing they were prepared in the cold, alcohol-precipitated fibrinogens were extremely stable.

It was later observed that glycine in high concentration substantially decreased the solubility of fibrinogen but increased the

solubility of contaminating plasma proteins (Blembäck and Blombäck, 1956). Human and bovine fibrinogens coagulable to 100% were obtained by a variation of the ethanol precipitation procedure (Morrison, Edsall and Miller, 1948) which included extraction with buffers containing 1 M glycine. The complete removal of prothrombin and lytic factors gave an extremely stable product. Direct precipitation of fibrinogen from plasma with glycine has also been described (Kazal, Amsel, Miller and Tocantins, 1963), yielding an extremely pure, undenatured sample.

Throughout this entire investigation it was necessary to obtain stable fibrinogens of high purity, and in as natural a state as possible. Of all the procedures considered, ethanolic precipitation in the presence of glycine appeared to be the method of choice, and seemed sufficiently flexible for adaptation to different species.

The N-terminal amino acids of ox fibrinogen and the changes occurring during its conversion to fibrin have been cited in the General Introduction. In all the mammalian species which have been reported upon two of the three fibrinogen peptide chains (considering the monomeric and not the dimeric form) are attacked by thrombin, which exposes two new glycine residues. Tyrosine, the N-terminal residue of the third fibrinogen chain, is also found in fibrin. The

glycine/tyrosine ratio in fibrin is thus 2:1 (Blombäck, 1958 b).

The two most widely used reagents for identification and quantitative estimation of N-terminal amino acid residues of proteins are 1-fluoro-2:4 dinitrobenzene (FDNB) and phenylisothiocyanate (PITC), both having been applied to mammalian fibrinogens (Bettelheim and Bailey, 1952; Blombäck and Yamashina, 1956). At alkaline pH, both compounds couple with a free α-amino group at the end of an open peptide chain. When FDNB is used as the end-group reagent, the dinitrophenyl amino acid is obtained after complete hydrolysis of the protein chain with constant boiling-point hydrochloric acid.

Using PITC as the coupling reagent, the resulting phenylthiocarbamyl protein (PTC-protein) can be subjected to milder acid conditions, the terminal PTC-amino acid (until this stage still peptide bound) undergoing cyclization to a phenylthiohydantoin (PTH-amino acid) with concomitant cleavage from the remainder of the peptide chain. The sequence of reactions is shown in Figure 2.

PITC has been used extensively in the determination of N-terminal residues. The PTH-acids are easily separated by chromatography (Sjöquist, 1953; Edman and Sjöquist, 1956) and because of their sharp absorption peak at 268 mμ, they may be readily estimated spectrophotometrically. The main disadvantage

Figure 2. Reaction of the N-terminal amino acid residue of a peptide chain with phenylisothiocyanate and subsequent cleavage and conversion of the phenylthiocarbamyl derivative (PTC-peptide) to a phenylthiohydantoin (PTH-amino acid).

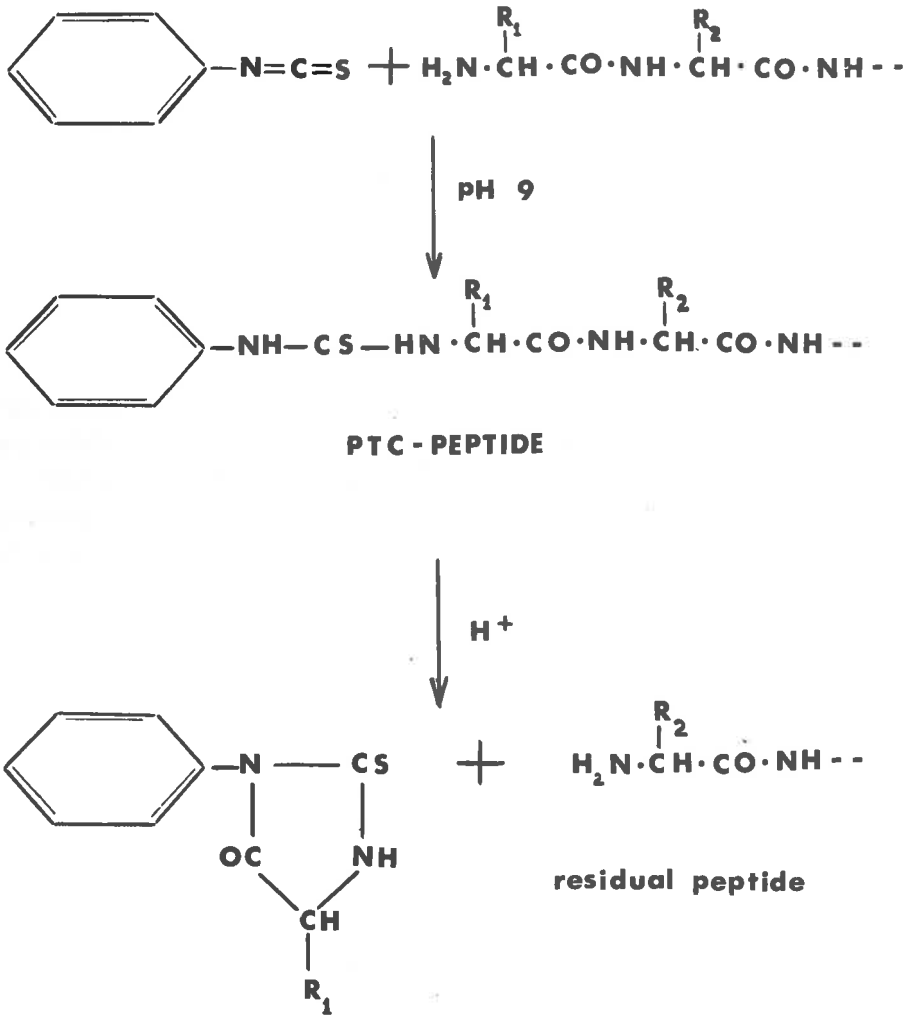

—N=C=S + H₂N·CH·CO·NH·CH·CO·NH--
R₁
R₂
pH 9
—NH—CS—HN·CH·CO·NH·CH·CO·NH--
PTC-PEPTIDE
H⁺
—N—CS
OC
NH
CH
R₁
H₂N·CH·CO·NH--
residual peptide
PTH-amino acid

FIGURE 2

inherent in this method is that some destruction of the PTH's may be expected during cyclisation and cleavage (Erickson and Sjöquist, 1960; Sjöquist, 1960) although such losses are of more significance with some derivatives than with others.

Protein chains cross-linked to one another by disulphide bridges can be separated by a variety of techniques which include performic acid oxidation, reduction with mercaptans or sodium borohydride, or by reaction with sulphite. Performic acid oxidation alters not only the disulphide bonds but also methionine, tryptophan and tyrosine residues, while with mercaptan reduction (e.g. mercaptoethanol) it is essential to ensure that reoxidation does not occur.

Cleavage with sulphite (Pechère, Dixon, Maybury and Neurath, 1958) is generally considered to be the gentlest and the most specific procedure. The reaction with cupric ion reoxidation proceeds according to the scheme (Kolthoff and Stricks, 1951):

$$3RSSR + 3SO_3^{--} \rightleftharpoons 3RSSO_3^- + 3RS^-$$

$$3RS^- + 2Cu^{++} + SO_3^{--} \longrightarrow RSSO_3 + 2RSCu$$

$$2RSCu + 4Cu^{++} + 2SO_3^{--} \longrightarrow RSSO_3^- + 6Cu^+$$

Sulphitolysis has been used in the separation of the chains of mammalian fibrinogens and fibrin (Clegg and Bailey, 1962; Haschmeyer and Nadeau, 1963; Henschen, 1963). Relative

non-reactivity of the disulphide bonds of human fibrinogen except in the presence of 8M urea indicates that many of the bridges may be of the intra- rather than inter- chain type (cf. Cecil and Wake, 1962).

After sulphitolysis, mammalian fibrinogen chains have been separated by electrophoresis and ion-exchange chromatography in the presence of 8 M urea. Chain separation of mammalian fibrinogen and fibrin has been achieved on CM-cellulose in the pH range of 5-6 using ionic strength gradients (Henschen, 1963; 1964 b).

The author undertook the purification of the fibrinogen of one reptilian and four avian species. Fibrinogen and fibrin N-terminals were investigated using PITC in the case of Cairina, Anser and Anas and experiments attempting to isolate the individual peptide chains of Cairina fibrinogen and fibrin after sulphitolysis will be described.

INVESTIGATION

MATERIALS AND METHODS

Collection of Blood.

Stumpy-tail lizards (Trachydosaurus rugosus rugosus) were taken locally by us or by paid collectors. They were deeply anaesthetized by an intrathecal injection of pentobarbitone (60 mgms)

given between the palatine bones in the roof of the opened mouth. The skin and pectoral girdle were cut cleanly with sharp instruments and blood was withdrawn from one of the large aortic trunks using a syringe containing 1.5 ml of 4% trisodium citrate. This was sufficient to anticoagulate 15 ml of blood; more was added after if required. Trachydosaurus blood shows little tendency to clot spontaneously in vitro even in the absence of citrate, due to a potent circulating antithrombin (Hackett and Hann, 1967). Whole blood was pooled during collection.

The blood was chilled and centrifuged twice at 5,000 G to ensure it was cell free.

Blood from each of the four bird species (Cairina, Anas and Meleagris) was obtained from poultry slaughter houses. The head was cut off cleanly and the arterial blood allowed to spurt into a graduated polythene beaker containing sufficient 4% trisodium citrate to make a final 9/1 mixture. Blood was pooled during collection, and centrifuged as described for Trachydosaurus.

Fibrinogen Purification: Fraction I-O.

53.3% ethanol (A.R. grade).

Extracting buffer: 0.06M trisodium citrate, 1.05M glycine was adjusted to the pH value selected for each species. Absolute ethanol was then added to the buffer to the concentration required.

The compositions are given in Table I.

The methods used were modifications of that described for the isolation of Fraction I-O (Blombäck and Blombäck, 1956). Pilot experiments were performed to determine the optimum conditions for fibrinogen isolation from each species, a compromise being reached between total yield and purity. The following general procedure was adopted.

The cell-free plasma (usually between one and two litres) was chilled to -3°C. and maintained at this temperature throughout. The pH of the plasma was adjusted to 7.0 with glacial acetic acid and 53.5% ethanol (pre-chilled) added to the selected final concentration. The volumes added for each species are listed in Table II. After 30 minutes' equilibration the precipitate was collected by centrifugation (5,000 G) and made into a smooth paste with a small volume of extracting buffer (also pre-chilled to -3°C.). Additional extracting buffer was added so that the total amount was approximately one-third that of the original plasma volume. After 30 minutes of gentle stirring the precipitate (containing the fibrinogen) was collected by centrifugation, and the supernatant discarded. Extraction was repeated once. The final precipitate, quite white in appearance, was made into a paste with distilled water and freeze-dried.

TABLE I

Composition of extracting buffers used in fibrinogen purification.

Species	Volume absolute ethanol added per litre glycine-citrate buffer	Final ethanol concentration	pH
Trachydosaurus	69.5 ml	6.5%	6.3
Cairina	52.5 ml	5.0%	6.4
Anas	69.5 ml	6.5%	6.4
Anser	64.0 ml	6.0%	6.4
Meleagris	52.5 ml	5.0%	6.2

TABLE II

Ethanol concentrations used for precipitation of plasma fraction I.

Species	Volume 53. 3% ethanol added. (ml/litre plasma)	Final ethanol concentration
Trachydosaurus	177	8%
Cairina	151	7%
Anas	177	8%
Anser	204	9%
Meleagris	133	6. 5%

Further Purification of Fraction I-O.

Fibrinogen of high purity was prepared by dissolving fraction I-O in 0.06M sodium citrate brought to pH 7.0 with hydrochloric acid to a protein concentration of 1% and precipitating directly with glycine added to 2.0M concentration (Kazal et al., 1963; Hann, 1966).

Attempted Purification of Gallus Fibrinogen.

Extensive denaturation of Gallus fibrinogen occurred when precipitated either with ethanol or glycine. No alternative more satisfactory method was devised.

Purity of Fibrinogens.

The content of coagulable protein in a fibrinogen preparation was determined by the synerēsis method (Morrison, 1947; Ferry and Morrison, 1947).

Phosphate buffer: pH 6.35 (Blombäck and Blombäck, 1956).

Alkaline urea: 40% urea in 0.2N NaOH.

Thrombin: Parke Davis Thrombin Topical (bovine origin) was purified by chromatography on Amberlite CG-50 (Rasmussen, 1955) and made up as a solution containing 100 NIH units/ml.

A weighed quantity of the freeze-dried fibrinogen preparation (usually 50 mg, of which about 15 mg was protein, the remainder

being salts and glycine) was dissolved in 6 ml of 0.3M saline. 1 ml aliquots were measured into each of two small beakers, diluted with 2 ml of phosphate buffer, and coagulated at room temperature with 10 units of thrombin. After 2 hours the clots were carefully transferred to silk squares placed on top of several thicknesses of filter paper and repeatedly washed with 0.15M saline. After expression of the clot liquor and when all non-coagulable protein had been washed away, the fibrin was transferred to test tubes and dissolved in 10 ml of alkaline urea and 1 ml of saline. The extinction, representing the content of coagulable protein was read at 280 mμ in 1 cm silica cuvettes against a blank of 10 ml alkaline urea and 1 ml of saline.

Total protein was determined by adding 1 ml of the original fibrinogen solution to 10 ml of alkaline urea, and reading spectrophotometrically against the same blank.

End-group analysis.

PTH amino acids: Authentic derivatives of eighteen naturally occurring amino acids were synthesised in the laboratory by methods described (Edman, 1950; Sjöquist, 1957). PTH serine and PTH-threonine were prepared according to Ingram (1953) and the PTH's of glutamine and cysteic acid were synthesised following the method of Edman and Lauber (1956). No sample of PTH-arginine

or PTH-histidine could be made.

Chromatography: Solvents D, E, F (Edman and Sjöquist, 1956) and Solvent G (Blombäck and Yamashina, 1956) were used as described.

PTH's were located on the dried chromatograms by spraying with iodine/azide reagent, when they appeared as bleached spots on a dark background (Sjöquist, 1953).

PITC: Eastman-Kodak.

Pyridine: A.R. grade.

Benzene: A.R. grade, shaken with concentrated sulphuric acid to remove thiophene (Vogel, 1961).

Ether: A.R. grade, treated with aqueous sodium sulphite to remove peroxides (Vogel, 1961).

Ethyl acetate: redistilled.

Urea: 40% urea, made alkaline by the addition of a few drops of normal NaOH.

Fibrinogen: Glycine-precipitated fraction I-O.

Phosphate buffer and thrombin: As described under "Purity of Fibrinogens".

The fibrinogen, dissolved in 0.3M saline to an arbitrary protein concentration between 0.3% and 1.0% was dialysed at 2°C. for 24 hours against several changes of 5 litres of 0.25M saline.

Any material which flocculated out of solution and remained undissolved on warming to room temperature was removed by centrifugation.

Fibrinogen N-terminals were determined on a 10 ml sample of the dialysed fibrinogen; fibrin N-terminals were estimated after coagulation of an equivalent volume of the same fibrinogen solution (i. e. 10 ml) with thrombin (10 ml of the fibrinogen was diluted with 50 ml of phosphate buffer and bovine thrombin added to a final concentration of 2. 5 units/ml. After 2 hours at room temperature, the clot was synerised, washed and dissolved in 10 ml of alkaline urea.

The fibrinogen solution and urea-solubilised fibrin were coupled with PITC following the method of Blombäck and Yamashina (1958). 10 ml of pyridine was added with vigorous stirring and the pH of the mixture adjusted to 9 with 1N NaOH. 1 ml of PITC was introduced and the tube stoppered and incubated at room temperature for two hours during which time occasional additions of NaOH were made to keep the pH in the vicinity of 9. The coupling mixture was stirred vigorously at intervals during the incubation.

Pyridine and excess PITC were removed by five extractions with benzene. The PTC-protein in the aqueous phase was completely precipitated with ten volumes of acetone and then washed three times

with 80% acetone. The vacuum-dried PTC protein was refluxed with 8 ml of 1N HCl for 1 hour, after which PTH-amino acids were extracted from the acidic phase with several lots of ethyl acetate. After evaporation of the ethyl acetate with a stream of nitrogen the PTH's were dissolved in 0.25 ml of ethyl acetate/acetone (8:2) for chromatography in solvents D, E, F and G.

Unmeasured samples for qualitative PTH determinations were withdrawn from the tubes which were kept immersed in iced water to reduce evaporation. Care was taken to ensure that the tubes were tightly stoppered at all other times.

Quantitative Estimations.

Semiquantitative estimations of the PTH amino acids from fibrinogen and fibrin samples were performed by chromatographing 20 µl of the PTH solution, using solvents E and F. The spots, the positions of which were determined by spraying another chromatogram run in parallel, were cut out and the PTH amino acids eluted with 3.5 ml of ethanol. The extinctions of the solutions were read at 269 mµ in 1 cm quartz cuvettes against suitable blanks prepared by cutting a disc of equal size from the chromatogram alongside the PTH-spot.

Sulphitolysis.

The method adopted for sulphitolysis of Cairina fibrinogen and

fibrin was essentially that of Pechère, Dixon, Maybury and Neurath (1958), as applied to mammalian fibrinogen (Henschen, 1963). Cairina fibrinogen coagulable to 98% was exhaustively dialysed against 0.3 M saline prior to reaction with sulphite, or for preparation of fibrin. Sodium sulphite, copper sulphate and urea of reagent grade were used. After sulphite cleavage, the product was exhaustively dialysed against buffers described by (Pechère et al., 1958).

Electrophoretic Analysis of Peptide Chains.

The dialysed sulphitolysed material was concentrated 5-10 fold using a "Diaflo" membrane filter (UM-2). The buffer used for routine analysis was 0.1M glycine, 0.07M EDTA, containing 6M urea, brought to pH 10.2 with NaOH. Samples were applied to strips of Whatman No. 3 MM paper, 36 cm x 4 cm, which were then impregnated with the buffer, quickly blotted on filter paper, and placed in a tent-type electrophoresis tank.

Electrophoresis was carried out for 21 hours with a potential of 140 volts, and current 8 m-amps. (The figures are valid for four such paper strips.) After drying, the papers were stained with Amido black as described by Henschen (1964).

Chromatographic Separation of Fibrinogen Chains.

Preliminary experiments indicated that sulphitolysed Cairina

chains could not be separated on CM-cellulose between pH's 4.6-5.6 since some degree of aggregation of the sulphitolysed protein was observed. The chains generally emerged as a single peak from CM-cellulose columns under conditions described in the literature (Henschen, 1964 b). A modified method was adopted.

CM-cellulose: Whatman CM-11 was washed successively with 0.5 N HCl, water, 0.2 N NaOH water, and finally with several litres of equilibrating buffer of the pH and ionic strength selected.

Buffer solutions: For equilibration of the resin, 0.02M sodium acetate brought to pH 4.2 with acetic acid was used.

Chromatography buffers for elution of column: Chromatography was carried out with sodium acetate buffers containing 8M urea. A concomitant linear pH and ionic strength gradient was employed, viz.: 0.02M sodium acetate pH 4.2 - 0.1M sodium acetate pH 4.8. 250 ml of each was used in a two-chambered gradient mixer.

A typical experiment is described. CM-cellulose, equilibrated with sodium acetate, 0.02M pH 4.2 (urea-free) was packed under nitrogen pressure into a column 2 x 10 cm. Before application of the sample, 100 ml of 0.02M sodium acetate at pH 4.2 containing 8M urea was run through the column. 50 mg of freeze-dried sulphitolysed fibrinogen (or fibrin) in 2.5 ml of the same urea-containing buffer was dialysed against a further 50-60 ml of buffer

for 2 to 3 hours.

The sample was then applied to the column and gradient elution begun using the buffer described. The flow rate was adjusted to 20 ml/hour, and 5 ml fractions collected.

Protein peaks were located by measuring the optical densities of fractions at 280 mμ.

N-terminal Amino Acid Analysis of Cairina Fibrinogen Chains.

Fractions embracing each of the separate peaks of sulphitolysed Cairina fibrinogen after chromatographing were pooled, dialysed and freeze-dried. The material of each peak was dissolved in 6 ml of 8M urea and coupled with PITC in the manner already described for fibrin N-terminal investigations. PTH's were identified chromatographically.

RESULTS

Purity of Fibrinogen Samples.

The ratio of the O. D. readings of coagulable protein/total protein expressed as a percentage was taken as a measure of purity (Blomback and Blomback, 1956). Coagulabilities of fraction I-O ranged from 82% (Meleagris) to 92% (Cairina) (Table III). Reprecipitation of fibrinogen fraction I-O with glycine greatly increased the coagulability (Table III).

After freezing and thawing several times fibrinogen solutions

TABLE III

Estimation of the purity of Avian and Reptilian fibrinogen preparations.

Species	Coagulability of fraction I-O	Coagulability of glycine - ppt'd fibrinogen
Trachydosaurus	88%	97%
Cairina	92%	98%
Anas	90%	95%
Anser	90%	96%
Meleagris	82%	88%

Figures represent the ratio of coagulable protein to total protein, expressed as a per cent.

lost only one or two per cent of their coagulability, neither was there any tendency for fibrin clots to show an apparent decrease in coagulable protein when allowed to stand for up to 8 hours. Spontaneous fibrinolysis thus did not occur.

The total yield of fraction I-O protein averaged 1.8 g/litre of plasma.

Fibrinogen and Fibrin N-terminals.

Chromatography in several solvents revealed that tyrosine was the only N-terminal amino acid detectable in Cairina fibrinogen (Figure 3) while, in the corresponding fibrin, tyrosine and glycine were present (Figure 4). Results for Anser were the same.

It is possible that the conditions of cleavage and cyclization used (viz.: 1N HCl for 1 hour) could have caused excessive destruction of PTH-glutamine were it present. However, no glutamine was detectable by chromatography when 0.01N HCl at 37°C. for 24 hours was applied. The yield of PTH-tyrosine under these conditions was very low.

PTH-arginine and PTH-histidine are reportedly not extracted from an acidic solution with ethyl acetate. However, when the acidic phase was neutralized and re-extracted, no PTH-spot which would correspond with PTH-histidine (Edman and Sjöquist, 1956) was subsequently seen on chromatograms, nor did the aqueous phase

Figure 3. Chromatographic identification, in two solvent systems, of PTH-tyrosine derived from the N-terminus of Cairina fibrinogen.
Solvent D: O-Xylene; Solvent F: Upper phase of *n*-heptane/ethylene dichloride/formic acid/water 100: 200: 150: 50 by volume.

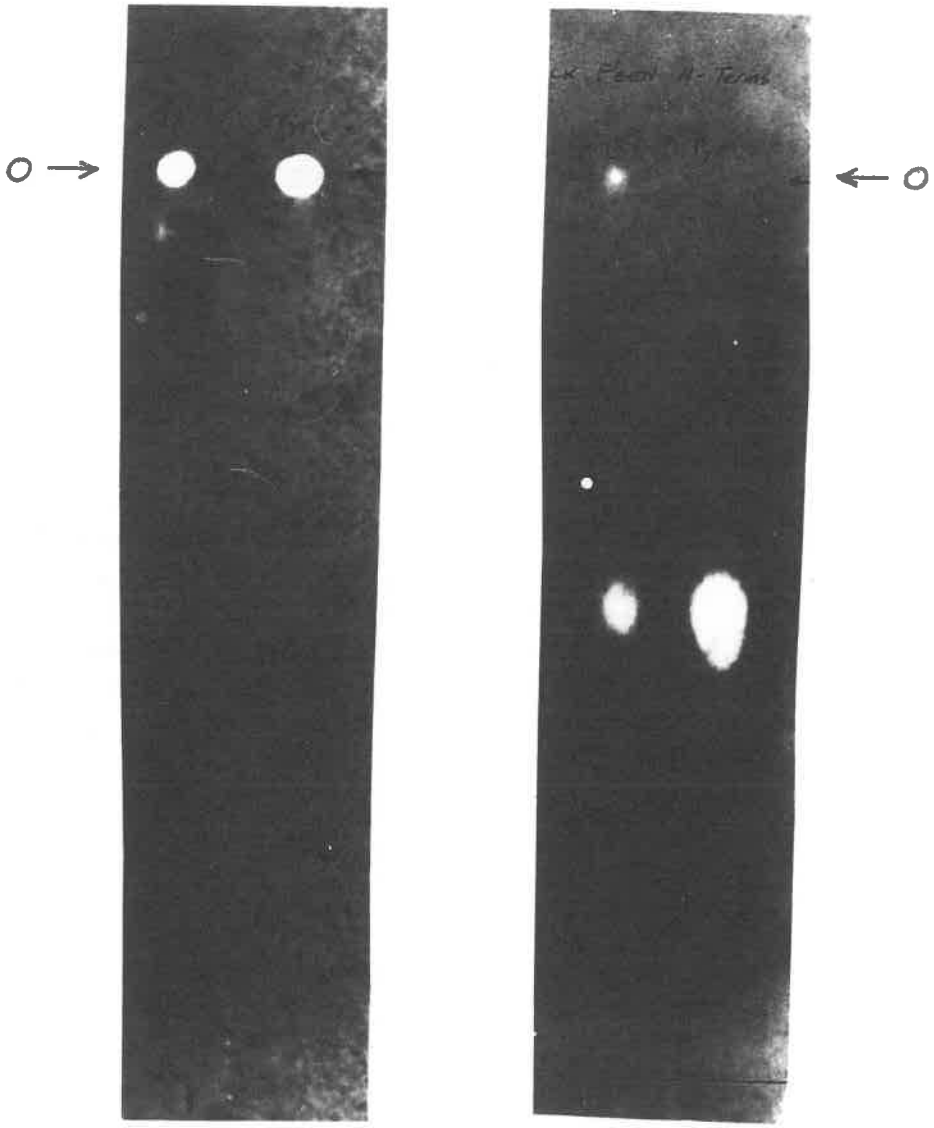


FIGURE 3

Figure 4: Chromatograms identifying PTH-glycine and PTH-tyrosine derived from the N-terminus of Cairina fibrin. For Solvent F, refer to Figure 3. Solvent G: Upper phase of Formic acid/water/ethylene dichloride/*n*-heptane 150: 50: 100: 100 by volume.

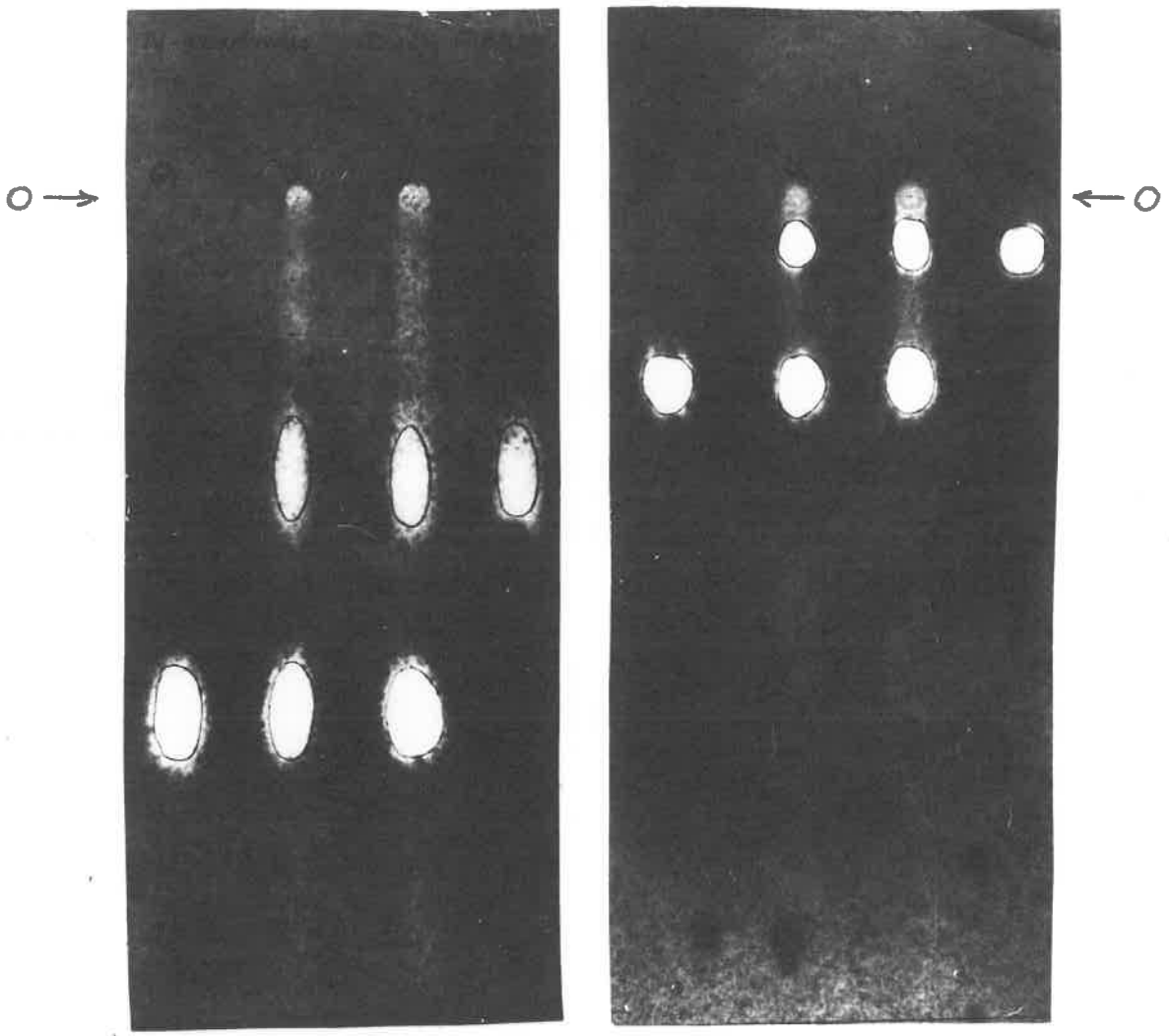


FIGURE 4

itself give a positive phenylthiohydantoin reaction.

Coupling of Cairina and Anser fibrinogens with PITC in the presence of 8M urea did not reveal any N-terminal amino acids which might have otherwise been masked.

Chromatography of the N-terminals for Anas fibrinogen and fibrin revealed tyrosine together with a number of weaker spots. Glycine, phenylalanine, serine and asparagine were tentatively identified. This could be attributed either to impurity or to heterogeneity of the fibrinogen chains. No determination on Meleagris was performed because of the comparative impurity of the fibrinogen preparations.

Quantitative PTH Determinations.

The results of four Cairina and two Anser samples are given in Table IV.

Electrophoretic Patterns of Sulphitolysed Cairina Fibrinogen and Fibrin.

The results of an electrophoretic analysis are shown in Figure 5. Three bands of Cairina fibrinogen were clearly resolved at pH 10.0, while in the corresponding fibrin only two bands were seen. A scan of the electrophoretogram indicated that in sulphitolysed Cairina fibrin, two of the protein bands were superimposed.

TABLE IV

N-terminal amino acids of fibrinogen and fibrin.

	Fibrinogen OD Tyr	Fibrin OD Tyr	Fibrin OD Gly	Gly:Tyr
Cairina				
Sample 1	0.080	0.077	0.135	1.7:1
Sample 2	0.275	0.285	0.427	1.5:1
Sample 3	0.120	0.105	0.140	1.3:1
Sample 4	-	0.259	0.410	1.6:1
Anser				
Sample 1	0.099	0.094	0.144	1.55:1
Sample 2	0.120	0.110	0.185	1.7:1

Quantitative estimations of PTH-amino acids derived from samples of Cairina and Anser fibrinogen (left-hand column) and fibrin (centre). The figures given are the optical densities at 269 mμ after elution of the pertinent PTH spots from paper chromatograms. The right-hand column shows the ratio of PTH-glycine : PTH-tyrosine in the fibrin of each sample. See text for further details.

Figure 5: Paper electrophoretic patterns of sulphitolysed Cairina fibrinogen and fibrin. 0.1M glycine-NaOH buffer, pH 10.2 containing 0.07M EDTA and 6M urea. 4 volts/cm for 21 hours.

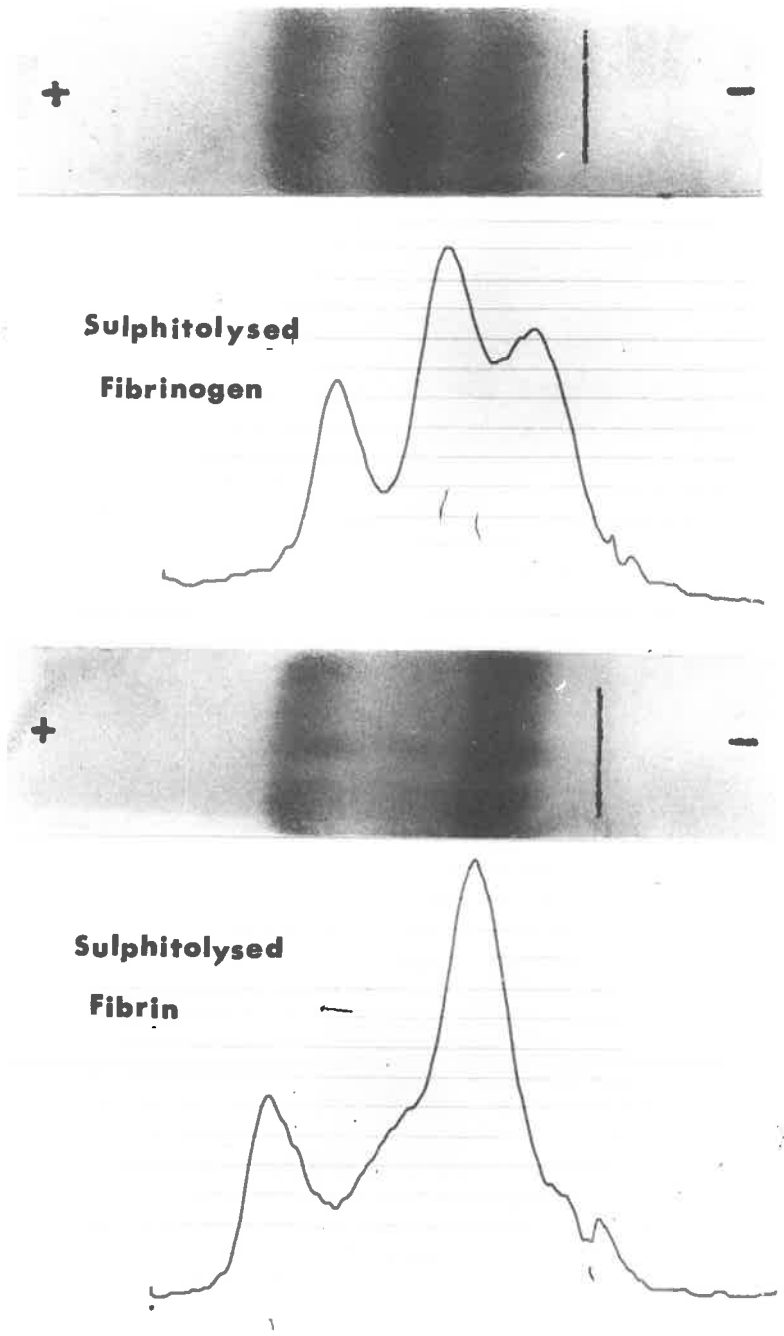


FIGURE 5

Chromatographic Separations of Cairina Fibrinogen and Fibrin Peptide Chains.

Separations achieved for Cairina fibrinogen (Figure 6) and fibrin (Figure 7) are shown. Duplication of runs was, for inexplicable reasons, difficult; varying degrees of overlap of the protein peaks were observed.

End Groups of Isolated Cairina Fibrinogen Chains.

The results of three separate analyses of peaks I, II and III (Figure 6) are shown in Table V.

DISCUSSION

The appearance of N-terminal glycine in fibrin chains of Cairina and Anser, which prior to coagulation had no detectable end groups, was consistent with the release of two fibrinopeptides with no free α-amino group. The results shown in Table IV indicate that no N-terminal tyrosine is lost during coagulation. It seemed that coagulation in these two avian species could be represented thus:

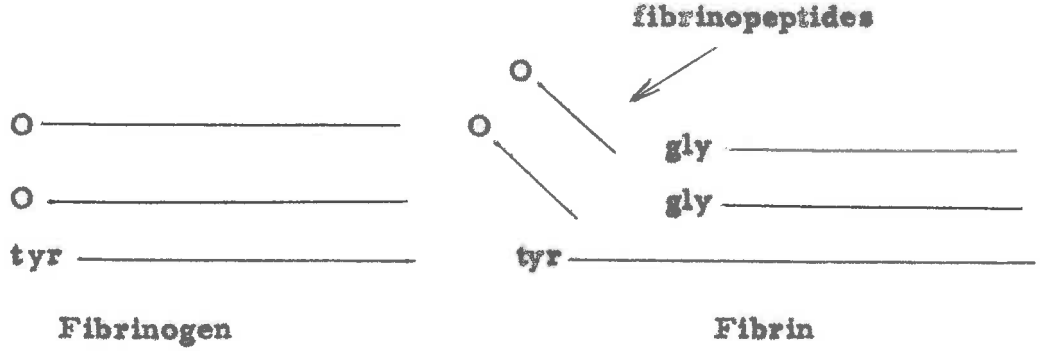


Figure 6. Resolution of Cairina fibrinogen chains on CM-cellulose using sodium acetate buffer gradients. (0.02M, pH 4.2 - 0.1M, pH 4.7.) The N-terminals of each peak (Table V) are also indicated.

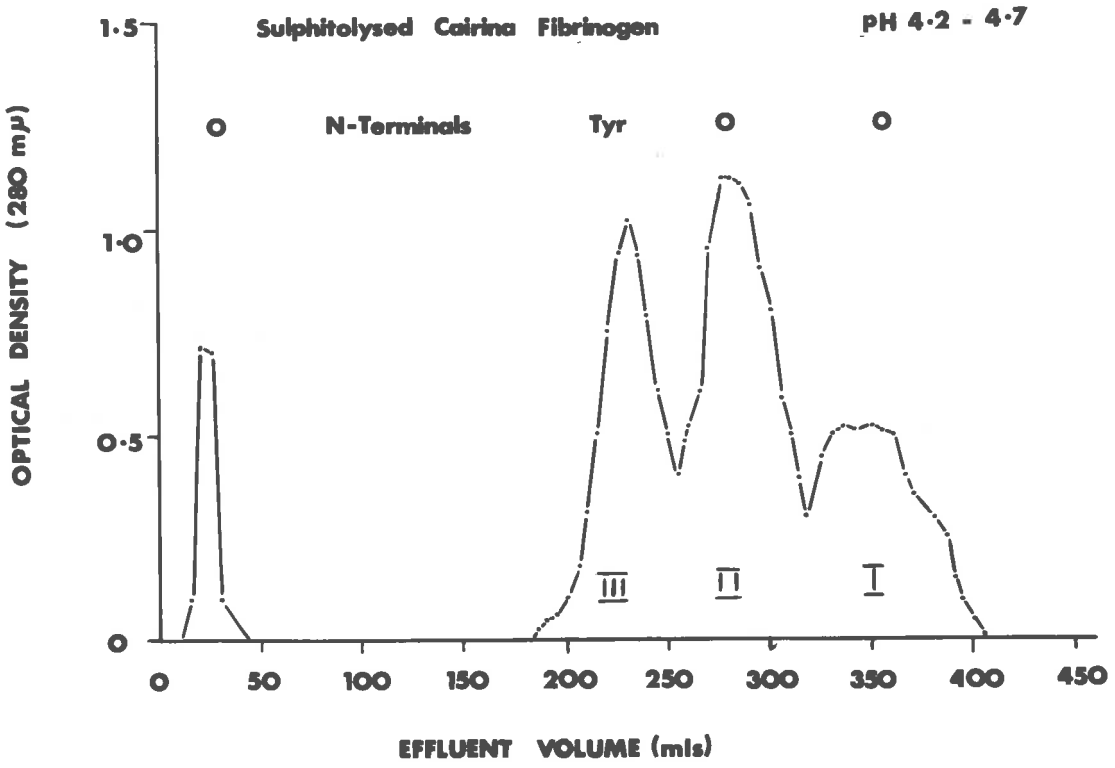

Sulphitolysed Cairina Fibrinogen
pH 4·2 - 4·7
N-Terminals
Tyr
III
II
I
OPTICAL DENSITY (280 mμ)
1·5
1·0
0·5
0
0
50
100
150
200
250
300
350
400
450
EFFLUENT VOLUME (mls)

FIGURE 6

Figure 7. Chromatographic separation of Cairina fibrin chains on CM-cellulose.

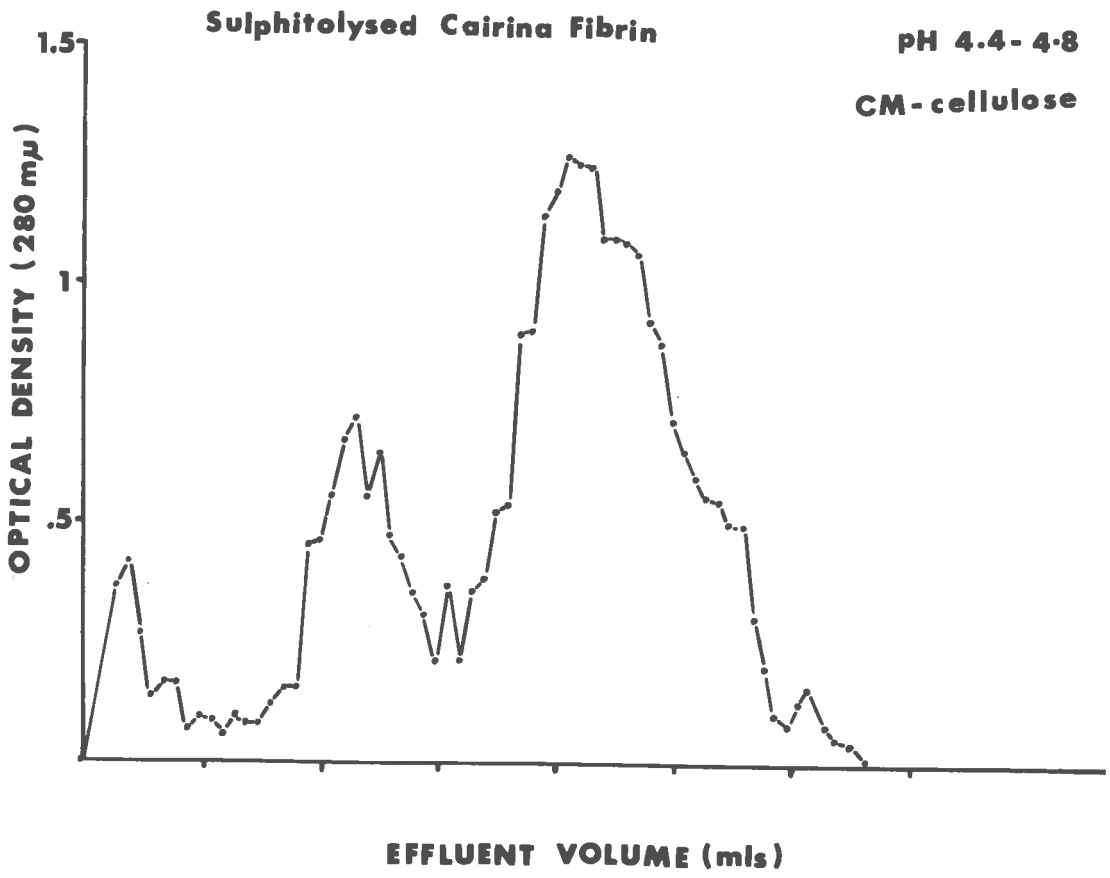

Sulphitolysed Cairina Fibrin
pH 4.4 - 4·8
CM - cellulose
1.5
1
.5
OPTICAL DENSITY (280 mμ)
EFFLUENT VOLUME (mls)

FIGURE 7

TABLE V

Identification, as the PTH derivatives of the N-terminal amino acids of separated chains of sulphitolysed *Cairina* fibrinogen. (See Figure 6.)

Experiment	Peak I	Peak II	Peak III
1	0	tyrosine (trace)	tyrosine
2	0	0	tyrosine
3	0	glycine) trace tyrosine)	tyrosine

The glycine/tyrosine ratio in fibrin was variable and never, in fact, reached the value of 2:1 which such a model would ideally require. However, incomplete cleavage of one or both of the fibrinopeptides from the parent fibrinogen molecule would reduce the expected 2:1 ratio.

It is notable that tyrosine has been found as a free end-group in Cairina, Anser and Anas fibrinogens and fibrins. With the exception of the lamprey eel Petromyzon marinus which has serine instead (Doolittle, Lorand and Jacobsen, 1963), tyrosine has been found constantly as an N-terminal in all other fibrinogens and fibrins so far examined (Blombäck, 1958; Doolittle and Blombäck, 1964). It is possible that this residue is in some way necessary for eventual fibrin polymerisation and that evolutionary replacement has been undesirable. Furthermore, in view of recent considerations of peptide chain initiation and synthesis at the ribosomal level, restrictions may be imposed on what amino acids are permissible in the N-terminal position (Nell, 1966).

Taking Cairina as a representative avian species, the author investigated the number and characteristics of the polypeptide chains of its fibrinogen and fibrin after sulphitolysis. N-terminal studies on the isolated fibrinogen chains confirmed that two of these bore no free end-groups detectable with PITC. Electrophoretic analysis

(Figure 5) revealed that one of the peptide chains of sulphitolysed Cairina fibrin showed a much reduced anodic mobility when compared with its migration in sulphitolysed fibrinogen. The data were consistent with a net loss of negative charge and suggested the cleavage of a highly acidic fibrinopeptide by thrombin from this fibrinogen polypeptide chain. The mobility of the other two chains was similar in both sulphitolysed fibrinogen and fibrin (Figure 5).

CHAPTER II

ISOLATION OF AVIAN AND REPTILIAN FIBRINOPEPTIDES.

CHAPTER II

INTRODUCTION

Mammalian fibrinopeptides are small straight-chain molecules with a molecular weight in the region of 1,500. An extensive and fragmentary literature has accumulated on their structure and properties, and for simplicity minor references are made below to some of these properties without mentioning all the original works. Articles from which information is drawn, and which are not specifically cited in the text, are given in Appendix I. On a structural basis the fibrinopeptides are generally designated as 'A' or 'B'-types, one of each being released by thrombin from the fibrinogen of all mammalian species so far examined (Blombäck and Sjöquist, 1960; Folk and Gladner, 1960; Blombäck and Doolittle, 1963). Lesser amounts of two other human peptides have been discovered, these being the phosphorus-containing "A-P peptide" (Blombäck, Blombäck and Searle, 1963) and the "Y-peptide"(Blombäck, Blombäck, Doolittle, Hessel and Edman, 1963). Both bear a resemblance to the major A-peptide.

Fibrinopeptides have been isolated from the supernatant liquor of purified fibrin clots by numerous techniques. After

precipitation of incoagulable protein with trichloracetic acid (TCA) bovine fibrinopeptides remained in solution (Lorand, 1952). Alternatively, it was found that the fibrinopeptides of most species could be adsorbed from an acidified clot supernatant (adjusted to pH 3. 1) on to a short column of 2% cross-linked Dowex-50, from which they were eluted with ammonium acetate buffers of higher pH and ionic strength (Blombäck and Vestermark, 1958; Blombäck, Boström and Vestermark, 1960).

Variations of these methods were later devised. To improve the overall yield of the human B-peptide, for example, which showed peculiar solubility characteristics at acid pH the freeze-dried clot liquor was extracted with aqueous pyridine, desalted on Sephadex G-25, and the B-peptide precipitated isoelectrically from solution at pH 3. 0 (Blombäck, Blombäck, Edman and Hessel, 1966).

Bovine fibrinopeptides were resolved by chromatography on DEAE-cellulose (Gladner, Folk, Laki and Carroll, 1959). Separation of the A and B-peptides of most mammalian species has, however, generally been accomplished on columns of Dowex-50 of low cross-linkage. The mixed fibrinopeptides, adsorbed from solution at low pH, can be eluted using buffer gradient or stepwise increments of pH.

Mammalian fibrinopeptides all have arginine as a C-terminal

amino acid, presumably dictated by the high specificity of mammalian thrombins. The same arginyl-glycyl bonds appear to be hydrolysed in both homologous and heterologous coagulation systems (Osbahr, Gladner, Laki and Irreverre, 1964), although some degree of species specificity with respect to rate of reaction may exist.

In this phase of the investigation, the author examined the nature of the fibrinopeptides released by bovine thrombin from the purified fibrinogens of the lizard and four bird species. In Cairina and Anser, where N-terminal amino acid studies had been performed, it was anticipated that two peptides each with a "blocked" amino terminus would be found. For the other species, a number of criteria were used for fibrinopeptide identification, viz. that material isolated from the clot supernatant should be peptide in nature (e.g. soluble in 7% trichloracetic acid, or dialysable); that it should be present after and not before coagulation; and that it must be Sakaguchi-positive (arginine in C-terminal position). It was expected in the first instance that these fibrinopeptides would most likely bear some resemblance in size and structure to their mammalian counterparts, since the fibrinogens from which they derived were readily coagulable with mammalian (bovine and human) thrombins.

Experiments will be described, and evidence presented that

the coagulation mechanism in Trachydosaurus is completely analagous to that of mammals, but that thrombin coagulation of the fibrinogens of the four bird species shows marked differences. Schemes for fibrinopeptide isolation and separation will be given in some detail since classical methods applied to each of the bird species led not only to large losses but to a complete failure to recover the main peptides present.

INVESTIGATION

BATCH PREPARATION OF FIBRINOPEPTIDES: MATERIALS AND METHODS

Trachydosaurus fibrinogen fraction I-O, coagulable to 90%, was used without further purification. Total weight of coagulable protein was 2.1 g.

Glycine-precipitated fibrinogen of each of the four bird species was processed in lots of 1-10 g. The purity of the preparations is indicated in Table III.

Thrombin: Parke Davis Thrombin Topical (Bovine origin) was purified by chromatography on Amberlite CG-50 (Rasmussen, 1955) to an activity of 100-150 NIH units per mg.

Folin Reagent: Combined Biuret-Phenol reaction (Lowry, Rosebrough, Farr and Randall, 1951) was routinely used to trace

fibrinopeptides after sub-sampling fractions of the column eluent. The colour was read at 650 mμ. This reagent is not necessarily dependent on the presence of tyrosine (Herriott, 1941).

Sakaguchi reagent: The dip reaction (Jepson and Smith, 1953) was applied after spotting fractions on to filter paper by passing the paper through 0.1% 8-hydroxyquinoline in acetone, followed by 0.2% bromine in N/2 NaOH. Arginine-positive material gave a strong orange-pink colouration.

Ninhydrin reaction: The reaction described by Rosen (1957) using acetate buffer and potassium cyanide activation was employed.

Coagulation of Trachydosaurus fibrinogen.

2.1 g of Trachydosaurus fibrinogen was dissolved in 150 ml of 0.3M ammonium acetate, against which it was dialysed exhaustively for 22 hours at 4°C. On warming to 25°C. any flocculated protein was removed by centrifugation, the clear supernatant diluted to 400 ml with ammonium acetate and bovine thrombin added to a final concentration of 1.5 NIH units/ml. Clotting was allowed to proceed for 3 hours at room temperature, whereupon the clot liquor was expressed from the fibrin by squeezing through silk cloth. The solution was freeze-dried, the last traces of ammonium acetate being removed by raising the temperature in the drying chamber to 38°C.

Isolation of Trachydosaurus Fibrinopeptides.

Fibrinopeptides were recovered from the dried residue essentially according to the method of Blombäck, Boström and Vestermark (1960), by extracting into 0.05M ammonium formate, adjusting the pH to 3.2 with formic acid and adsorbing fibrinopeptide material on to a column of Dowex-50-X2 (3.5 x 8 cm, NH_4^+ form) pre-equilibrated with ammonium formate at the same pH and ionic strength. Most contaminating protein passed through the column.

Fibrinopeptides were eluted from the column with 0.2M ammonium acetate at pH 6.5 and concentrated by freeze-drying for further separation.

Coagulation of Avian Fibrinogens.

After dissolving in 0.3M ammonium acetate to a protein concentration of approximately 1%, the fibrinogen was exhaustively dialysed, diluted and coagulated with bovine thrombin as described for the preparation of Trachydosaurus fibrinopeptides. Coagulation was carried out at room temperature for between 1 and 6 hours in different experiments.

The clot supernatant was concentrated by freeze-drying and the fibrinopeptides recovered by one of the several methods set out

below.

(i) Isolation of Avian Fibrinopeptides using Trichloracetic acid (TCA).

This method is a modification of that described by Lorand (1952) and was initially used for all four bird species. The dried clot liquor was suspended in 30-60 ml of 0.05M ammonium acetate (pH 6.5) and 25% TCA added to give a final concentration of 7%. Protein contaminating the fibrinopeptide preparation was precipitated and removed by centrifugation. On chilling to 2°C., TCA was removed from the supernatant by several extractions with cold ether. Peptide material was precipitated in the cold by addition of acetone to a final concentration of 90%.

(ii) Isolation of Avian Fibrinopeptides using Dowex-50.

The crude fibrinopeptide preparation was dissolved in 0.05M ammonium formate at pH 6.0-6.5. After clarifying the suspension by centrifugation, the supernatant was acidified to pH 3.4 with formic acid and run on to a column of Dowex-50-X2 (4 x 8 cm, NH_4^+ form) equilibrated with 0.05M ammonium formate at the same pH.

The column was washed successively with 100 ml of ammonium formate pH 3.1 then 200 ml of ammonium acetate 0.02M, pH 6.5 and finally with 1N ammonium hydroxide to remove the more strongly bound material. Fractions of 5-8 ml were collected with a flow rate

of 120 ml/hr. Emerging material was detected by the Folin reaction after sub-sampling, and by spotting fractions on to filter paper and applying the Sakaguchi dip-reaction.

(iii) Isolation of Cairina Fibrinopeptides using Sephadex G-25 and G-50.

The crude clot liquor was dried, extracted with 0.05M ammonium formate pH 6.5, clarified by centrifugation and filtered through a column of Sephadex G-25 or Sephadex G-50 (medium grades). The size of the columns used depended on the amount of fibrinogen originally coagulated and the volume of ammonium formate used to dissolve the peptides. (For the fibrinopeptides from 5 grams of fibrinogen a column of G-25, 2.5 x 40 cm, was employed; of G-50, 1.5 x 35 cm).

Elution was performed with ammonium formate of the same pH and ionic strength, with a flow rate of 60 ml/hour. Fractions (5 ml) were sub-sampled and analysed with Folin and ninhydrin reagents. The samples (0.2 ml) withdrawn from each fraction were taken to dryness under high vacuum to remove the volatile ammonium buffer salt before carrying out the ninhydrin reaction.

Electrophoretic Analysis of Fibrinopeptides.

Fibrinopeptides isolated by the procedures described were routinely analysed by electrophoresis in 0.05M phosphate buffer at

pH 7.0 on Whatman 3MM paper strips (4 volts/cm, 6½ hours). The peptides were located (a) by application of the Sakaguchi reaction, (b) by chlorination of the strip followed by spraying with 1% starch-1%potassium iodide (Jepson and Smith, 1953) or (c) by staining with 0.1% ninhydrin in acetone.

BATCH PREPARATION RESULTS

Electrophoretic patterns.

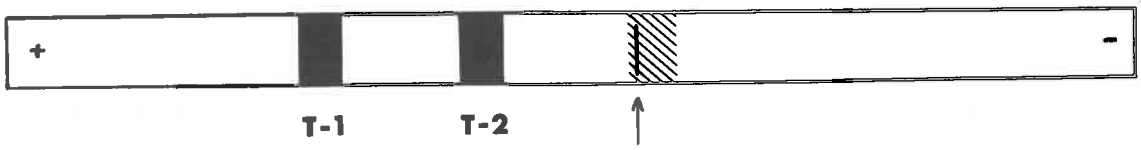
Analysis of Trachydosaurus fibrinopeptides revealed two major bands (T-1, T-2) migrating towards the anode, shown diagrammatically in Figures 8a and 8b.

The more acidic band (T-1) was negative to ninhydrin, indicative of a 'blocked' N-terminus. Some ninhydrin-positive material was seen as a diffuse band near the origin. This proved to be of low molecular weight, only weakly Sakaguchi-positive, and representing only a small amount of the total.

Electrophoretic separations of the crude fibrinopeptides of each avian species isolated by the method using TCA are shown in Figure 9. All bands were located by the Sakaguchi reaction or by chlorination, the most acidic band of each species (designated C-1, AN-1, A-1 and M-1) staining very much more strongly than the rest. This band was the only one negative to ninhydrin (Hann, 1966). A

Figure 8. Electrophoretic separation of Trachydosaurus fibrinopeptides on Whatman 3MM paper. 0.05M phosphate buffer, pH 7.0. 4 volts/cm for 6½ hours. (A) Sakaguchi stain (B) Ninhydrin stain.

(A) Sakaguchi stain.



(B) Ninhydrin stain.

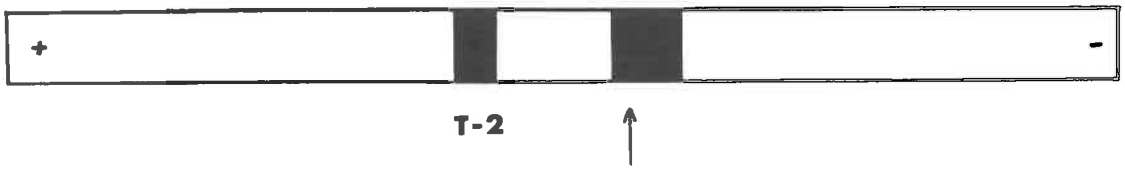


FIGURE 8

Figure 9. Electrophoretic analysis of Avian fibrinopeptides. Conditions as in Figure 8. Sakaguchi stain.

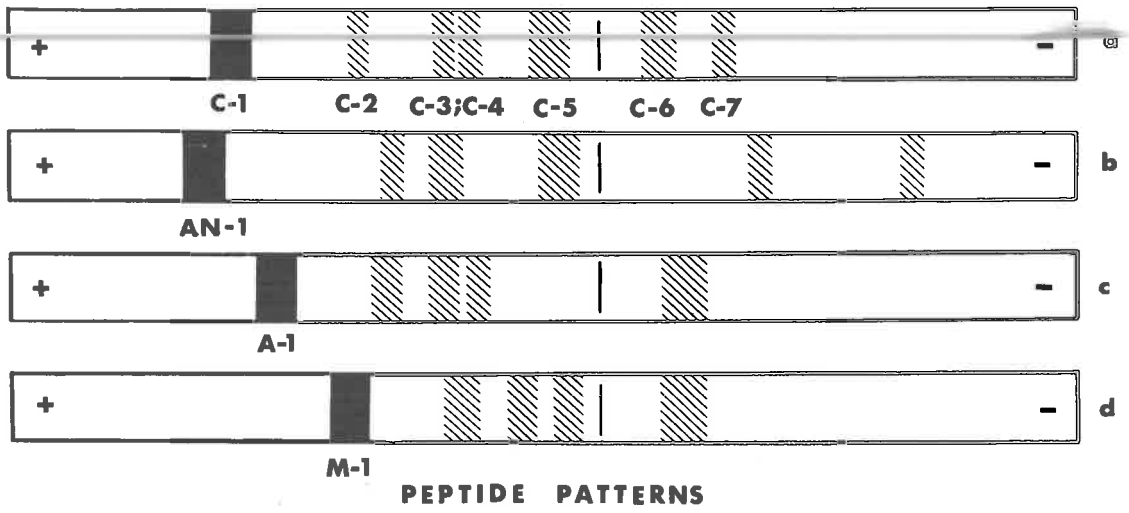

+
-
a
C-1
C-2
C-3;C-4
C-5
C-6
C-7
+
-
b
AN-1
+
-
c
A-1
+
-
d
M-1
PEPTIDE PATTERNS
(a) Cairina (b) Anas (c) Anser (d) Meleagris

FIGURE 9

strong ninhydrin colour was present near the origin, but this material was only weakly positive for the Sakaguchi reaction.

Isolation with Dowex-50.

The elution pattern for Cairina is shown in Figure 10. Much of the material was not adsorbed and passed straight through the column. Electrophoresis of each of the three main peaks denoted I, II and III revealed that the acidic peptide C-1 (see Figure 9a) was present in the first peak, being unretained by the resin. Peaks II and III comprised the remainder of the peptides (C-2 to C-7) seen in the multibanded electrophoretic pattern.

The fibrinopeptide C-1 emerging with the buffer front was contaminated with protein derived from incoagulable material present in the original fibrinogen. This protein showed as a diffuse blur near the origin after electrophoresis and gave only a weakly positive arginine reaction.

Entirely analagous results were seen for Anser, Anas and Meleagris. The single main fibrinopeptide (Figures 9b, 9c, 9d) was not retained by Dowex-50-X2 under the conditions described. No additional material of peptide nature which might have escaped detection using the TCA procedure was apparent.

Gel-filtration of Cairina Peptides.

The emergence of material through Sephadex G-25 and

Figure 10. Preliminary isolation of Cairina fibrinopeptides on Dowex 50-X2 (NH_4^+ form) using ammonium formate (pH 3.1) and ammonium acetate (pH 6.5) buffers. The main acidic component (C-1) was not retained by the resin. Fractions subsampled and peaks located by Folin reaction.

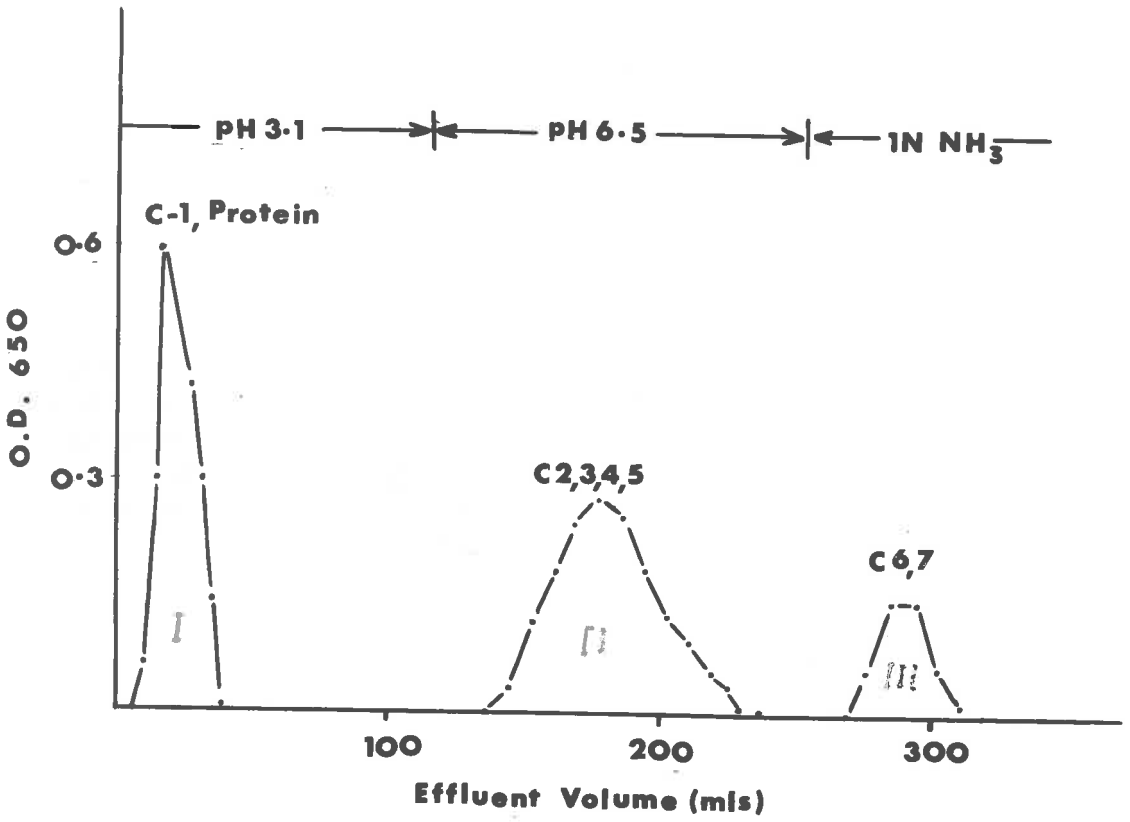

PH 3·1
PH 6·5
1N NH_3
C-1, Protein
0·6
0·3
O.D. 650
C2,3,4,5
C6,7
I
II
III
100
200
300
Effluent Volume (mls)

FIGURE 10

Sephadex G-50 is shown in Figures 11a and 11b, respectively.

Electrophoresis revealed the bulk of the fibrinopeptides to be in the trailing portion of the large Folin-positive peak of the G-25 eluate, still contaminated with non-coagulable protein.

A greater degree of separation was achieved on Sephadex G-50, although electrophoresis indicated here that some of the fibrinopeptide material (particularly the very acidic peptide) emerged with the protein peak. Exactly the same spectrum of peptides found by the other methods was present.

Yield of Fibrinopeptides.

Both Trachydosaurus fibrinopeptides were obtained in approximately equal amounts by weight (4 mg per gram of fibrinogen) but the overall yield of peptides from the bird species was much lower. Only the most acidic peptides of Cairina and Anser (C-1 and A-1 respectively) were recovered in substantial amounts (2-5 mg/gram of fibrinogen), the yield of the others being only fractional.

Because of the complexity of these procedures, it has not yet been possible to obtain comparable data for Anas or Meleagris.

Figure 11. Gel filtration of crude Cairina fibrinopeptides through (A) Sephadex G-25 and (B) Sephadex G-50. Fibrinopeptide material, traced by the Folin reaction, was eluted as indicated.

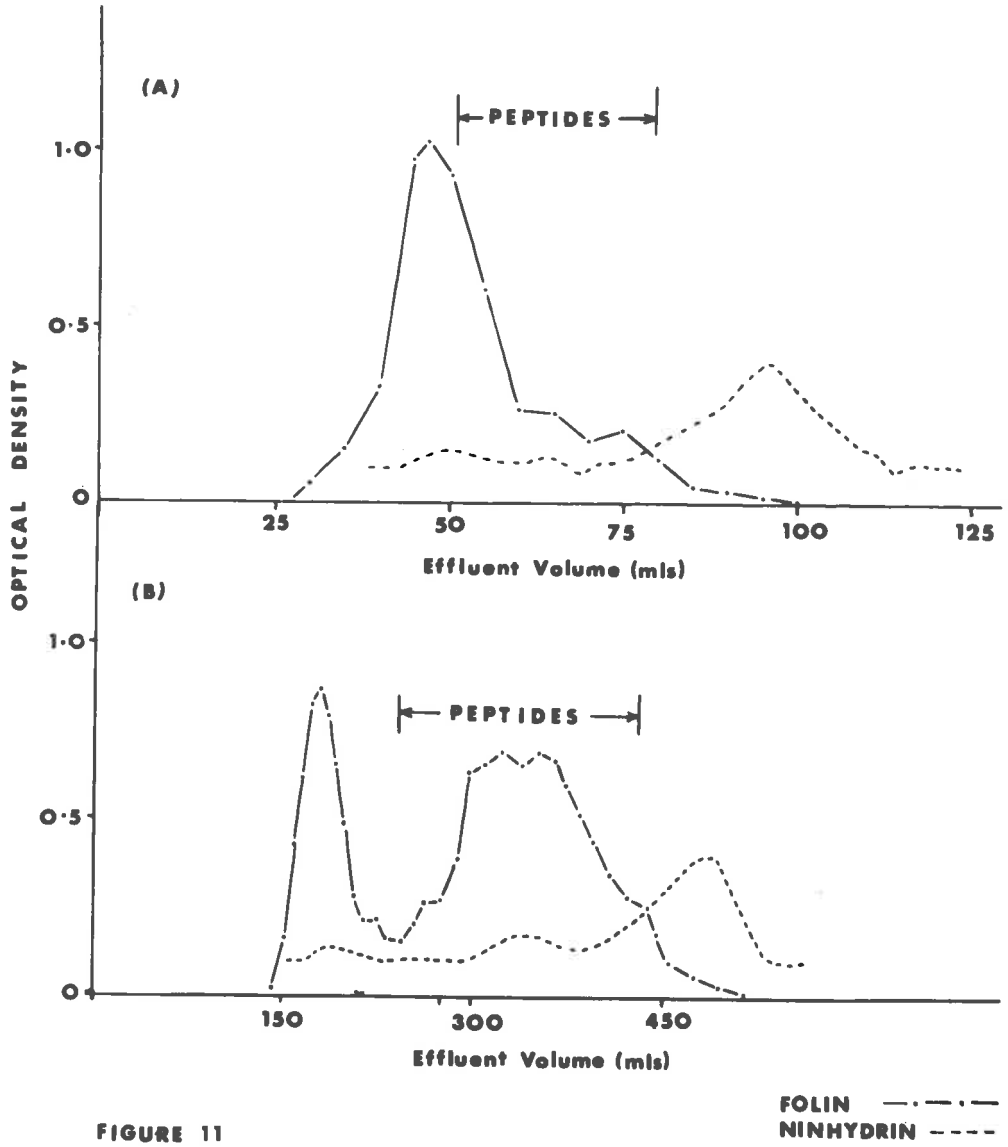

(A)
PEPTIDES
1·0
0·5
0
25
50
75
100
125
OPTICAL DENSITY
Effluent Volume (mls)
(B)
PEPTIDES
1·0
0·5
0
150
300
450
Effluent Volume (mls)
FOLIN
NINHYDRIN

FIGURE 11

CHROMATOGRAPHIC SEPARATION OF FIBRINOPEPTIDES: MATERIALS AND METHODS

Batches of fibrinopeptides of Trachydosaurus and Cairina prepared by the methods described were separated on columns of Dowex-50 using gradients of ammonium formate - ammonium acetate buffer salts (Moore and Stein, 1951; Hirs, Moore and Stein, 1952; Blombäck and Vestermark, 1958).

Separation of Trachydosaurus Fibrinopeptides.

The mixed fibrinopeptides (T-1 and T-2) in 1.5 ml of 0.05M ammonium formate (pH 3.2) were applied to a column of Dowex-50-X2, NH_4^+-form, 1.0 x 45 cm, equilibrated with the same buffer. After washing successively with 50 ml of buffer, and 50 ml of 0.05M ammonium formate pH 3.7, a total of 500 ml of a linear gradient of ammonium formate (0.05M, pH 4.2) - ammonium acetate (0.2M, pH 7.8) was used for elution. Fractions of 5 ml were collected, with a flow rate of 20-25 ml/hour. 0.2 ml of each fraction was sampled for a Folin reaction, and peaks pooled for freeze-drying.

Separation of Cairina Fibrinopeptides.

Cairina fibrinopeptides C-2 through C-7 were retained by Dowex-50 at pH 3.1 (Figure 10) and could be eluted by increasing pH and ionic strength as described for Trachydosaurus. The most basic fragment C-7 was eluted with 1N NH_4OH.

Purification of Cairina Fibrinopeptide C-1.

Final purification of this fibrinopeptide was achieved by adsorbing on to 2% cross-linked Dowex-1 (formate form) equilibrated with 0.05M ammonium formate at pH 4.4. The peptide was strongly retained. The column was washed successively with N/2 and 1N formic acid, then the peptide eluted with 3N formic acid.

RESULTS

Trachydosaurus peptides.

The elution curve with separation of peptides T-1 and T-2 is shown in Figure 12.

Cairina peptides C-2 to C-7.

An elution curve obtained from a typical experiment is shown in Figure 13. Fibrinopeptide C-7 which was eluted with 1N NH_4OH was impure as judged by electrophoresis. Two of the peptides (C-3 and C-4) emerged as a single symmetrical peak.

In other experiments fibrinopeptides C-3 and C-4 were obtained by elution from electrophoresis strips.

Purification of Cairina peptide C-1 on Dowex-1.

The electrophoretic mobility of the peptide was unaltered by this treatment. It was still ninhydrin negative. The pure fibrino-

Figure 12. Separation of Trachydosaurus fibrinopeptides on Dowex 50-X2 (column 1.0 x 45 cms) employing a linear ammonium acetate buffer gradient. Folin reaction.

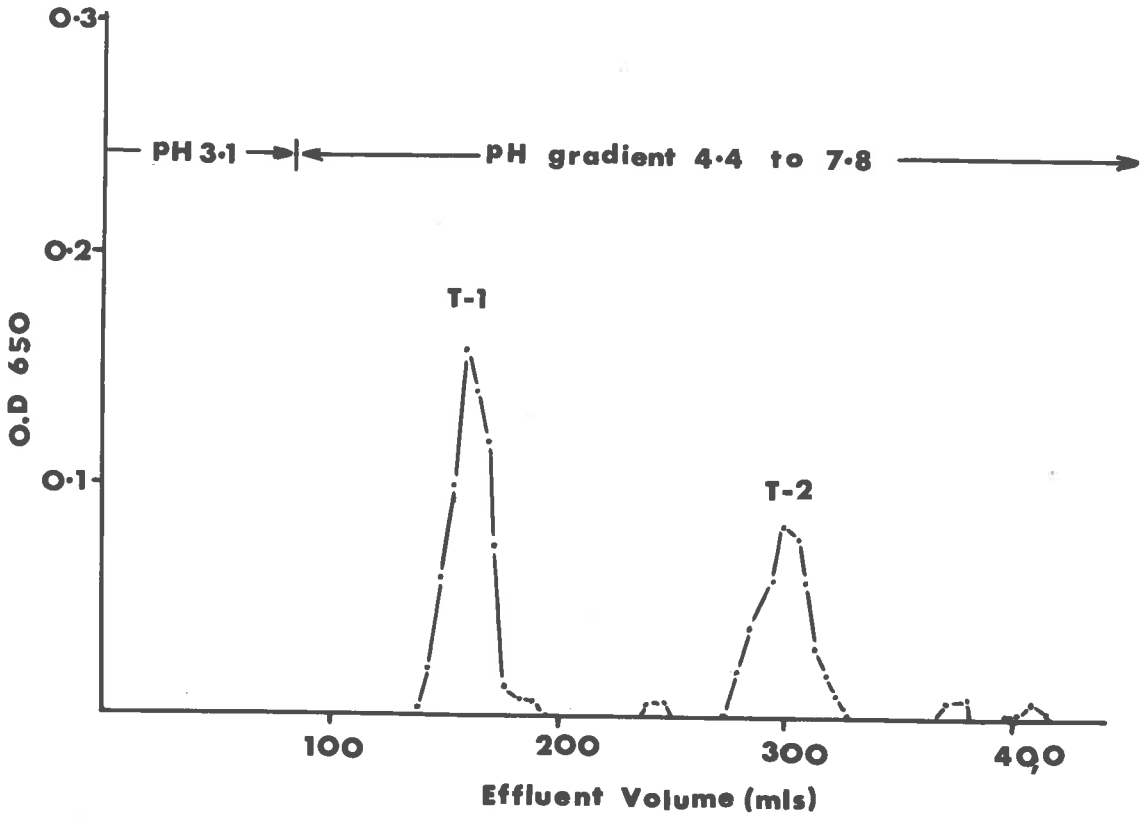

0·3
0·2
0·1
O.D 650
PH 3·1
PH gradient 4·4 to 7·8
T-1
T-2
100
200
300
400
Effluent Volume (mls)

FIGURE 12

Figure 13. Separation of certain Cairina fibrinopeptides on Dowex 50-X2 using a linear ammonium acetate buffer gradient. Folin reaction.

pH gradient 4 ⟶ 7.8

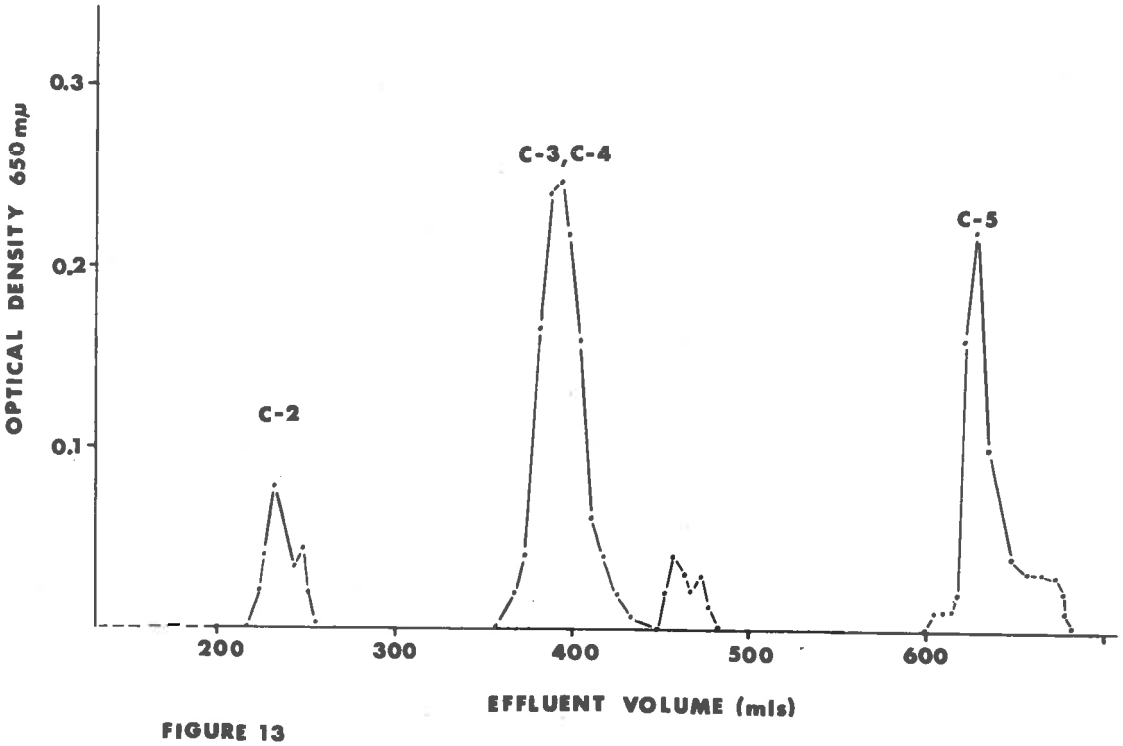


FIGURE 13

peptide was white and very hygroscopic.

RELEASE OF FIBRINOPEPTIDES IN A HOMOLOGOUS BIRD COAGULATION SYSTEM AND INVESTIGATIONS OF FIBRINOGEN HETEROGENEITY: MATERIALS AND METHODS

Preparation of Cairina Prothrombin.

Prothrombin was isolated from 1,680 ml of oxalated Cairina plasma following a method outlined by Biggs and Macfarlane (1957).

$BaSO_4$: X-ray powder, suspended in water several times to remove extra fine particles.

Citrate-saline for dialysis: 0.85% sodium chloride/3.8% trisodium citrate were mixed in the ratio 9/1.

168 grams of dried $BaSO_4$ was stirred into the plasma at 37°C. After 15 minutes the barium sulphate, on to which plasma prothrombin was adsorbed, was collected by centrifugation and washed twice with 500 ml of ice-cold distilled water. Prothrombin was eluted from the $BaSO_4$ by stirring gently with 5% trisodium citrate at 37°C. for 20 minutes.

After centrifugation, the supernatant solution was dialysed for 16 hours against a total of 20 litres of citrate/saline at 2°C. The final volume was 550 ml.

Generation of Thrombin from Prothrombin.

No thrombin activity appeared in the prothrombin solution during preparation. Thrombin was produced by addition of tissue thromboplastin.

Cairina lung, washed free of blood by perfusing with copious volumes of saline, was homogenised in 25 ml of 0.85% saline. Cellular debris was removed by centrifugation at 15,000 G and discarded, the supernatant being used as a source of thromboplastin.

To the entire 550 ml of dialysed prothrombin solution was added 30.0 ml of M/4 $CaCl_2$ and 8 ml of the tissue thromboplastin. Thrombin generation was followed by adding 0.1 ml of the reaction mixture to 1 ml aliquots of an 0.1% human fibrinogen solution at intervals of one minute and recording the clotting time.

Measurement of Cairina Thrombin Activity.

An estimate of the total thrombin activity in terms of N.I.H. units was made in the following manner: 1/1, 1/2, 1/3, 1/4 dilutions of the incubation mixture were made in 0.85% saline, and the clotting times of 1 ml portions of the human fibrinogen measured after addition of 0.1 ml of each dilution.

Purification of Cairina Thrombin.

Pilot experiments were conducted to test the stability of Cairina thrombin to acetone precipitation, freezing and thawing, and

freeze-drying. 10 ml of the crude thrombin (40 units/ml) was precipitated with 10 ml of acetone at room temperature, collected by centrifugation and re-dissolved in 10 ml of 0.85% saline. Clotting activities of 0.1 ml of thrombin solution was estimated before and after precipitation.

Before precipitation: 4.3 secs.

Precipitated (and reconstituted): 4.5 secs.

Precipitated, frozen and thawed once: 13.5 secs.

Great loss of activity was observed after freezing and thawing, but it was noted that thrombin dissolved in 0.5M ammonium acetate at pH 6.0 was perfectly stable to this treatment.

Accordingly, the bulk of the thrombin was precipitated with acetone added to a final concentration of 50%. Portions of the precipitate were dissolved in 0.1M ammonium acetate at pH 6, clarified by centrifugation and purified on Amberlite CG-50 (NH_4^+ form) (Rasmussen, 1955).

Coagulation of Cairina Fibrinogen with Cairina Thrombin.

500 mg of dialysed fibrinogen in 200 ml of 0.3M ammonium acetate were coagulated with purified Cairina thrombin (final concentration 1 unit/ml). The fibrinopeptides were isolated by the method using TCA and acetone precipitation, and subjected to paper electrophoresis.

Isolation of Fibrinopeptides from Individual Bird.

Five Cairina drakes were bled separately into citrate and the fibrinogen and ultimately the fibrinopeptides isolated (TCA method). After concentration each batch of peptides was analysed by electrophoresis.

RESULTS

Cairina Thrombin Production.

The results in Table VI show that maximum thrombin production was reached after 8 minutes.

Activity Measurements.

A dilution of 1/4 of the crude thrombin solution gave a clotting time of 15 seconds, and, by definition, represents the activity of 1 NIH unit. The total thrombin activity of the original solution was thus 40 units/ml.

(The NIH thrombin unit is strictly defined as that amount of thrombin activity which, when added to 1 ml of an 0.1% solution of standardized fibrinogen will give a clotting time of 15 seconds. The human fibrinogen used in these tests was not a standardized sample, but when cross-checked with bovine thrombin preparations of known potency gave a coagulation time of 15.5 seconds with 1 NIH unit. The assay with Cairina thrombin did not take into account

TABLE VI

Cairina thrombin generation.

Incubation Time (minutes)	Coagulation Time of Substrate Fibrinogen
0	> 60 secs
1	> 60 secs
2	> 60 secs
3	25 secs
4	14 secs
5	9.5 secs
6	7.5 secs
7	6.5 secs
8	4.5 secs
9	4.5 secs
10	5.0 secs
15	4.5 secs

Activation of Cairina prothrombin by incubation with Cairina tissue thromboplastin in the presence of Ca^{++} (final concentration M/80). Thrombin generation was followed by adding 0.1 mls of the incubation mixture to 1 ml aliquots of an 0.1% human fibrinogen solution and recording the coagulation time.

possible species specificity, but it was felt that gross errors arising from this would be unlikely.)

Fibrinopeptide Analysis.

Electrophoresis of the peptides released in the homologous Cairina coagulation system showed a multibanded pattern identical to that depicted in Figure 9a. As assessed visually from the Sakaguchi stain the most acidic peptide (C-1) was present in a larger amount than the "minor" peptides.

Also, the same spectrum of fibrinopeptides was evident when they were prepared from the individual fibrinogens of Cairina drakes.

DISCUSSION

Two major fibrinopeptides were isolated after the coagulation of Trachydosaurus fibrinogen with bovine thrombin. In direct contrast, the electrophoretic patterns of fibrinopeptides recovered in each of the four bird species were remarkable. Thrombin coagulation of the reptilian fibrinogen appeared to follow the typical mammalian pattern in every respect, where in most instances only two fibrinopeptides are released.

The appearance of "extra" fibrinopeptides in mammals has been documented in two cases. Coagulation of human fibrinogen

results in the recovery of a phosphorylated "AP" peptide (Blombäck, Blombäck and Searle, 1963) and a 'Y' peptide (Blombäck, Blombäck, Doolittle, Hessel and Edman, 1963) besides the major 'A' and 'B' peptides. It has been shown that the 'AP' and 'A' peptides are identical in amino acid sequence apart from the phosphorylated state of the serine residue in the former. The human 'Y' peptide is similar in amino acid sequence to the 'A'-peptide save that it is missing one amino acid residue at the N-terminal end, bearing aspartic acid in this position. Aspartic acid is difficult to identify as an N-terminal in human fibrinogen, but as the Y-peptide is released in low yields, this is not surprising.

A second report pertaining to an 'abnormal' mammalian fibrinopeptide was made by Doolittle and Blombäck (1964) who discovered an extra B-type fibrinopeptide in a batch prepared from the mixed bloods of three reindeer. The yield was consistent with one animal being homozygous for the trait, or two of the animals being heterozygous.

Avian fibrinopeptides were routinely prepared from the bloods of many birds pooled during collection. The possibility that the spectrum of peptides was due to the existence of a number of genetically different polymorphic fibrinogen molecules occurring in the pooled bird blood was investigated in Cairina by isolating peptides

from individual drakes. Here the fibrinopeptide patterns could not be attributed to such heterogeneity. It would seem reasonable to assume that this is true of Anser, Anas and Meleagris as well. Thus, while it is evident that some degree of fibrinopeptide heterogeneity does exist in at least two mammalian species it is doubtful whether this would account for the wide spectrum of fibrinopeptides released in any of the four bird species the individuals of which are derived from highly-inbred domestic flocks.

The same range of fibrinopeptides was released from Cairina fibrinogen when either bovine or the homologous thrombin was used.

Perhaps because of their size or acidic character the main peptide of each bird species was unretained by Dowex-50-X2 at pH 3.1 in contrast to the fibrinopeptides of mammalian species and the lizard. Oversight of this phenomenon led to a total loss of the peptide in the earlier stages of the investigation. Experimental difficulties were encountered in freeing the main peptide both from protein and from an unidentified green pigment which contaminated all preparations. TCA-precipitation of protein was not entirely satisfactory as it was apparent that this resulted in some loss of the peptide as well. Repeated gel-filtration through Sephadex G-50, or elution from Dowex-1 was considered best.

The appearance of a single main fibrinopeptide in Cairina

and Anser negative to ninhydrin indicating a probable blocked amino terminus was in partial agreement with the original N-terminal amino acid analyses of the fibrinogen and fibrin (Chapter I) which suggested that two such peptides would be found. On the other hand, electrophoretic analysis of the sulphite-cleaved chains of Cairina fibrinogen and fibrin (Figure 5) showed that only one was substantially altered by thrombin. The reduced anodic migration that this chain showed in sulphitolysed fibrin as contrasted with sulphitolysed fibrinogen was in accord with the loss of a large net negative charge during coagulation. The negative charge residing on Cairina fibrinopeptide C-1 could well account for this loss.

The origin of the minor fibrinopeptides is not clear. All were dialysable through Visking cellulose tubing and since the highly purified fibrinogen was exhaustively dialysed before coagulation the possibility that some of the peptide material was inherent in the fibrinogen preparation was excluded. Thrombin itself was chromatographically pure. Other explanations must be sought and several possibilities are that (i) they are the products of a more extensive but less intense proteolysis of the fibrinogen molecule by thrombin, perhaps 'artifacts' in the sense that the fibrinogen, although still highly coagulable may undergo some conformational change during isolation, to varying extents exposing to thrombin a number of bonds

not normally hydrolysed in the native molecule, or (ii) they are derived from the main peptide by secondary proteolytic action either by thrombin or by another enzyme present in the avian fibrinogen preparations, or (iii) they are, in fact, released by the action of thrombin on several 'minor' fibrinogen chains of different molecular structure.

Secondary proteolysis by thrombin would seem unlikely because of the enzyme's limited specificity in so many animal species. Because of the complexity of the peptide patterns appearing many phases of the investigation were necessarily restricted to a single species. Cairina was chosen because of relative availability.

CHAPTER III

THE N-TERMINALS AND AMINO ACID COMPOSITIONS OF CERTAIN FIBRINOPEPTIDES.

CHAPTER III

INTRODUCTION

Inspection of the amino acid compositions of the mammalian fibrinopeptides listed in Appendix II reveals that those belonging to series 'A' have a number of features which distinguish them from the fibrinopeptides 'B'. While phenylalanine and leucine, for example, are constantly found in mammalian peptides A their occurrence in B-peptides is variable, and histidine and tyrosine are characteristic of the latter.

The mammalian fibrinopeptides listed in Appendix II, with the exception of man, all contain tyrosine, the hydroxyl group of which is sulphated. The occurrence of this ester was first reported by Bettelheim (1954) and subsequently confirmed by others (Blombäck and Vestermark, 1958; Von Korff and Bronfenbrenner, 1958; Jevons, 1963). The bovine fibrinopeptide B, although giving rise to tyrosine on acid hydrolysis, showed ne ither of the characteristic U-V absorption peaks at 275 mμ and 293 mμ in neutral and alkaline solution respectively. It was shown (Blombäck, Boström and Vestermark, 1960) that after mild acid hydrolysis of the rabbit fibrinopeptide B, the tyrosine absorption peak appeared. From $^{35}[S]$ sulphate incorporation studies corroborative evidence was

presented by these authors that acid hydrolysis did desulphate the tyrosine ester.

A second feature of some, but not all mammalian B-peptides is the absence of a free ∝-amino group at the N-terminus. Some controversy has existed regarding the nature of the blocked residue of the bovine B-peptide for it has been reported to be N-acetyl-threonine (Folk, Gladner and Laki, 1959; Folk and Gladner, 1960) and cyclic (pyro-) glutamic acid (Blombäck and Doolittle, 1963a, b). Evidence for the latter is far more substantial. Pyroglutamic acid has now been unequivocally confirmed as the "blocked" amino acid residue of the human B-peptide (Blombäck, Blombäck and Edman, 1963).

The existence of an amino acid without a free ∝-amino group prevents reaction with PITC, FDNB or 1-dimethylamino-naphthalene-5-sulphonyl chloride (Dansyl-chloride, DNS-Cl). This latter very sensitive fluorescent end-group reagent has been used to identify terminal residues of proteins and peptides requiring only 10^{-3} to 10^{-4} µmoles of material (Gray and Hartley, 1963). After hydrolysis of the coupled peptide with 6N HCl, the DNS-amino acids have been identified by high voltage electrophoresis (Gray and Hartley, 1963), paper chromatography (Boulton and Bush, 1964), or by chromatography on silica gel (summarized by Pataki, 1966). The methods described require either lengthy development of

chromatograms or give only partial resolution.

The N-terminal residues of Trachydosaurus and certain Cairina fibrinopeptides were investigated using PITC and DNS-Cl. The observed non-reactivity of the most acidic peptides with ninhydrin implied that they had no free α-amino group. In connection with the identification of the cleaved DNS-amino acids, new chromatographic techniques for rapid and positive qualitative estimation were devised by the author. TheU-V absorption curve of Cairina fibrinopeptide C-1 was investigated, and the total amino acid compositions of some fibrinopeptides are reported.

INVESTIGATION

MATERIALS AND METHODS

Amino Acid Composition of Trachydosaurus Fibrinopeptide T-1.

This fibrinopeptide, obtained by the chromatographic procedures described in the previous chapter was homogeneous by chromatography and electrophoresis.

1.3 mg of the peptide was hydrolysed in constant boiling point hydrochloric acid for 22 hours at 105°C. in a pyrex tube flushed with nitrogen before sealing. The hydrolysate, twice taken to dryness under high vacuum was dissolved in 2.5 ml of a solution 0.1N HCl containing 12.5% sucrose and 0.2 μ moles/ml norleucine.

1.0 ml was loaded on to the resin column of a Technicon amino acid analyser.

Amino Acid Composition of Cairina Fibrinopeptides.

A highly purified sample of the ninhydrin-negative Cairina fibrinopeptide (C-1) was obtained by elution from Dowex-1.

Peptide C-2: The peptide eluted from Dowex-50-X2 was homogeneous by chromatography and electrophoresis.

Peptide C-3 and C-4: These emerged as a single peak from Dowex-50 (Figure 13). Separation was achieved by electrophoresis in phosphate buffer at pH 7 on Whatman 3MM paper. (5 volts/cm for 10 hours.) The peptides were eluted with water, concentrated by freeze-drying and desalted on Sephadex G-15. The total yields were 2.6 mg of peptide 3 and 1.8 mg of peptide 4.

Peptides 5 and 6: No satisfactory preparation of these minor fibrinopeptides was achieved. Under a variety of electrophoretic conditions, the fibrinopeptides appeared to be inhomogeneous and consequently no amino acid analysis of this material was attempted.

Peptide 7: This peptide was obtained by elution from Whatman 3MM paper and desalted on Sephadex G-10.

Approximately 1.5 mg of each of the fibrinopeptides was hydrolysed and analysed as described for Trachydosaurus.

U-V Absorption Curves.

2 mg of the purified Cairina peptide was dissolved in 3 ml of distilled water for a spectrophetometric scan in the U-V range 225 mμ - 300 mμ. A second sample of the peptide in 3 ml of 1N HCl was heated at 100°C. for 5 minutes before scanning. The hydrolysed sample was then made alkaline by the addition of 1 ml of 4N NaOH and re-examined at 290-300 mμ.

Qualitative Estimation of Dansyl-amino Acids.

DNS-amino acids as markers were synthesized following the method of Boulton and Bush (1964). However, after coupling, the reaction mixture was evaporated to dryness and the DNS-amino acid hydrolysed in 5.7N HCl for 8-16 hours at 105°C. to simulate the conditions used for end-group detection of peptides and proteins. They were again dried and dissolved in 50% aqueous acetone for chromatography.

Paper chromatography: Solvent E was used after impregnation of Whatman No. 1 paper with formamide (Edman and Sjöquist, 1956).

Silica gel chromatography: 0.5 mm thick plates (8 inches square) of Silica gel G were heated to 100°C. for 30 minutes before use. O-DNS tyrosine and ε-DNS-lysine (coupled only in the hydroxyl and ε-amino positions respectively) were prepared by reacting the dipeptides glycyl-tyrosine and glycyl-lysine with DNS-Cl and

hydrolysing as described.

The single phase solvent system used was water-saturated n-butyl acetate (50 ml), propionic acid (4 ml) and dimethylformamide (5 ml).

DNS-amino acids were spotted on a line 2 cm from the bottom of the plate. The solvent front was allowed to run a total distance of 15 cm from the base of the plate.

DNS-amino acids were located using an ultra violet light with an output wavelength of 230-310 mμ.

N-terminal Analyses with PITC.

The end groups of Trachydosaurus fibrinopeptides T-1 and T-2, the Cairina peptide C-1 and Anser peptide A-1 were investigated using PITC.

Reagents were purified in the manner described for N-terminal studies on fibrinogen and fibrin.

The original method (Edman, 1950 b) was slightly modified, incorporating the use of trifluoracetic acid (TFA) for cleavage and cyclisation of the terminal PTC-amino acid to the corresponding phenylthiohydantoin.

The peptides were coupled with PITC using a reaction mixture described by Margoliash (1962). 3 mg of each fibrinopeptide was dissolved in 0.4 ml of ammonia-free water. 0.4 ml of 2% PITC

in pyridine was added, followed by 40 µl of triethylamine. The mixture was incubated at 37°C. for 1½ hours, during which time it took on a yellow colouration. Excess PITC and pyridine were removed by five extractions with benzene, the aqueous phase dried in vacuo and cleavage achieved by addition of 0.3 ml of TFA. After 60 minutes at 37°C., TFA was removed by vacuum drying, the residue suspended in 0.5 ml of 0.01N HCl and the PTH amino acid extracted with three lots of 0.5 ml ethyl acetate and one lot of 0.5 ml of peroxide-free ether. The extracts were pooled and evaporated to dryness using a stream of nitrogen. The PTH's were re-dissolved in 0.1 ml of ethyl acetate/acetone (8/2) for qualitative chromatography.

Chromatography: Solvents D, E and F of Edman and Sjöquist (1956) were used as described.

N-terminal Analyses with DNS-Cl.

Peptides T-1, T-2, C-1, C-2, C-3, C-4 and C-7 were coupled with DNS-Cl following the method of Gray and Hartley (1963). About 1.5 mg of the material was dissolved in 2 ml of water and 20 µl measured into a test tube measuring 4" x ¼". (Predicting a molecular weight in the vicinity of 1,500 this corresponded to 10^{-2} µmoles.) The material was dried and the residue dissolved in 20 µl of 0.2N $NaHCO_3$ and 20 µl of DNS-Cl in acetone (1 mg/ml) added.

After flushing the tube with nitrogen, coupling was carried out at room temperature for 2-3 hours.

After drying in vacuo, 50 µl of constant-boiling point HCl was added, the tube sealed under nitrogen and incubated for 12-16 hours at 105°C. HCl was removed by drying and the coupled DNS-amino acids identified by chromatography in the systems described.

RESULTS

Amino Acid Compositions.

Total amino acid compositions showing the minimum number of residues of the lizard fibrinopeptide T-1 (Table VII) and Cairina peptides C-1, C-2 (Table VIII) and C-3 (Table IX) are given.

U-V Absorption Curve.

The graph (Figure 14) shows that no absorption peak at 275 mµ characteristic of tyrosine was evident in the aqueous solution of Cairina fibrinopeptide C-1. However, a peak appeared in this region after mild hydrolysis. A typical shift to 293 mµ in alkaline solution was observed.

Chromatography of Dansyl-Amino Acids.

A chromatogram of the DNS-derivatives of seventeen of the commonly occurring amino acids separated on silica gel is illustrated

TABLE VII

Molar ratios of amino acids in Trachydosaurus fibrinopeptide T-1.

Aspartic Acid	8
Threonine	1
Glutamic Acid	3
Proline	3
Glycine	3
Alanine	2
Valine	2
Tyrosine	1
Arginine	3
Total	26

TABLE VIII

Molar ratios of amino acids in Cairina fibrinopeptides C-1 and C-2.

	C-1	C-2
Aspartic Acid	5	5
Threonine	1	1
Serine	2	2
Glutamic Acid	5	5
Proline	2	2
Glycine	0	1
Alanine	1	1
Valine	1	1
Tyrosine	1	0
Arginine	1	1
Ammonia	(7)	(6)
Total	19	19

TABLE IX

Molar ratios of amino acids in Cairina fibrinopeptide C-3.

Aspartic Acid	2
Threonine	1
Serine	2
Glutamic Acid	5
Glycine	4
Alanine	1
Valine	1
iso-Leucine	1
Leucine	1
Phenylalanine	1
Lysine	2
Arginine	1
Ammonia	High
Total	22

Figure 14. U-V absorption spectra of Cairina fibrinopeptide C-1. After mild acid hydrolysis (viz. 1N HCl at 100°C. for 5 mins.) a peak with an absorption maximum at 275 mμ characteristic of tyrosine appeared. (Blombäck, Boström and Vestermark, 1960.)

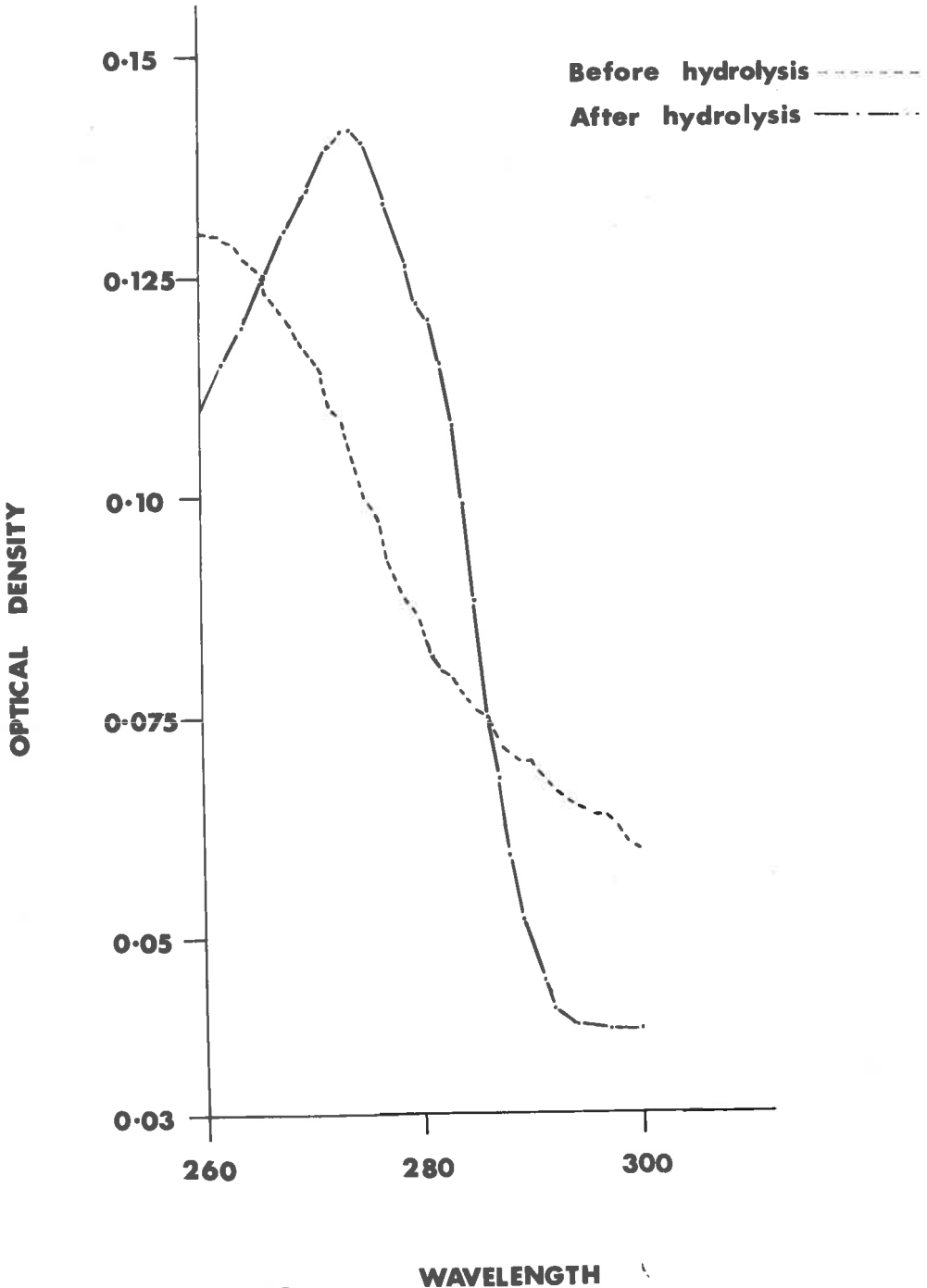

0·15
0·125
0·10
0·075
0·05
0·03
OPTICAL DENSITY
260
280
300
WAVELENGTH
Before hydrolysis
After hydrolysis

FIGURE 14

(Figure 15) and the R_F values given in Table X. The R_F values obtained by chromatography in Solvent E are also included.

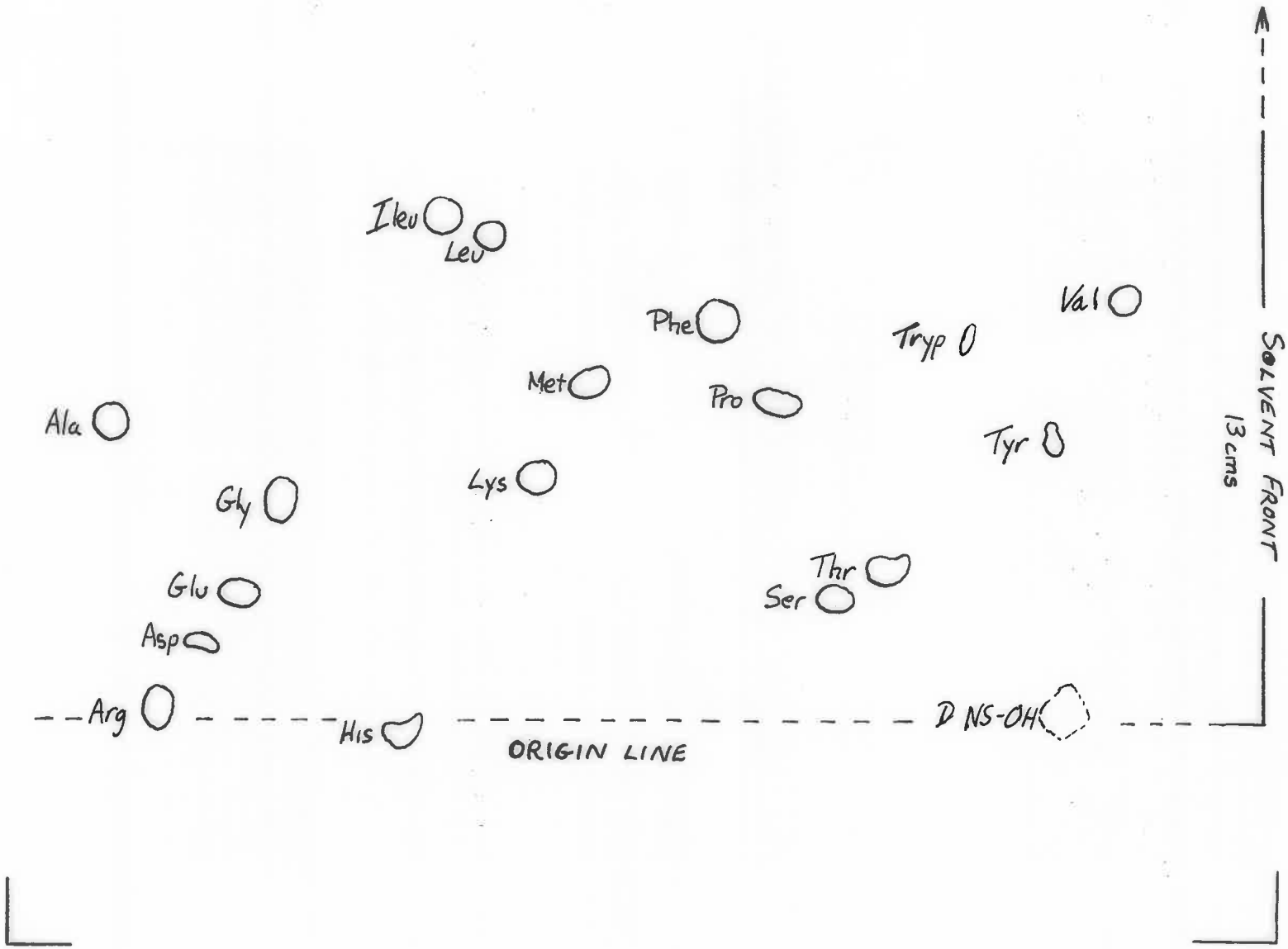
Excess reagent converting to 1-dimethylamino-naphthalene-5-sulphonic acid (DNS-OH) during hydrolysis did not move and fluoresced blue-green at the origin. A faintly fluorescent spot with R_F 0.9 apparently due to ammonia contamination was sometimes seen.

The results indicate that the separation of all the DNS-amino acids except DNS-arginine and DNS-histidine can be achieved by using a single run on both paper and silica gel. The time for development of the chromatograms was approximately 1¾ hours. Distinction between DNS-glutamic acid and DNS-serine and DNS-methionine and DNS-tyrosine on silica gel was critically dependent on running conditions, but with the use of markers differentiation could be achieved. DNS-arginine and DNS-histidine were easily distinguishable by low voltage electrophoresis at pH 4.4.

Fibrinopeptide N-terminals.

No PTH-amino acid was detected after coupling fibrinopeptides T-1, C-1 or A-1 with PITC. Trachydosaurus fibrinopeptide T-2 had glutamic acid as N-terminal residue. (The R_F values of the phenylthiohydantoin derived from this peptide in solvents D, E and F were 0, 0.46 and 0.27 respectively; when compared with

Figure 15. Thin layer chromatography of 'Dansyl' amino acids in n-butyl acetate/propionic acid/dimethylformamide 50: 4: 5.


Ileu
Leu
Phe
Tryp
Val
Met
Pro
Ala
Tyr
Lys
Gly
Thr
Glu
Ser
Asp
Arg
His
DNS-OH
ORIGIN LINE
SOLVENT FRONT
13 cms

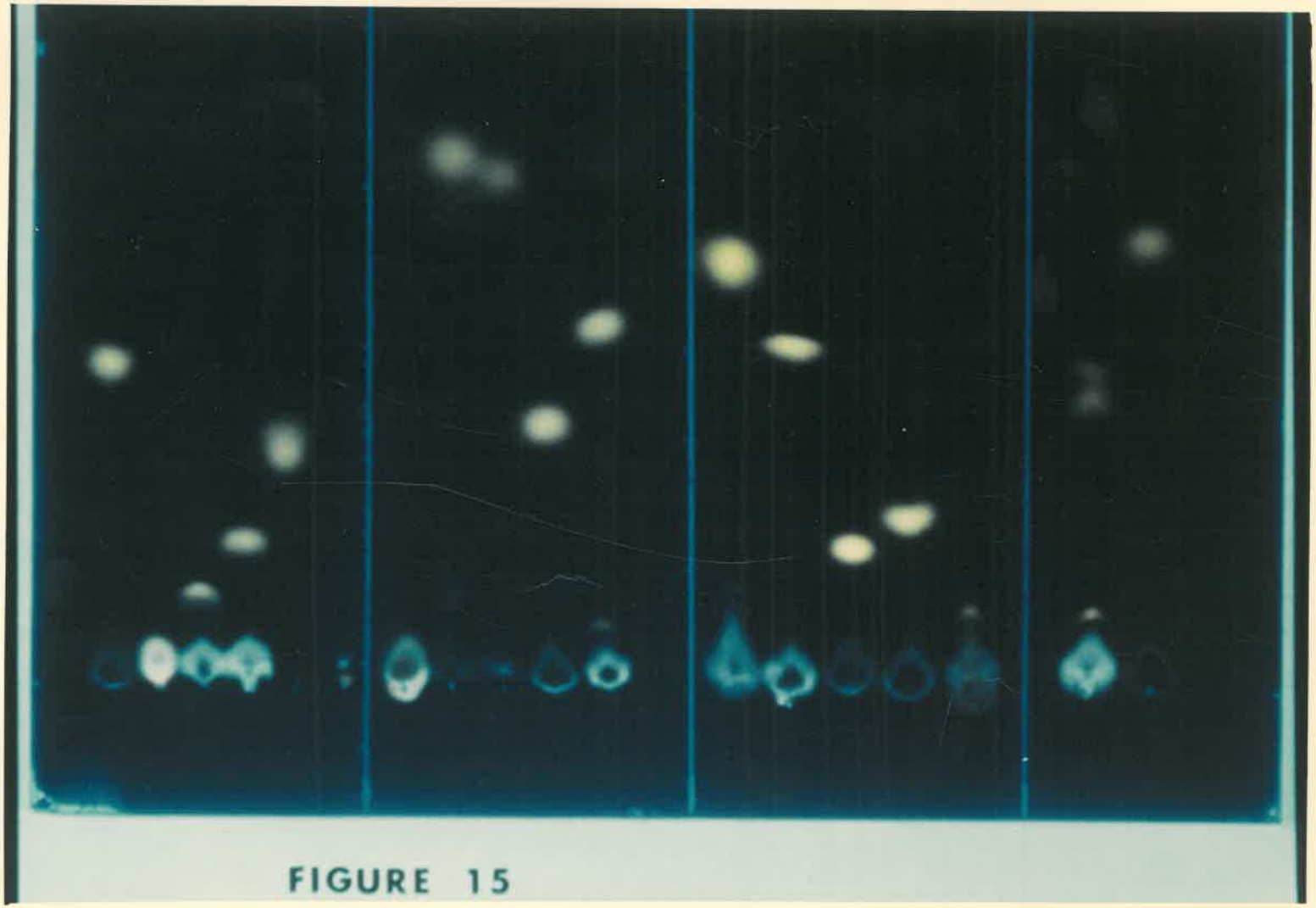


FIGURE 15

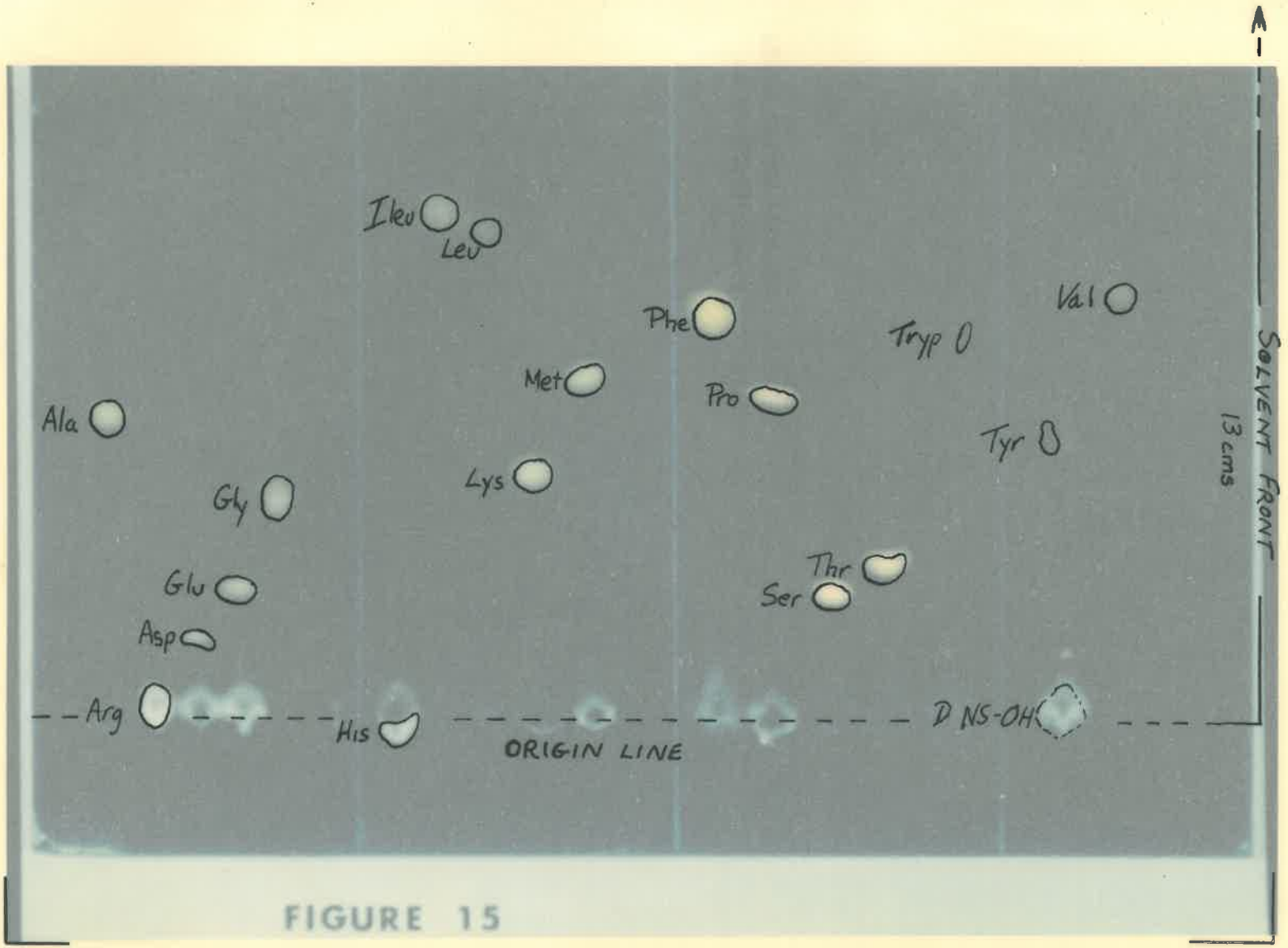

Ileu
Leu
Phe
Tryp
Val
Met
Pro
Ala
Tyr
Lys
Gly
Thr
Glu
Ser
Asp
Arg
His
DNS-OH
ORIGIN LINE
SOLVENT FRONT
13 cms

FIGURE 15

TABLE X

Rf values of DNS-amino acids on silica gel. and paper.

	Silica Gel	Paper
Alanine	0.44	0.48
Arginine	0.01	0.03
Aspartic acid	0.11	0.10
Glutamic acid	0.17	0.15
Glycine	0.31	0.27
Histidine	0	0.03
Isoleucine	0.67	0.75
Leucine	0.65	0.73
Lysine	0.47, 0.70*	0.38, 0.65*
Methionine	0.46	0.53
Phenylalanine	0.55	0.60
Proline	0.45	0.57
Serine	0.16	0.12
Threonine	0.22	0.21
Tryptophan	0.49	0.57
Tyrosine	0.40*	0.39*
Valine	0.61	0.67
DNS-OH	0	0.02
-DNS-Lysine	0	0
O-DNS-Tyrosine	0.04	0.04

Average of eight observations. Solvent: n-butyl acetate/propionic acid/dimethyl formamide 50:4:5.

* Sometimes gave double spots.

an authentic sample of PTH-glutamic acid, the unknown ran in parallel.)

The main fibrinopeptides T-1, C-1 and A-1 had no N-terminal residue detectable with dansyl chloride. Although the amino acid compositions showed that the peptides T-1 and C-1 contained tyrosine the orange-yellow fluorescence of O-DNS tyrosine on silica-gel plates was not seen, further evidence that the hydroxyl group of tyrosine is 'blocked'.

DNS-glutamic acid was identified as the end group of the lizard peptide T-2, corroborating the PITC result.

Of the minor Cairina fibrinopeptides, no end group was detectable in peptides C-2 or C-3. C-4 had glutamic acid in the N-terminal position, and C-7 had glycine.

DISCUSSION

Comparison of the amino acid compositions of Trachydosaurus fibrinopeptide T-1 and Cairina fibrinopeptide C-1 with mammalian B-peptide clearly establishes them as members of this class. The preponderance of aspartic and glutamic acids (some of which, however, may exist as the monoamides) and the presence of only one basic residue would account for the high net negative charge exhibited by the main Cairina peptide. No sample

of the second lizard peptide bearing N-terminal glutamic acid was available for analysis in this laboratory, but it is reported that in both amino acid composition and partial sequence it is entirely analagous to a mammalian A-type fibrinopeptide (B. Blombäck, Personal Communication).

The U-V absorption curves of Cairina peptide C-1 are consistent with the sulphate esterification of the tyrosine residue.

The release by thrombin of the B-peptide from mammalian fibrinogens is not a prerequisite for eventual fibrin polymerization. An enzyme from the venom of the South American Pit Viper (Bothrops jararaca) is capable of causing coagulation by cleaving only the A-peptide (Blombäck, 1958 b, c). There are no reports known to the author of mammalian fibrinogens being coagulated by a thrombin-like enzyme which selectively removes only the B-peptide. However, it seems that only a B-peptide is removed by thrombin during the coagulation of the fibrinogen of the lamprey eel Petromyzon marinus (Blombäck and Lorand, Personal Communication).

The composition of the minor Cairina peptide C-2 indicates the replacement of the tyrosine residue with a glycine, suggesting that intraspecific heterogeneity of the fibrinogen molecule from which it derives does exist. Peptide C-3 contains residues not found in C-1 (viz. leucine, isoleucine, glycine) thus precluding it from being a

degradation product of the main peptide. Its composition is more akin to a mammalian A-peptide, but it must be stressed that the yield of C-3 is both low and variable.

Those fibrinopeptides unreactive with ninhydrin (T-1, C-1, A-1) had as anticipated no N-terminal residue coupling with PITC or DNS-Cl. The positive reaction of C-2 and C-3 with ninhydrin but failure to detect a free end-group with DNS-Cl suggests that a trace of a ninhydrin-positive contaminant is responsible.

The possibility of an extensive proteolysis of a second major Cairina fibrinopeptide (which may not be released quantitatively in the first place) cannot be completely excluded. Peptide C-3 does bear resemblance to a mammalian A-type fibrinopeptide. It carries no free α-amino acid which is a condition demanded by the original end-group analyses of Cairina fibrinogen and fibrin. The peptide material designated C-5 and C-6, while appearing homogeneous under the routine electrophoretic conditions could be partially resolved into several ill-defined components at pH 4.4. Whether or not these are smaller fragments of a second main peptide (of which C-3 is either the residual undigested piece or the N-terminal portion) cannot yet be decided.

The known specificity of thrombin for only a few particular arginyl-glycyl linkages in mammalian and also Trachydosaurus

fibrinogens would make it improbable that this is the active hydrolytic enzyme. For thrombin itself to cleave an A-type peptide into many fragments a first presumption would be that the peptide must contain several internal arginyl-glycyl linkages. Each fragment should thus have glycine as an N-terminal. This is not true of C-4 which has glutamic acid in this position. Should thrombin be cleaving to limited but varying extents arginyl-glycyl linkages within the polymerized fibrin chains similar conjecture would apply.

A proteolytic enzyme contaminating the fibrinogen preparations of each bird species and which is in some way activated during coagulation might be responsible for the 'secondary' digestion of the fibrinogen or fibrinopeptides. Its substrate requirements may be such that the main B-type peptide (C-1, A-1) is immune to its action.

CHAPTER IV

PARTIAL AMINO ACID SEQUENCES OF CERTAIN *CAIRINA* AND *TRACHYDOSAURUS* FIBRINOPEPTIDES.

CHAPTER IV

INTRODUCTION

Besides the chemical end-group reagents such as PITC, FDNB and DNS-Cl, N-terminals may be detected enzymatically. Leucine aminopeptidase, an enzyme of relatively low specificity, liberates free amino acids from the N-terminal end of a susceptible peptide chain. Semiquantitative analysis of the amino acids appearing in the digest at intervals may yield some information on the order of their release. In this manner it has been possible to determine the sequence of the first six residues of the B chain of oxidized insulin (Hill and Smith, 1957).

Carboxypeptidases which hydrolyse the peptide bond adjacent to a free α-carboxyl group are generally used to detect C-terminal amino acids. Carboxypeptidase-A shows preferential specificity for aromatic amino acids, while carboxypeptidase-B removes only basic residues. By the use of either or both enzymes the sequence of a few residues at the C-terminus might be elucidated.

There are limitations to such methods. Endopeptidase contamination can make assessment of chromatograms impossible, the amino- or carboxypeptidases may be unable to cleave newly exposed residues for reasons of specificity, or the eventual appearance

of several amino acid spots on chromatograms may make it impossible to decide the order of release. Endopeptidase activity can be eliminated by prior treatment of the enzymes with diisopropylphosphofluoridate.

Anhydrous hydrazine, in the presence of hydrazine sulphate which acts as an acidic catalyst is the most used chemical method for carboxy terminal detection (Bradbury, 1958 a, b). The C-terminal residue is released as a free amino acid in semiquantitative yield while the internal residues are converted to amino acid hydrazides.

Direct sequential breakdown with PITC (the Edman degradation) is undoubtedly the preferred procedure in the field of sequence analysis and a great number of modifications and variations of the original method (Edman, 1950 b) have been described. Emerging from the experience of many workers are a number of conditions of practical importance for the satisfactory application of the technique. The scheme outlined in Figure 2 shows that coupling of the peptide with PITC takes place at alkaline pH. Buffers used include aqueous pyridine-trimethylamine (Acher, Laurila and Fromageot, 1956), N-allylpiperidine-pyridine (Niall and Edman, 1962), N-ethylmorpholine-acetate (Konigsberg and Hill, 1962) and pyridine-dimethylallylamine (Blombäck, Blombäck, Edman and Hessel, 1966).

It is essential that coupling with PITC proceed to 100%, otherwise after only one or two degradative cycles "carry over" of uncoupled material from preceding steps will cause multiple PTH spots to appear on chromatograms. Positive identification of the PTH's produced in successive steps then becomes impossible. Reports suggest that N-ethyl morpholine-acetate or pyridine-dimethylallylamine buffers are ideal for ensuring complete reaction with PITC.

Cleavage of the terminal PTC-amino acid from the remainder of the peptide chain and its subsequent conversion (through cyclization) to the phenylthiohydantoin can be achieved under acid conditions which do not cause the hydrolysis of internal peptide bonds. Glacial acetic acid saturated with hydrogen chloride was once favoured to bring about conversion. However, the pertinent work of Smyth, Stein and Moore (1963) has shown that (1) newly exposed serine and threonine residues are acetylated in acetic acid/HCl and the extent to which this occurs reduces yields in subsequent degradative steps (2) glutamine undergoes cyclization to pyrollidone carboxylic acid which "blocks" further reaction of the peptide with PITC (3) internal serine or threonine residues are O-acetylated, and when stepwise degradation brings these residues to the N-terminal position an O→N acetyl shift may occur again preventing further reaction with PITC.

The use of anhydrous TFA for cleavage and cyclization obviated

these difficulties (Edman, 1957, 1960; Konigsberg and Hill, 1962). While it is possible to carry out both reactions in the one step with TFA, separation of the two stages is to be preferred. Cleavage is rapid, but conversion is much slower (Edman, 1960; Ilse and Edman, 1963). In the "three-stage technique" (Ilse and Edman, 1963) the peptide is exposed to TFA for fifteen minutes at 40°C. to cleave the PTC-amino acid, thus minimizing the risk of breaking internal peptide bonds. The intermediate thiazolinone derivative (not indicated in Figure 2 for the sake of clarity) is extracted with ethylene dichloride and recovered by evaporation. Conversion to the PTH-amino acid is achieved by treating the thiazolinone at pH 1 for sixty minutes at 80°C. These conditions reputedly give high yields of all PTH's except serine, whose yield is only 20%. In a sequential analysis of the human fibrinopeptide A total yields of PTH's over the first twelve steps were reported to be 91% (Blombäck, Blombäck, Edman and Hessel, 1966), but thereafter it decreased. Increased solubility of the residual peptide in ethylene dichloride seemed to account for this.

The problem of multiple spots appearing after chromatography of the PTH's was overcome by Konigsberg and Hill (1962) by purifying the residual peptide on a small column of Dowex-50 before the next degradative cycle.

In the "subtractive procedure" total amino acid analysis is carried out on the residual peptide after each step of the Edman degradation. Confirmation of the PTH identification can thus be made.

Other variations of the Edman technique have been described. For example, the peptide can be supported on paper during the entire procedure (Fraenkel-Conrat, 1954; Fraenkel-Conrat, Harris and Levy, 1955) or on silica gel (Wieland and Gebert, 1963).

Establishing the amino acid sequence of larger polypeptides or of peptides without free α-amino groups requires prior hydrolysis with proteolytic enzymes and the determination of the sequences of the residues of each of the smaller fragments. It is customary to cleave the protein with enzymes of different specificity and obtain overlapping sequences so that unequivocal assignment of the order of the fragments may be made. Trypsin is the enzyme of choice for proteolytic cleavage as it has the greatest specificity breaking in the main peptide bonds involving the carboxyl groups of arginine and lysine. Other bonds are sometimes attacked, e.g. -phenylalanyl-threonyl- and -tyrosyl-leucyl- of glucagon (Bromer, Staub, Sinn and Behrens, 1957). Chymotrypsin preferentially attacks peptide bonds on the carboxyl side of aromatic amino acids but its action is not always restricted to these. In ribonuclease, a -leucyl-threonyl-

sequence is split (Hirs, Stein and Moore, 1956). Pepsin cleaves -phenylalanyl-phenylalanyl- sequences rapidly (Baker, 1951), but many other bonds are susceptible to its action, e.g. -phenylalanyl-valyl-, -leucyl-valyl, and glutaminyl-histidyl- of the oxidized B chain of insulin (Sanger and Tuppy, 1951). Protease (subtilisin) elastase and papain are all used when more extensive digestion is necessary.

The author is currently engaged in amino acid sequence investigations of certain Trachydosaurus und Cairina fibrinopeptides. Experience with both the direct and three-stage Edman technique has shown that difficulties arise in the identification of some of the PTH's, in particular, glutamine, asparagine, serine and threonine. Some experiments are described attempting to "supplement" the Edman technique by making use of the flexible and reliable DNS-Cl method to detect the N-terminal after each step of the PITC degradation. Preliminary amino acid sequences are reported.

INVESTIGATION

MATERIALS AND METHODS

Edman Degradation of Trachydosaurus Peptide T-2.

TFA: Fluka-Buchs.

Benzene: Thiophene-free; redistilled.

Triethylamine, ethylene dichloride, ethyl acetate and pyridine were redistilled.

Glacial acetic acid was saturated with hydrogen chloride generated from 11.7 N HCl by dropwise addition of concentrated H_2SO_4. It was dried by bubbling through concentrated H_2SO_4, and then passing through a column of crystalline sodium alumino silicate (Union Carbide Molecular Sieve, type 4A).

The following methods, basically combinations of those of Acher, Laurila and Fromageot (1956) and Konigsberg and Hill (1962) were adopted.

Experiment 1.

To about 3 mg of the peptide in 0.5 ml of water was added 0.5 ml of 2% PITC in pyridine and 0.1 ml of triethylamine. Coupling was carried out at 37°C. for 2½ hours. The incubation mixture which took on a yellow coloration was extracted four times with benzene (discarded) and the aqueous phase was dried from the frozen state. Cleavage and conversion were performed by treatment with 0.5 ml of acetic acid/HCl for two hours at room temperature. After freeze-drying, the residue was suspended in 0.5 ml of 0.01 N HCl and the PTH-amino acid extracted with four lots of 0.75 ml of ethyl acetate.

The ethyl acetate extracts were pooled, made up to 3.5 ml with additional solvent and the extinction of the solution read at 269 mμ

and 320 mμ in 1 cm quartz cuvettes. The ethyl acetate was then evaporated to dryness in a stream of nitrogen, and the PTH identified in solvents D, E and F (Edman and Sjöquist, 1956).

The acidic phase containing the residual peptide was freeze dried and subjected to the next degradative cycle.

Experiment 2.

The procedure was the same as in Experiment 1 except that cleavage and cyclization were carried out using 0.4 ml of TFA for 2 hours.

Enzymatic Digestion of Cairina Peptide C-1.

About 20 mg of the highly purified (but very hygroscopic) fibrinopeptide was heated in 4 ml of 1 N HCl for five minutes at 100°C. After freeze-drying, the peptide was dissolved in 5 ml of 0.1 M triethylamine containing 5 mM $CaCl_2$ and brought to pH 8.0 with acetic acid. 1 mg of α-chymotrypsin (Sigma, three times crystallised) was added and digestion carried out at 37°C. The pH of the solution was kept in the vicinity of 7.8-8.0 by the occasional addition of aqueous ammonia. After four hours the pH was adjusted to 6.0 with acetic acid and the digest was stored frozen.

Electrophoretic Analysis of Chymotryptic Digest.

Analysis was performed in 0.1 M pyridine-acetate buffer

pH 4.0, 5.5 volts/cm for nine hours on Whatman 3 MM paper strips. Bands were located with ninhydrin or with the Sakaguchi reagent.

N-terminal Analysis of Chymotryptic Fragments.

0.05 ml of the digest applied as a band to each of two paper strips was subjected to electrophoresis as described above. The bands, located on one of the strips with ninhydrin were eluted from the second with 1M acetic acid. The dried fragments were further purified by adsorbing from a pyridine-formate buffer at pH 4.0 on to a short (5 x 1 cm) column of Dowex-1 (formate form) and eluting successively with 0.2M formate brought to pH 3.0 with pyridine, 0.2M formic acid, 0.5M formic acid, 1.0M formic acid, 2.5M formic acid. The main fragment was located by spotting the small fractions collected on to paper and staining with ninhydrin. After drying, the N-terminals of each fragment were determined with DNS-Cl as described in Chapter III.

Edman Degradation of Chymotryptic Fragments.

Sufficient of each of the main chymotryptic fragments for preliminary sequence analysis was obtained by eluting bands from several electrophoresis strips and repurifying on Dowex-1-X2 as described above.

The material was dissolved in 200 µl of water, and a

subsample of 20 µl was withdrawn for coupling with DNS-Cl. 180 µl of 2% PITC in pyridine and about 20 µl of triethylamine were then added. Coupling was carried out for 2 hours at 37°C., the reaction mixture extracted four times with 400 µl of benzene, and dried. Cleavage was achieved by treatment with 150 µl of TFA, the thiazolinone extracted with several lots of ethylene dichloride and converted to the PTH in 0.1 N HCl as described (Ilse and Edman, 1963).

For the second, third ..... cycles, the residual peptide was dissolved in 180 µl, 160 µl ..... of water, a 20 µl subsample withdrawn for reaction with DNS-Cl and the remainder subjected to the Edman procedure using proportionately less reagents. The DNS-amino acids were identified by silica gel chromatography and, where possible, the corresponding PTH's were also examined.

RESULTS

Preliminary Sequence of Fibrinopeptide T-2.

A summary of the PTH-amino acids identified in the first four degradative steps of experiments 1 and 2 is given in Tables XI and XII respectively. No amino acid was identifiable in either case after the fourth step, and some doubt remained about the amino acid in the third position.

TABLE XI

Sequence study of Trachydosaurus peptide.

Edman Step	O. D. PTH solution		Rf			PTH
	269 mμ	320 mμ	D	E	F	
1	0.66	0.060	0	0.47	0.29	Glu.
2	0.69	0.035	0	0.45	0.30	Glu.
				0.22	0.16	Asp.
3	0.62	0.040	0	-	-	?
4	0.215	0.020	0	0.62	0.64	Gly. (weak)
			0.1	0.20	0.18	Asp.
5	0.30	0.14	0	-	-	Multiple spots

Partial Edman degradation of Trachydosaurus peptide T-2 (Experiment 1). Yields of PTH's after each step were followed by determining the optical density of the solution at 269 mμ and 320 mμ. The R_F values in three solvent systems, and the identification of PTH spots are listed.

TABLE XII

Sequence study of Trachydosaurus peptide.

Edman Step	O. D. PTH solution		Rf			PTH
	269 mμ	320 mμ	D	E	F	
1	0.60	0.090	0	0.43	0.30	Glu.
2	0.55	0.045	0	0.45	0.28	Glu. (weak)
				0.24	0.15	Asp.
3	0.41	0.158	0	0.24	0.18	Asp.
			0.08	0.45	0.37	Thr.
				0.22	0.14	?
4	0.42	0.0	0	0.47	0.33	Thr. (weak)
			0.09	0.62	0.60	Gly. (weak)
5	0.30	0.0	-	-	-	No PTH spot

Edman degradation of Trachydosaurus peptide T-2 (Experiment 2). Details as for Table XI.

The proposed amino acid sequence for the first four residues determined in this laboratory is

glu - asp - thr - gly -

Electrophoretic Analysis of Chymotryptic Digest of Fibrinopeptide C-1.

The pattern revealed by ninhydrin staining, with each of the fragments numbered, is shown in Figure 16. Undigested fibrinopeptide was detectable as a weakly Sakaguchi-positive band slightly in advance of the main fragment Ch-3, with which it overlapped. It appeared that digestion was very nearly complete.

Fragments Ch-3 and Ch-5 were Sakaguchi-positive.

N-terminals of Chymotryptic Fragments.

The results are given in Table XIII.

Stepwise Degradation of Fibrinopeptide C-1 Fragments Ch-3 and Ch-4.

The results of several degradative steps of fragments Ch-3 and Ch-4 are summarized in Tables XIV and XV. The following partial amino acid sequences are proposed:

Ch-3: asp - tyr - asp -

or

asp - tyr - asp NH_2 -

Ch-4: glu - val -

Figure 16. Paper electrophoretic analysis of chymotryptic digest of Cairina fibrinopeptide C-1. 0.1M pyridine-acetate buffer pH 4.0, 5.5 volts/cm for nine hours.

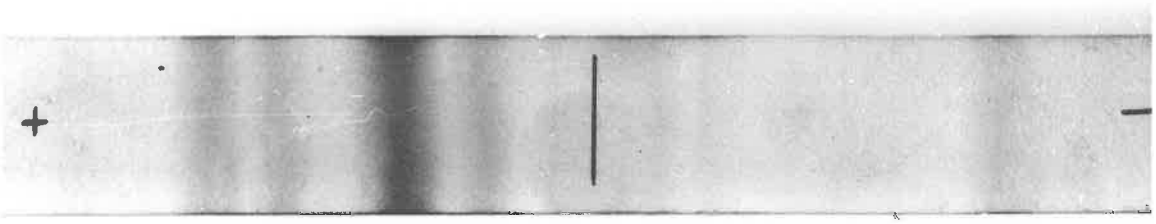


FIGURE 16

TABLE XIII

N-terminals of chymotryptic fragments of Cairina fibrinopeptide C-1, identified as the 'Dansyl' derivatives.

Chymotryptic Fragment	DNS-amino acids identified	
	N-terminal	Internal
Ch-1	None	--
Ch-2	None	O-DNS-Tyr
Ch-3	Asp	O-DNS-Tyr
Ch-4	Glu	O-DNS-Tyr (trace)
Ch-5	--	--

TABLE XIV

Edman Degradation of C-1 Fragment Ch-3

Edman Step	DNS-amino acids identified	PTH-amino acids identified
1	asp; O-DNS-tyr	asp
2	tyr	-
3	asp	-
4		

After each step of phenylisothiocyanate degradation a small sample of the residual peptide was coupled with Dansyl chloride for identification of its N-terminal residue.

TABLE XV

Dansyl-Edman Degradation of C-1 Fragment Ch-4

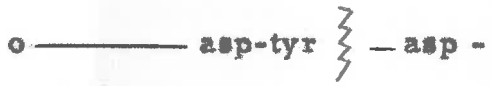
Edman Step	DNS-amino acids identified	PTH-amino acids identified
1	glu	streaking
2	val	val
3	0	-
4	0	-

Details as in Table XIV.

DISCUSSION

Sequential analysis of the open chain Trachydosaurus fibrinopeptide T-2 yielded the order of the first four residues only. It was evident that either coupling or cleavage was incomplete as PTH-amino acids from preceding steps were appearing on the chromatograms. No explanation for the total breakdown of the reaction after the fourth step can be forwarded.

The Cairina peptide C-1 was insusceptible to tryptic digestion, and it was observed that, even after prolonged incubation, only a slight degree of hydrolysis with chymotrypsin took place, despite the existence of a tyrosine residue. From the supporting evidence that the hydroxyl group was sulphated, mild acid hydrolysis was carried out. As judged by electrophoresis, this left the peptide chain intact but at the same time rendered it susceptible to chymotrypsin. It was anticipated that primary cleavage into two main fragments would occur at the tyrosyl position. However, the partial sequence of the main ninhydrin positive fragment, Ch-3, (asp-tyr-asp-) indicates that this did not happen.



expected primary cleavage

actual cleavage

In the native peptide, the tyrosine residue is sulphated. The sequence of residues around the tyrosine is entirely analagous to that found in mammalian fibrinopeptides-B. The glu-val-segment of Ch-4 is also found in these fibrinopeptides (Appendix II) and it may prove that the main avian fibrinopeptide will be quite similar in primary structure to its mammalian counterparts. Further amino acid composition and sequence studies of each chymotryptic fragment, as well as more extensive digestion with hydrolytic enzymes such as subtilisin will be necessary before the entire structure can be elucidated.

APPENDIX I

Additional References relating to Fibrinopeptides, not cited in text.

Bettelheim, F. R. (1956). Biochim. Biophys. Acta 19, 121.

Lorand, L. and Middlebrook, W. R. (1952). Biochem. J. 52, 196.

Osbahr, A. J., Gladner, J. A. and Laki, K. (1964). Biochim. Biophys. Acta 86, 535.

Sjöquist, J. (1959). Acta Chem. Scand. 13, 1727.

Sjöquist, J., Blombäck, B. and Wallen, P. (1960). Arkiv Kemi 16, 425.

APPENDIX II

AMINO ACID COMPOSITION OF SOME MAMMALIAN FIBRINOPEPTIDES A

	Human	Ox	Sheep/ Goat	Reindeer	Pig	Rabbit
alanine	2	0	2	3	2	1
arginine	1	1	1	1	1	1
aspartic acid (and asparagine)	2	3	3	2	1	2
glutamic acid (and glutamine)	2	2	2	2	4	2
glycine	5	5	5	6	4	3
leucine	1	1	1	1	1	1
lysine	-	-	-	-	1	-
phenylalanine	1	1	1	1	1	1
proline	-	2	1	1	-	1
serine	1	2	1	1	-	1
threonine	-	1	-	-	-	2
valine	1	1	2	1	2	1
	16	19	19	19	17	16

Data compiled from various sources.

APPENDIX II (cont.)

AMINO ACID COMPOSITIONS OF SOME MAMMALIAN FIBRINOPEPTIDES B

	Human	Ox	Sheep/ Goat	Reindeer	Pig	Rabbit
alanine	1	1	2	3	2	2
arginine	1	2	2	2	2	1
aspartic acid (asparagine)	3	4	6	4	5	5
glutamic acid (glutamine)	3	3	1	2	2	1
glycine	2	3	1	-	1	-
histidine	-	-	-	3	1	-
isoleucine	-	-	-	-	1	-
leucine	-	1	3	3	-	1
lysine	-	1	1	1	1	-
phenylalanine	2	1	-	-	-	-
proline	-	2	1	-	1	1
serine	1	-	-	-	-	-
threonine		1	-	-	-	-
tyrosine	-	1	2	1	1	1
valine	1	1	1	1	2	1
	14	21	20	20	19	13

Data compiled from various sources.

APPENDIX II

FIBRINOPEPTIDES A.

	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Man													H-	Ala-	Asp-	Ser-	Gly-	Glu-	Gly-	Asp-	Phe-	Leu-	Ala-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH
Ox	H-	Glu-	Asp-	Gly-	Ser-	Asp-	Pro-	Pro-	Ser-	Gly-	Asp-	Phe-	Leu-	Thr-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH									
Sheep	H-	Ala-	Asp-	Asp-	Ser-	Asp-	Pro-	Val-	Gly-	Gly-	Glu-	Phe-	Leu-	Ala-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH									
Goat	H-	Ala-	Asp-	Asp-	Ser-	Asp-	Pro-	Val-	Gly-	Gly-	Glu-	Phe-	Leu-	Ala-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH									
Reindeer	H-	Ala-	Asp-	Gly-	Ser-	Asp-	Pro-	Ala-	Gly-	Gly-	Glu-	Phe-	Leu-	Ala-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH									
Pig								H-	Ala-	Glu-	Val-	Gln-	Asp-	Lys-	Gly-	Glu-	Phe-	Leu-	Ala-	Glu-	Gly-	Gly-	Gly-	Val-	Arg-OH				
Rabbit													H-	Val-	Asp-	Pro-	Gly-	Glu-	Thr-	Ser-	Phe-	Leu-	Thr-	Glu-	Gly-	Gly-	Asp-	Ala-	Arg-OH	

After Doolittle and Blombäck, 1964.

APPENDIX II

FIBRINOPEPTIDES B.

	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1
Man													Pyr-	Gly-	Val-	Asn-	Asp-	Asn-	Glu-	Glu-	Gly-	Phe-	Phe-	Ser-	Ala-	Arg-OH
Ox	Pyr-	Phe-	Pro-	Thr-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Gly-	Gln-	Asp-	Asp-	Arg-	Pro-	Lys-	Val-	Gly-	Leu-	Gly-	Ala-	Arg-OH
Sheep						Gly-	Tyr-	Leu-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Val-	Asp-	Asp-	Asn-	Arg-	Ala-	Lys-	Leu-	Pro-	Leu-	Asp-	Ala-	Arg-OH
Goat						Gly-	Tyr-	Leu-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Val-	Asp-	Asp-	Asp-	Arg-	Ala-	Lys-	Leu-	Pro-	Leu-	Asp-	Ala-	Arg-OH
Reindeer						Pyr-	Leu-	Ala-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Val-	Glu-	His-	Asp-	Arg-	Ala-	Lys-	Leu-	His-	Leu-	Asp-	Ala-	Arg-OH
Pig						Ala-	Leu-	Asp-	Tyr(OSO_3H)-	Asp-	Glu-	Asp-	Glu-	Asp-	Gly-	Arg-	Pro-	Lys-	Val-	His-	Val-	Asp-	Ala-	Arg-OH	
Rabbit																Ala-	Asp-	Asn-	Tyr(OSO_3H)-	Asp-	Glu-	Pro-	Leu-	Asp-	Val-	Asp-	Ala-	Arg-OH

After Doolittle and Blombäck, 1964.

REFERENCES

Acher, R., Laurila, U. R. and Fromageot, C. (1956). Biochim. Biophys. Acta 19, 97.

Astrup, T. and Darling, S. (1942). Acta Physiol. Scand. 4, 45.

Bailey, K. and Bettelheim, F. R. (1955). Biochim. Biophys. Acta 18, 495.

Bailey, K., Bettelheim, F. R., Lorand, L. and Middlebrook, W. R. (1951). Nature 167, 233.

Baker, L. E. (1951). J. Biol. Chem. 193, 809.

Bettelheim, F. R. (1954). J. Am. Chem. Soc. 76, 2838.

Bettelheim, F. R. and Bailey, K. (1952). Biochim. Biophys. Acta 9, 578.

Biggs, R. and MacFarlane, R. G. (1962). "Human Blood Coagulation and its Disorders", Blackwell Scientific Publications, Oxford.

Blombäck, B. (1958 a). Arkiv Kemi 12, 99.

Blombäck, B. (1958 b). Acta Physiol. Scand. 43, Supp. 148.

Blombäck, B. (1958 c). Arkiv Kemi 12, 321.

Blombäck, B. and Blombäck, M. (1956). Arkiv Kemi 10, 415.

Blombäck, B., Blombäck, M., Doolittle, R. F. Hessel, B. and Edman, P. (1963). Biochim. Biophys. Acta 78, 563.

Blombäck, B., Blombäck, M. and Edman, P. (1963). Acta Chem. Scand. 17, 1184.

Blombäck, B., Blombäck, M., Edman, P. and Hessel, B. (1962). Nature 193, 883.

Blombäck, B., Blombäck, M., Edman, P. and Hessel, B. (1966). Biochim. Biophys. Acta 115, 371.

Blombäck, B., Blombäck, M. and Searle, J. (1963). Biochim. Biophys. Acta 74, 148.

Blombäck, B., Boström, H. and Vestermark, A. (1960). Biochim. Biophys. Acta 38, 502.

Blombäck, B. and Doolittle, R. F. (1963 a). Acta Chem. Scand. 17, 1816.

Blombäck, B. and Doolittle, R. F. (1963 b). Acta Chem. Scand. 17, 1819.

Blombäck, B. and Sjöquist, J. (1960). Acta Chem. Scand. 14, 493.

Blombäck, B., Sjöquist, J. and Wallen, P. (1959). Acta Chem. Scand. 13, 819.

Blombäck, B. and Vestermark, A. (1958). Arkiv Kemi 12, 173.

Blombäck, B. and Yamashina, A. (1958). Arkiv Kemi 12, 299.

Boulton, A. A. and Bush, I. E. (1964). Biochem. J. 92, 11P.

Bradbury, J. H. (1958 a). Biochem. J. 68, 475.

Bradbury, J. H. (1958 b). Ibid. 482.

Bremer, W. W., Staub, A., Sinn, L. G. and Behrens, G. K. (1957). J. Am. Chem. Soc. 79, 2801.

Buettner-Janusch, J. and Hill, R. L. (1965). Science 147, 836.

Caspary, E. A. and Kekwick, R. A. (1954). Biochem. J. 56, xxxv.

Cecil, R. and Wake, R. G. (1962). Biochem. J. 82, 401.

Chargaff, E. and Bendich, A. (1943). J. Biol. Chem. 149, 93.

Clegg, J. B. and Bailey, K. (1962). Biochim. Biophys. Acta 63, 525.

Cohn, E. J. Streng, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M. and Taylor, H. L. (1946). J. Am. Chem. Soc. 68, 459.

Doolittle, R. F. and Blombäck, B. (1964). Nature 202, 147.

Doolittle, R. F., Lorand, L. and Jacobsen, A. (1963). Biochim. Biophys. Acta 69, 161.

Eagle, H. (1935). J. Gen. Physiol. 18, 531.

Eck, R. V. (1963). Science 140, 477.

Edman, P. (1950 a). Acta Chem. Scand. 4, 277.

Edman, P. (1950 b). Ibid. 283.

Edman, P. (1957). Proc. Roy. Aust. Chem. Inst. 24, 434.

Edman, P. (1960). Ann. N. Y. Acad. Sci. 88, 602.

Edman, P. and Lauber, K. (1956). Acta Chem. Scand. 10, 466.

Edman, P. and Sjöquist, J. (1956). Acta Chem. Scand. 10, 1507.

Eriksson, S. and Sjöquist, J. (1960). Biochim. Biophys. Acta 45, 290.

Fantl, P. (1961). Aust. J. Exp. Biol. and Med. Sci. 39, 403.

Ferry, J. D. and Morrison, P. R. (1947). J. Am. Chem. Soc. 69, 388.

Florkin, M. (1930). J. Biol. Chem. 87, 629.

Folk, J. E. and Gladner, J. A. (1960). Biochim. Biophys. Acta 44, 383.

Folk, J. E., Gladner, J. A. and Laki, K. (1959). J. Biol. Chem. 234, 67.

Folk, J. E., Gladner, J. A. and Levin, Y. (1959). J. Biol. Chem. 234, 2317.

Fraenkel-Conrat, H. (1954). J. Am. Chem. Soc. 76, 3606.

Fraenkel-Conrat, H., Harris, I. J. and Levy, A. L. (1955) in "Methods of Biochem. Analysis", Interscience, N. Y.

Gladner, J. A., Folk, J. E., Laki, K. and Carroll, W. R. (1959). J. Biol. Chem. 234, 62.

Gray, W. R. and Hartley, B. S. (1963). Biochem. J. 89, 59P.

Hackett, E. and Hann, C. S. (1967). J. Comp. Path. In the Press.

Hackett, E. and Le Page, R. (1961 a). Aust. J. Exp. Biol. 39, 57.

Hackett, E. and Le Page, R. (1961 b). Ibid. 67.

Hall, C. A. and Slayer, H. S. (1959). J. Biophys. Biochem. Cytol. 5, 11.

Hammarsten, O. (1875). Uppsala Lakareforen XI, 538. Quoted by Blombäck and Blombäck, 1956.

Hann, C. S. (1966). Biochim. Biophys. Acta 124, 398.

Haschmeyer, R. H. and Nadeau, R. E. (1963). Biochem. Biophys. Res. Commun. 11, 217.

Henschen, A. (1963). Arkiv Kemi 22, 1.

Henschen, A. (1964 a). Arkiv Kemi 22, 355.

Henschen, A. (1964 b). Arkiv Kemi 22, 375.

Herriott, R. M. (1941). Proc. Soc. Exp. Biol. Med. 46, 642.

Hill, R. L. and Buettner-Janusch, J. (1964). Fed. Proc. 23, 1236.

Hill, R. L. and Smith, E. L. (1957). J. Biol. Chem. 228, 577.

Hirs, C. H. W., Moore, S. and Stein, W. H. (1952). J. Biol. Chem. 195, 669.

Hirs, C. H. W., Stein, W. H. and Moore, S. (1956). J. Biol. Chem. 221, 151.

Ilse, D. and Edman, P. (1963). Aust. J. Chem. 16, 411.

Ingram, V. M. (1953). J. Chem. Soc. 3717.

Jepson, J. B. and Smith, I. (1953). Nature 172, 1100.

Jevons, F. R. (1963). Biochem. J. 89, 621.

Kazal, L. A., Amsel, S., Miller, O. P. and Tocantins, L. M. (1963). Proc. Soc. Exp. Biol. and Med. 113, 989.

Kolthoff, I. M. and Stricks, W. (1951). J. Am. Chem. Soc. 73, 1728.

Königsberg, W. and Hill, R. J. (1962). J. Biol. Chem. 237, 2547.

Laki, K. (1951). Arch. Biochem. 32, 317.

Laki, K. (1953). Blood 8, 845.

Lamy, F. and Waugh, D. F. (1953). J. Biol. Chem. 203, 489.

Lorand, L. (1952). Biochem. J. 52, 200.

Lorand, L. and Middlebrook, W. R. (1952). Biochim. Biophys. Acta 9, 581.

Lowry, O. H., Rosebrough, N. J., Farr, A. C. and Randall, R. J. (1951). J. Biol. Chem. 193, 265.

Lyons, R. N. (1945). Aust. J. Exp. Biol. Med. Sci. 23, 131.

Marciniak, E., Cole, E. R. and Seegers, W. H. (1962). Nature 195, 1305.

Margoliash, E. (1962). J. Biol. Chem. 237,2161.

Margoliash, E., Needleman, S. B. and Stewart, J. W. (1963). Acta Chem. Scand. 17, S250.

Mellanby, J. (1909). J. Physiol. 38, 28.

Mihalyi, E. (1954). J. Biol. Chem. 209, 723.

Mommaerts, W. F. H. M. (1946 a). J. Gen. Physiol. 29, 103.

Mommaerts, W. F. H. M. (1946 b). Ibid. 113.

Moore, S. and Stein, W. H. (1951). J. Biol. Chem. 192, 663.

Morrison, P. R. (1947). J. Am. Chem. Soc. 69, 2723.

Morrison, P. R., Edsall, J. T. and Miller, S. G. (1948). J. Am. Chem. Soc. 70, 3103.

Niall, H. and Edman, P. (1962). J. Gen. Physiol. 45, 8185.

Noll, H. (1966). Science 151, 1241.

Osbahr, A. J., Gladner, J. A., Laki, K. and Irreverre, F. (1964). Nature 201, 707.

Pataki, G. (1966). "Dünnschichtchromatographie in der Aminosäure und Peptid Chemie", Walter de Gruyter and Co., Berlin.

Pechère, J.-F., Dixon, G. H., Maybury, R. H. and Neurath, H. (1958). J. Biol. Chem. 233, 1364.

Quick, A. J. (1938). J. Biol. Chem. 123, 99.

Rasmussen, P. S. (1955). Biochim. Biophys. Acta 16, 157.

Rosen, H. (1957). Arch. Biochem. Biophys. 67, 10.

Sanger, F. (1945). Biochem. J. 39, 507.

Sanger, F. and Tuppy, H. (1951). Biochem. J. 49, 481.

Scheraga, H. A. (1958). Ann. N.Y. Acad. Sci. 75, 189.

Seegers, W. H. (1955). Adv. Enzymology 16, 23.

Seegers, W. H., Casillas, G., Shepard, R. S., Thomas, W. R. and Halick, P. (1959). Canad. J. Biochem. 37, 775.

Sjöquist, J. (1953). Acta Chem. Scand. 7, 447.

Sjöquist, J. (1957). Arkiv Kemi 11, 129.

Sjöquist, J. (1960). Biochim. Biophys. Acta 41, 20.

Smith, E. L. and Margoliash, E. (1964). Fed. Proc. 23, 1243.

Smith, H. P., Warner, E. D. and Brinkhous, K. M. (1934). Am. J. Physiol. 107, 63.

Smyth, D. G., Stein, W. H. and Moore, S. (1962). J. Biol. Chem. 237, 1845.

Smyth, D. G., Stein, W. H. and Moore, S. (1963). J. Biol. Chem. 238, 227.

Soulier, J.-P., Wartelle, O. and Ménaché, D. (1959). Brit. J. Haem. 5, 121.

Steiner, R. F. and Laki, K. (1951). J. Am. Chem. Soc. 73, 882.

Thomas, W. R. and Seegers, W. H. (1962). Biochim. Biophys. Acta 42, 556.

Vogel, A. I. (1961). "Practical Organic Chemistry", Longmans.

von Korff, R. W. and Bronfenbrenner, A. (1958). J. Am. Chem. Soc. 80, 5575.

Ware, A. G., Guest, M. M. and Seegers, W. H. (1947). Arch. Biochem. 13, 231.

Wieland, T. and Gebert, U. (1963). Anal. Biochem. 6, 201.

Reprinted from
Biochimica et Biophysica Acta
Elsevier Publishing Company
Amsterdam
Printed in The Netherlands

BBA 23262

Fibrinopeptides in one lizard and four bird species

The localised proteolytic action of thrombin on the fibrinogen molecule releases peptide fragments, the fibrinopeptides, which have been isolated and characterised in a number of mammalian species[1–5]. In mammals, 2 major components appear when the purified fibrinogens are clotted with thrombin. These peptides, usually designated "A" and "B", arise by the cleavage of arginyl–glycine bonds near the N-terminal end of the parent fibrinogen molecule and carry a net negative charge at neutral pH. Regularities of amino acid sequences of the A and B peptides in different mammals are apparent.

There may be a relation between the adjacent sequences and the narrow specificity that thrombin shows towards the particular arginyl–glycine bonds hydrolysed[6]. Although the interaction between non-homologous mammalian thrombins and fibrinogens shows some species specificity with respect to rate of fibrinogen–fibrin transformation[7], eventual coagulation occurs, and at present one presumes that the same peptides are released both in homologous and heterologous systems[8]. All avian and reptilian fibrinogens that have been examined in this laboratory are coagulable with human and bovine thrombins. The peptide materials released from one reptilian and four avian fibrinogens have been investigated, with the following results. (The species reported are the Muscovy duck *Cairina moschata*, the Peking duck *Anas platyrhynchos*, the domestic goose *Anser anser*, the domestic turkey *Meleagris gallopavo* and the stumpy-tail lizard *Trachydosaurus rugosus rugosus*.)

The fibrinogen corresponding to BLOMBÄCK AND BLOMBÄCK[9] fraction I-O was isolated from citrated plasma, and the products so obtained were coagulable with thrombin to about 92 %. Further purification was achieved by the method of KAZAL *et al.*[10] by dissolving in 0.06 M citrate buffer at pH 7 and precipitating with glycine added to a final concentration of 2.0 M. The product, which contained 98–100 % clottable protein, was then exhaustively dialysed against 0.3 M saline or 0.3 M ammonium acetate. Semi-quantitative N-terminal studies of the fibrinogens of Cairina and Anser, using phenylisothyocyanate[11], demonstrated tyrosine only, but after coagulation with either bovine or human thrombins tyrosine and glycine N-terminals were found in a ratio of 1:2, respectively. No N-terminal tyrosine was lost during coagulation. The appearance of N-terminal glycine in two fibrin protein chains which had no demonstrable N-terminal residue before coagulation seemingly indicated the release of two fibrinopeptides with no free α-amino group in these species.

Fibrinopeptide isolated from the clot supernatants of all species after coagulation with bovine thrombin was freed from non-coagulable protein by gel filtration through Sephadex G-25 and subjected to paper electrophoresis in 0.05 M phosphate buffer at pH 7.0. Application of the Sakuguchi reaction for arginine, or chlorination of the strip followed by spraying with starch-iodide revealed two peptides in the lizard, but multiple bands appeared in all four bird species (Fig. 1).

Both lizard peptides were obtained in approximately equal yield (6–7 mg/g fibrinogen) by chromatography on Dowex 50-X2 (ammonium form) using an ammonium formate–ammonium acetate buffer gradient. The more acidic peptide (denoted 1 in Fig. 1) had no N-terminal amino acid reacting with phenylisothiocyanate, while the second peptide proved to be a single chain with N-terminal glutamic acid

(B. BLOMBÄCK AND M. BLOMBÄCK, personal communication and C. S. HANN, unpublished results). Of the four bird species, only the most acidic peptide was obtained in substantial quantities (5 mg/g fibrinogen), the yields of the "secondary" peptides being very much lower. The single main peptide of each avian species had a blocked N-terminal. (In the specific instances of Cairina and Anser this was partly in agreement with the original N-terminal analyses of the fibrinogen and fibrin, which indicated that two such "blocked" peptides would be found.)

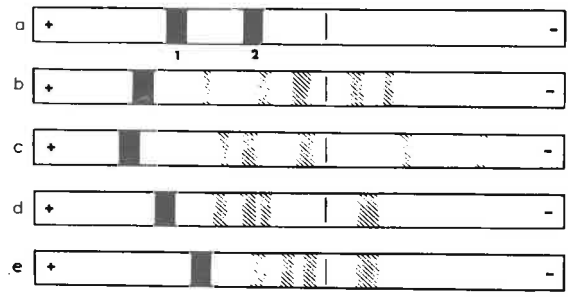


Fig. 1. Electrophoretic analyses of fibrinopeptides. a, Trachydosaurus; b, Cairina; c, Anas; d, Anser; e, Meleagris. 4 V/cm for 6.5 h at pH 7.0. For further details see text.

The absence of a second main peptide, and the range of secondary products appearing in each of the bird species, suggested that this might be due to the heterologous coagulation system used, *i.e.* mammalian thrombin on avian fibrinogen. Accordingly, Cairina thrombin was prepared by $BaSO_4$ adsorption, tissue thromboplastin activation and purified by acetone precipitation or chromatography on Amberlite CG-50. Coagulation of Cairina fibrinogen with the homologous thrombin yielded peptides, the electrophoretic pattern of which was identical with that obtained using bovine or human thrombin. Although the fibrinogens of all species (including lizard) were prepared from pooled samples of plasma, polymorphism of the fibrinogen chains of the birds seems unlikely, for peptides isolated from five individual Cairina drakes showed the same multibanded patterns.

Thrombin coagulation of the fibrinogens of these four avian species may be involving the cleavage of one peptide chain intact, with more extensive proteolysis of a second fibrinogen chain being responsible for the secondary peptides produced.

Institute of Medical and Veterinary Science, Adelaide (Australia) C. S. HANN

1 L. LORAND AND W. R. MIDDLEBROOK, *Biochem. J.*, 52 (1952) 196.
2 F. R. BETTELHEIM AND K. BAILEY, *Biochim. Biophys. Acta*, 9 (1952) 578.
3 J. A. GLADNER, J. E. FOLK, K. LAKI AND W. R. CARROLL, *J. Biol. Chem.*, 234 (1959) 62.
4 J. E. FOLK, J. A. GLADNER AND K. LAKI, *J. Biol. Chem.*, 234 (1959) 67.
5 B. BLOMBÄCK AND J. SJÖQUIST, *Acta Chem. Scand.*, 14 (1960) 493.
6 R. F. DOOLITTLE AND B. BLOMBÄCK, *Nature*, 202 (1964) 147.
7 A. J. QUICK, *J. Biol. Chem.*, 123 (1938) 99.
8 A. J. OSBAHR, J. A. GLADNER, K. LAKI AND F. IRREVERRE, *Nature*, 201 (1964) 707.
9 B. BLOMBÄCK AND M. BLOMBÄCK, *Arkiv Kemi*, 10 (1956) 415.
10 L. A. KAZAL, S. AMSEL, O. P. MILLER AND L. M. TOCANTINS, *Proc. Soc. Exptl. Biol. Med.*, 113 (1963) 989.
11 B. BLOMBÄCK AND I. YAMASHINA, *Arkiv Kemi*, 12 (1958) 299.

Received April 25th, 1966

Hackett, E., & Hann, C. (1964). Erythrocytes and the liquefying of clotted amphibian blood in vitro. *Nature, 204*(4958), 590-591.

SLOW CLOTTING OF REPTILE BLOODS

By

E. Hackett and C. Hann

Institute of Medical and Veterinary Science, Adelaide, South Australia

INTRODUCTION

It has been reported by Finlayson, Symons and T-W-Fiennes (1962) that atheromatous changes are rare in the arteries of reptiles and this view was confirmed on a larger number of species by Ardlie and Schwartz (1965).

Because of the hypothesis that atheroma is related to thrombosis (Duguid, 1946), we have investigated a tendency of the bloods of reptiles to clot slowly compared with that of mammals. We have worked mainly with *Trachydosaurus rugosus rugosus*, known as the Australian "shingle back" lizard and also called "stumpy-tail" or "sleepy".

MATERIALS AND METHODS

Reptiles were taken in Australia by us, or by paid collectors. They were anaesthetised by an intrathecal injection of pentobaritone, given through the palatine bones in the roof of the opened mouth. The skin and, where necessary, the pectoral girdle were cut cleanly with sharp instruments, avoiding excess damage to soft tissue and blood was drawn from an arterial trunk into a paraffined syringe containing enough sodium oxolate (0·1M) or tri-sodium citrate (0·12M) solution to make a nine to one mixture of blood with anticoagulant.

Trachydosaurus serum was prepared by stirring glass powder with natural plasma to produce excess thromboplastin generation and complete clotting. Chloroform-treated plasma or serum was made by stirring equal volumes together for 5 minutes at 2°C., lightly centrifuging for 60 seconds, and separating with a pipette.

Antithrombin experiments were performed either with human thrombin (special product) from the Commonwealth Serum Laboratories, Melbourne, or with Parke-Davis thrombin topical (bovine origin). Human fibrinogen (Cohn-Blomback fraction I-O, coagulable to 86 per cent.) was prepared as an 0·1 per cent. solution in Owren's buffer, pH 7·2.

Antithrombin measurements of *Trachydosaurus* serum were carried out by the method of Seegers (Seegers, Miller, Andrew and Murphy, 1952; Seegers, 1962), using the thrombins indicated above. One unit of thrombin was taken to be that which could coagulate 1 ml. of the fibrinogen solution in 15 seconds at 37°C.

All other general methods were as previously described for an investigation of blood clotting in a toad (Hackett and Le Page, 1961a, b).

RESULTS

Trachydosaurus *Clotting Times*

The whole blood of *Trachydosaurus* and that of most other species of reptile which we have examined is exceptionally slow to clot in vitro, when compared with many mammals or amphibia. The same was true of the recalcified clotting time of *Trachydosaurus* cell-free and reconstituted plasma (Table 1).

"Viscous metamorphosis" of *Trachydosaurus* spindle cells (thrombocytes) on glass could be seen. *Trachydosaurus* prothrombin, which converted quantitatively

to thrombin, was adsorbable from plasma on aluminium hydroxide or barium sulphate and recoverable. Dilution of the lizard's plasma did not accelerate the coagulation times, whereas the addition of excess tissue thromboplastin produced rapid clotting. These findings suggest that intrinsic thromboplastin generation in *Trachydosaurus* was poor, which confirms the findings of Fantl (1961). The weakness in intrinsic thromboplastin activity was not corrected by the addition of small amounts of human or toad plasma in the way that Hageman and PTA "deficiencies" in the bloods of some avian species can be corrected by the addition of normal human plasma (Didisheim, Hattori and Lewis, 1959; Soulier, Wartelle and Ménaché, 1959).

Treatment of the plasma with chloroform accelerated recalcified coagulation times. It appeared that chloroform either activated an accelerator, or removed or destroyed an inhibitor. No anticoagulant was recoverable from the chloroform. Small quantities (20 per cent.) of chloroform-treated plasma added to ordinary citrated lizard plasma did not accelerate its clotting time, which suggests that no

TABLE I

TRACHYDOSAURUS CLOTTING TIMES

Average of twenty observations, with the range shown in brackets

	Clotting time (min.)
Natural whole blood	> 60
Recalcified whole blood (diluted 1:3)	> 27
Natural plasma (leucocyte-rich)	19 (16–21)
Recalcified plasma (leucocyte-rich)	23 (20–27)
Cell-free plasma (with replaced leucocytes)	22 (19–27)
Natural cell-free plasma	> 120

convential accelerator, the activity of which would be expected to stand some dilution, was being activated. Although the effect of mixing plasmas from different species is often difficult to interpret, combinations of *Trachydosaurus* and human plasma, or toad plasma (*Bufo marinus*) gave no indication that *Trachydosaurus* plasma lacked an active accelerator which could be provided by these other species, but rather that it was relatively rich in a broadly acting inhibitor which was destroyed by chloroform. Other conventional factors in *Trachydosaurus* blood, such as prothrombin and fibrinogen, appeared to be relatively unaffected by chloroform treatment.

Since the inhibitor was not antagonised by protamine sulphate or toludine blue it was not heparin. As heparin itself was equally effective as an anticoagulant in both natural *Trachydosaurus* plasma and chloroform-treated plasma, the anticoagulant was not a heparin co-factor. It was not adsorbed by barium sulphate. Plasma so adsorbed, which contained no prothrombin, had no progressive neutralising effect on dilute tissue (lizard lung) thromboplastin incubated with it. When *Trachydosaurus* thrombocytes, removed before chloroform treatment, were returned to chloroform-treated plasma, they showed no enhanced rate of viscous metamorphosis on glass.

Trachydosaurus *Antithrombin Activity*

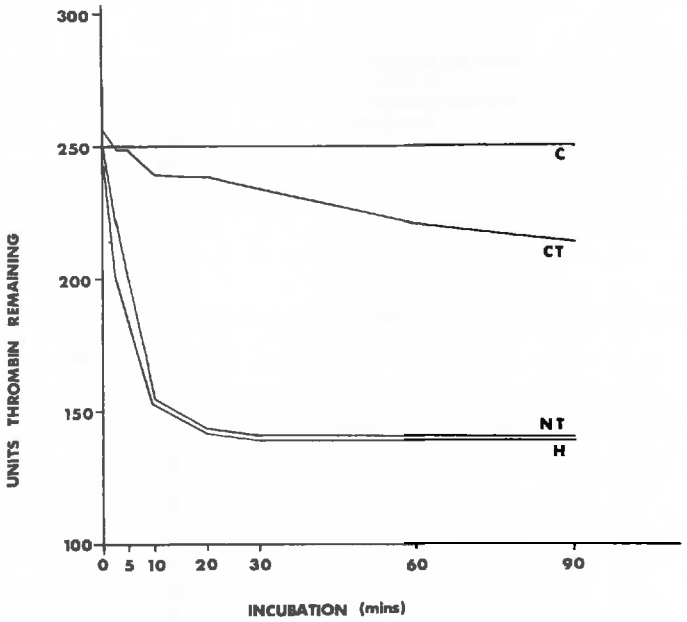
Adsorbed (prothrombin free) *Trachydosaurus* plasma antagonised bovine thrombin and *Trachydosaurus* thrombin, prepared by barium sulphate adsorption, citrate elution and tissue thromboplastin activation. Chloroform treatment of adsorbed plasma greatly reduced this effect (Table 2). A comparison using bovine thrombin was made between the antithrombin activities of *Trachydosaurus* and human serum prepared in the same way (Fig. 1). The levels of activity of both

TABLE 2

TRACHYDOSAURUS PLASMA ANTAGONIZING THROMBIN (28°C.)

Bovine thrombin units added	*Substrate clotting time (seconds)*	
	Adsorbed plasma	*Adsorbed chloroform-treated plasma*
8	42	8
6	60	13
5	63	14
4	77	15
2	130	25
1	140	35
Lizard thrombin (not standardized)	400	20

Fig. 1.



Neutralization of bovine thrombin by Trachydosaurus serum (average of seven experiments). Ordinate axis shows thrombin activity remaining in incubation mixture. Temperature 28°C.

C Control
CT Chloroform-treated Trachydosaurus serum
NT Natural Trachydosaurus serum
H Natural human serum

sera were similar. Human thrombin gave analagous results. Although the purest preparations of thrombin are desirable for quantitative antithrombin measurements (Seegers, 1962), these results obtained with the preparations available to us seemed sufficiently definite to justify a qualitative comparison. This does not take into account possible species antithrombin differences, but it seems unnecessary to suppose that a reptilian antithrombin should be more active than human antithrombin against the mammalian thrombins used. *Trachydosaurus* plasma was not defibrinated by heating to 56°C. for a few minutes and because the antithrombin activity was somewhat heat labile data could not be obtained showing a comparison between such activity in fibrin-free natural plasma and in similar plasma treated with chloroform.

TABLE 3

RECALCIFIED CLOTTING TIMES OF VARIOUS REPTILES

Common name	*Specific name*	*Plasma*		*Chloroform-treated plasma*	
		mins	*secs*	*mins*	*secs*
Long-necked Tortoise	Chelodina longicollis	6	30	2	15
Estuarine Crocodile	Crocodylus porosus	9		2	40
Brown Tree Snake	Boiga irregularis	30		12	30
		30		10	15
Scrub Python	Liasis amethistinus	30		9	
		30		8	20
		19		2	30
		18		3	30
		28		7	15
Children's Python	Liasis childreni childreni	30		11	
		25	30	8	15
		30		9	
Diamond Python	Morelia spilotes spilotes	30		10	10
Carpet Python	Morelia spilotes variegata	16		5	15
		30		6	30
		30		4	
		30		10	25
		30		5	25
		30		7	20
		30		7	5
		30		8	
		30		6	12
Olive Python	Liasis olivaceus	4	15	3	
		8	50	3	30
		5	20	3	15
		8		4	5
		5	55	3	30
Cunningham's Skink	Egernia cunninghami	18	30	8	20
		30		3	30
		30		7	
		27		17	
		30		8	20
		4	15	3	20
Blue Tongue Lizard	Tiliqua scincoides scincoides	30		8	30
		30		6	50
		30		7	5
		30		9	15
		30		7	45
		30		18	30
Bearded Dragon	Amphibolurus barbatus barbatus	21		4	35
		18	30	4	45
		30		5	15
Rock Monitor	Varanus (Odatria) acanthurus	8	30	5	50

These results suggest that the *Trachydosaurus* anticoagulant was antithrombin. We found that it was inactivated by heating to 54°C. for 10 minutes. It was not separable from plasma by dialysis and was precipitated, though with some loss of activity, at between 25 per cent. and 40 per cent. saturation with ammonium sulphate. Like the "progressive antithrombin" of human plasma, it was largely inactivated by ether, as well as by chloroform (Seegers, 1962).

An empirical survey of the bloods of other Australian reptiles recording the sensitivities of their plasmas to chloroform was made. Assuming that an accelerated clotting time after chloroform treatment indicated a predominance of antithrombin activity in their bloods, similar to that which we have found in *Trachydosaurus*, the results in Table 3 appear to confirm that this phenomenon is general in reptiles.

DISCUSSION

Fantl (1961) showed that the bloods of several Australian reptiles are poor generators of intrinsic thromboplastin. We confirm this, and note also that the blood of *Trachydosaurus rugosus rugosus*, compared to mammalian blood tested in vitro, is held in a relatively incoaguable state mainly by the activity of a circulating antithrombin. This antithrombin is, comparatively, no stronger than may be found in the blood of a mammal, but it has very free play in *Trachydosaurus* because of the accompanying poor intrinsic thromboplastin activity, which causes slow thrombin production from prothrombin, making anti-thrombin effects prominent. Our data suggests that this combination is widely present in the bloods of reptiles of different genera. It is not marked in the amphibians we have studied.

It may be that the evolutionary process has reduced thromboplastic factors in reptile bloods, allowing antithrombin activity to dominate their clotting processes. These animals have armoured skin, a low blood pressure, a slow circulation time and they are commonly lethargic in their movements. Their arterial blood moves in conditions more like those on the venous side of the mammal, which probably accounts for their freedom from atheroma. Nevertheless, intravascular clotting of mammalian-type blood would be possible, even likely, under such sluggish conditions. It is possible that a "naturally anticoagulated" blood, clotting only whenever thromboplastin activity rises slowly to become locally overwhelming, is particularly suitable for reptiles, especially as they have no delicate vascularised exposed surfaces such as gills or respiratory skin which would seem to require more prompt staunching mechanisms. One might even comment that reptilian blood is a natural example of the artificial state which physicians seek to bring about in human blood when they prescribe controlled anticoagulant treatment for venous thrombosis.

SUMMARY

An investigation of blood coagulation in *Trachydosaurus rugosus rugosus* and other Australian reptiles has confirmed that their intrinsic thromboplastin activity is poor and has also shown that, in consequence, the effect of a natural antithrombin is very prominent, much more so than in mammals. This may be related to the fact that intravascular flow conditions on both the arterial and venous sides of the reptilian circulation are sluggish.

ACKNOWLEDGMENT

This work was supported by the National Heart Foundation of Australia.

REFERENCES

Ardlie, N. G., and Schwartz, C. J. (1965). *J. Path. Bact.*, **90**, 487.
Didisheim, P. Hattori, K., and Lewis, J. (1959). *J. Lab. clin. Med.*, **53**, 866.
Duguid, J. B. (1946). *J. Path. Bact.*, **58**, 207.
Fantl. P. (1961). *Aust. J. exp. Biol.*, **39**, 403.
Finlayson, R., Symons, C., and T-W-Fiennes, R. N. (1962). *Brit. med. J., i*, 501.
Hackett, E., and Le Page, R. (1961a). *Aust. J. exp. Biol.*, **39**, 57; (1961b). *Ibid.*, 67.
Seegers, W. H., Miller, K. D., Andrews, E. B., and Murphy, R. C. (1952). *Amer. J. Physiol.*, **169**, 700.
Seegers, W. H. (1962). *Prothrombin*. University Press; Harvard.
Soulier, J. P., Wartelle, O., and Ménaché, D. (1959). *Brit. J. Haem.*, **5**, 121.

[*Received for publication, August 9th*, 1966]