STUDIES ON THE BLOOD COAGULATION MECHANISMS OF DIFFERENT SPECIES COMPARED TO MAN

Volume One

by

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Thesis submitted for the degree of Ph.D.
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February, 1964.

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SUMMARY

Blood coagulation has been investigated in lobsters, fowl, cattle, horses, and rabbits. Comparison has been made with the human blood coagulation mechanism, on the basis of current concepts of that system.

Blood coagulation in the lobsters was found to occur in one phase, namely the conversion of lobster fibrinogen from the state of sol into that of gel by the action of an active thrombin-like material, in the presence of calcium ions. This material is released directly from one particular kind of blood cell, the explosive cell, characterised by its great sensitivity to contact with foreign surfaces which causes it to disintegrate almost instantaneously. The thrombin-like material had no hydrolytic activity on arginine esters, but its property of coagulating lobster plasma or purified lobster fibrinogen was inhibited by large concentrations of heparin, which action was reversed by hexadimethrine bromide. The lobster blood did not contain any of the other coagulation factors known to occur in man. The lobster coagulation reagents were absolutely species specific and were not interchangeable with human coagulation reagents.

Fowl blood lacked completely factors IX, XI, and XII activities,

while factors V, VII, VIII, and X were present but species specific. Fowl thrombocytes possessed a weak thromboplastic activity even in homologous thromboplastin generation mixtures.

In the mammalian vertebrates, species specificity was only demonstrable in the case of factors VII and X in bovine and equine blood; all the other factors were not species specific and mostly present in high concentrations, especially bovine factors I, II, and VIII, equine factors II, V, and IX, and in the rabbit factors V and XII. The exception was factor IX which was partially deficient in both cattle and rabbit sera.

All vertebrate plasmas and the extracted fibrinogen solutions were coagulated most rapidly with the homologous thrombin solution, again revealing the species specificity of the thrombin-fibrinogen reaction.

Adsorbed plasmas from the fowl and horses had an inhibitory effect when added to human thromboplastin generation mixtures. This inhibitory property can be removed by heating the adsorbed plasma prior to its addition, to precipitate the fibrinogen which is believed to be, or associated with, the responsible agent, since both fowl and horse sera had no such inhibitory activity at all.

The presented findings reveal that during the process of evolution, several phenomena occur. Firstly, functions which were the sole property of the cells become attributed to factors freely

soluble in the extra-cellular plasma. Secondly, the coagulation mechanism becomes more complex due to the appearance of more coagulation factors; clotting then occurs after several stages of interactions, with the consequent differentiation of two thromboplastin systems, the more primitive extrinsic or tissue thromboplastin system, and the more intricate intrinsic or plasma thromboplastin system being acquired in higher animals. Thirdly, the mammals which are regarded as the most highly evolved species, have an increased concentration of the coagulation factors, thereby probably ensuring the efficiency of the coagulation mechanism.

Finally, factors which in lower animals were completely species specific gradually lose their specificity, eventually becoming fully interchangeable with human blood coagulation factors.

A C K N O W L E D G E M E N T S

The experimental work contained in this thesis was performed in the University Department of Medicine, The Royal Infirmary, Glasgow.

Grateful acknowledgement is made to Emeritus Professor L.J.Davis for giving me the opportunity of working in his department, and to Professor E.M.McGirr for allowing me to continue with this work, and for the generous facilities granted.

I am indebted to my supervisor Dr.A.S.Douglas for his ever helpful advice and constant encouragement.

To Professor W.L.Weipers, Dr.T.Douglas, Dr.A.Weaver, and Mr.J.Longstreath, members of the staff of Glasgow University Veterinary Hospital, I wish to express my thanks for their help in providing me access to horses under their care, to obtain horse blood samples.

Professor T.Symington and Dr.A.Mackay of the Pathology Department, Glasgow Royal Infirmary, kindly permitted me the use of their phase contrast microscope, and to them I express my deep appreciation.

I gratefully acknowledge the cooperation of Mr.C.H.Mortimer,

director of Millport Marine Station, and his assistants Mr.E.Latham and Mr.A.Elliott, for providing me with a constant supply of fresh live lobsters over a period of several months.

My thanks are due also to Mr.R.S.MacGregor and Mr.J.Leiper for the photographic reproduction of all the graphs, and to Mr.T.Parker for his help in photographing the colour transparencies from which the colour prints were made.

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INTRODUCTION

The object of this work is to study the blood coagulation mechanisms of different animal species as compared to that of man. A great deal has been done in the past by various workers on the same subject, when knowledge of the human blood coagulation mechanism was lacking. Except in few cases, usually each investigator has looked into one or two aspects of the coagulation mechanism, again only of a particular species of animals. Though there is fair agreement in the results of the different reported investigations, still on reviewing the voluminous literature on this subject one can discern many contradictions and wide variations among them.

Therefore, it was thought worthwhile to undertake such an investigation, covering the whole coagulation mechanism of several species, using the same methods and the same reference standard plasma or serum so as to be able to compare and contrast the results with a certain degree of accuracy and conformity.

The main interest was to see if the results would suggest the presence of any relation between the pattern of the blood coagulation mechanism of a certain class of animals and its position in the

evolutive zoological classification, or with special physiological peculiarities of the species. This is by no means a simple question to answer adequately, and I make no pretence to have done so.

Also, it was hoped that such an investigation would help in the fuller understanding of the evolution of the human coagulation mechanism, as well as throwing some light on the use of animal coagulation reagents in human coagulation studies.

Endeavour in the choice of animals was made to represent as widely varied species as feasible, subject to availability, ease of handling, and the possibility of obtaining reasonable amounts of blood from them.

I wish to point out that referring to chicken blood or plasma, rabbit blood, etc.., is only true with regard to the particular genus or strain investigated, namely chicken of the Light Sussex strain, rabbits of the Californian strain, and so on.

Since the coagulation mechanism as it occurs in the human blood forms the basis for comparison between the species, a detailed review and discussion of the processes and factors involved in it are presented in Part 1. In the subsequent parts discussion of the results of experiments made on the various species is presented. Details of the methods used and of the results obtained, as well as the illustrations are all contained in volume two.

GLOSSARY OF ABBREVIATIONS

Recourse has often been made to the use of the following abbreviations for the sake of brevity, especially in the construction of the tables and in the graphs' footnotes.

Chemicals were designated their agreed formulae and are not included in this list.

A.H.G. = Antihaemophilic globulin.

Ads.pl. = Adsorbed plasma. Unless otherwise stated, this usually means plasma adsorbed with alumina.

Alumina = Aluminium hydroxide gel.

A.R. = Analytical reagent.

B.D.H. = British Drug Houses Ltd.

Bov. = Bovine.

Br. = Brain.

B.W. & Co. = Burroughs Wellcome and Company.

Ceph. = Cephalin.

Ck. = Chicken.

Conc. = Concentration.

Def. = Deficient.

Dil. = Diluted, dilution.

Dist. = Distilled.

Expt. = Experiment.

F = Factor.

g = Acceleration of gravity.

Hse. = Horse.

Hum. = Human.

H.S.pl. = High-spun plasma, i.e. platelet-poor plasma.

I.C.I. = Imperial Chemical Industries.

Lob. = Lobster.

M = Molar solution, moles.

mμ = Milli-micron

μM = Micromoles.

μ = Ionic strength.

M.S.E. = Measuring and Scientific Equipment Limited.

N = Normal solution.

P.C.V. = Packed cell volume.

P.D. & Co. = Parke Davis and Company.

Phenind.pl. = Phenindione plasma, i.e. plasma from patients under phenindione therapy.

Pl. = Plasma.

Plat. = Platelets.

P.T.A. = Plasma thromboplastin antecedent.

P.T.C. = Plasma thromboplastin component.

Ppt. = Precipitate.

Rab. = Rabbit.

r.p.m. = revolution per minute.

R.V.V. = Russell's viper venom.

Ser. = Serum.

Sp.G. = Specific gravity.

S.W.G. = Standard wire gauge.

TAMe = p-Tosyl-L-Arginine Methyl ester.

T.C.A.a. = Trichloracetic acid.

Temp. = Temperature.

W.B.Cs. = White blood corpuscles.

Wt. = Weight.

X-mas F. = Christmas factor.

SYNONYMS OF BLOOD COAGULATION FACTORS

The Roman numerals used are in accordance with the recommendations of the International Committee on Nomenclature of Blood Clotting Factors.

Use was made of these Roman numerals whenever possible, except in few instances when the more familiar synonym was used, e.g. tissue thromboplastin, fibrinogen, prothrombin, etc..

Factor	Synonyms
I	: Fibrinogen.
II	:- Thrombogen, (Morawitz, 1905).
	- Thrombozyme,(Nolf, 1908).
	- Prothrombin.
III	:- Thrombokinase,(Morawitz, 1903).
	- Tissue thromboplastin,(Howell, 1935).
IV	: Calcium.
V	:- Labile factor,(Quick, 1943, 1951).
	- Thrombogen,(Nolf, 1945).
	- Prothrombin accelerator,(Fantl and Nance, 1946).
	- Factor V,(Owren, 1947).
	- Plasma accelerator globulin, (Ware and Seegers, 1948a and b).
	- Proaccelerin,(Owren, 1950).
VI	: Accelerin,(Owren, 1951).

Factor	Synonyms
VII	: - Inactive prothrombin,(Quick, 1943; Quick and Stefanini, 1949).
	- Stable factor,(Owren, 1947; Owen, Magath, and Bollman, 1951).
	- Co-thromboplastin, (Mann et al, 1947, 1950, 1951, and 1952; Hurn, Barker, and Mann, 1947; Hurn and Mann, 1947).
	- Prothrombin conversion factor,(Owen and Bollman, 1948).
	- Serum factor of Jacox,(Jacox, 1949; Jacox and Bays, 1949 and 1950).
	- Serum prothrombin conversion accelerator (SPCA), (Alexander et al, 1949, 1949a, 1949b, 1950, and 1951).
	- Proconvertin and Convertin,(Owren, 1947 and 1951; Owren and Bjerkelund, 1949; Owren and Aas, 1951).
	- Factor VII,(Koller, Loeliger, and Duckert, 1951).
	- Autoprothrombin I,(Seegers, Alkjaersig, and Johnson, 1955).
VIII	: - Antihaemophilic globulin (AHG),(Patek and Taylor, 1937).
	- Antihemophilic factor (AHF),(Brinkhous, 1947, and 1954; Brinkhous and Wagner, 1958).
	- Thromboplastinogen,(Quick, 1947, and 1951).
	- Facteur antihemophilique A,(Soulier et Larrieu, 1953a, and 1953b).

Factor	Synonyms
	- Plasma thromboplastic factor A.
	- Prothromboplastin.
	- Antihaemophilic globulin A,(Cramer et al, 1953).
	- Platelet co-factor I,(Seegers, 1954).
	- Factor VIII,(Koller, 1954).
IX	: - Christmas factor,(Biggs, Douglas, Macfarlane, Dacie, Pitney, Merskey, and O'Brien, 1952).
	- Plasma thromboplastin component (PTC),(Aggeler et al, 1952 a and b; White et al, 1952, and 1953).
	- Plasma thromboplastic factor B,(Cramer et al, 1953).
	- Facteur antihemophilique B,(Soulier et Larrieu, 1953a, and 1953b).
	- Platelet co-factor II,(Seegers, 1954).
	- Autoprothrombin II,(Seegers, 1954).
	- Factor IX,(Koller, 1954).
X	: - Factor X, (Koller, 1955).
	- Stuart factor,(Hougie et al, 1957).
	- Stuart-Prower factor,(Bachmann, Duckert, Geiger, Baer, and Koller, 1957).
XI	: Plasma thromboplastin antecedent (PTA),(Rosenthal et al, 1953, and 1955).
XII	: - Hageman factor,(Ratnoff, 1954; Ratnoff and Colopy, 1955).
	- Contact factor,(Margolis, 1958a).
XIII	: Fibrin stabilizing factor (FSF),(Lorand, 1950b).

PART I

HUMAN BLOOD COAGULATION MECHANISM

HUMAN BLOOD COAGULATION MECHANISM

H I S T O R I C A L

Man must have been mystified since time began by the simple act of the spontaneous change of shed blood from the fluid state to that of solid clot. Though many hypotheses may have been postulated, and possibly some experiments carried out earlier, the first comprehensive recorded account was that compiled by George Gulliver, F.R.S.(1846) in the introduction to his book "The Works of William Hewson".

Aristotle (1605) perhaps was the first to recognise the role played by fibrin in the coagulation of the blood. He realised that the removal of the fibrous matter from the blood rendered it incoagulable. However, it is not certain whether he considered this fibrous matter existed as such in the circulation, or as liquid, being formed in the blood after its extravasation.

The discovery of the circulation of blood in 1616 by Harvey, and his monograph "Motion of the Heart and Blood" published in 1628 were major factors in attracting many workers to this fascinating field of study. Harvey (1628, 1651) supposed that the living blood does not contain the parts found in it after death.

He described blood after death as a cruor of red and white portions, one dense and fibrous, the other watery and serous, with fibres connecting the whole. Willis in 1659 described the filaments of the crassamentum (solid clot) as joined together or connected into a parenchyma. He separated the "serum", identified it as the lightest part of the blood, coagulable by heat and by some acids. Malpighi (1666) washed away the colouring matter of the clot, and examined the whitish fibrous part under the microscope, which he found to be made up of a fibrous network. Borelli (1681) from microscopical observations on the fibres, was the first worker to conclude that what he described as white glutinous and spontaneously coagulable matter of the blood, is liquid in the living body. Collins (1685), and Bidloo (1685) both regarded the clot as made up of minute white tensile filaments enclosing the red particles.

The increasing practice of blood letting in the seventeenth and eighteenth centuries prompted more experiments as to the nature of the fibrin clot. It is interesting to note that Ruysch (1707) made the first attempt to separate the fibrous matter from fluid blood by agitating it with a twig.

Hewson's experiments and observations were perhaps the most precise ones performed up to that time. He separated a coagulable plasma from cooled blood by allowing the cells to settle, and demonstrated that this plasma contained a substance which could be

precipitated at temperatures above 50°C. He also ascribed the coagulation of the blood to the formation of this insoluble substance from a soluble precursor in the plasma, which he called "coagulable lymph", (Hewson, 1771).

Amidst the previous worthwhile reports, there were many others maintaining the importance of the red corpuscles as a factor in the coagulation of the blood. However, it was Davies (1760) who finally refuted this assumption. Furthermore, he declared the ability of the fibrous matter "gluten" to press out the serum from the clot, by its concreting force. Also, that the denser the structure of the "gluten" is, the more serum it presses out, attributing a tender coagulum to the weakened contraction of the glutinous parts.

Fordyce (1770) was able to prevent blood coagulation by saturating whole blood with common salt. He clearly distinguished between the coagulable lymph, the red corpuscles, and the serum. His observations that blood coagulation was not due to change of temperature, cessation of movement, or exposure to air, and that blood remains fluid for more than three hours when retained in the blood vessels, are in perfect agreement with those of Hewson (1771) who in fact said that these findings were mentioned in his lectures in 1767. Thackrah (1819) suggested that clotting of blood depends on the tone of the endothelial lining of the blood vessels.

Rudolph Virchow in 1821 coined the term "fibrinogen", stating that there was only one type of fibrin, which could not be detected in the blood stream or tissues, but only by the act of coagulation; and that most of the fibrin was fibrinogen which was formed in the lymphatic and connective tissue, (Macfarlane and Robb-Smith, 1961).

Professor Andrew Buchanan (1879), professor of physiology at Glasgow University, made very original contributions which unfortunately were not recognised till much later. In 1831 he demonstrated that clot washings can clot hydrocele fluid within few minutes (normally incoagulable), comparing this property to the enzyme action of rennin on milk. He carried out microscopical studies of fibrin, red cells, and of the colourless corpuscles, the platelets. Several experiments led him to conclude that the platelets were the clotting agent. His most important discovery was that emulsions of certain body tissues (skin and muscles) promoted clotting when added to cooled blood.

Brücke (1857) produced experimental evidence that blood vessels exert an active influence on the blood to maintain its fluid state. He transferred part of the turtle's fresh blood into the heart of a recently killed turtle, and part into an open dish, and observed that the former took much longer time to clot. He supposed that intact endothelial cells must possess the power of preventing clotting.

Richardson in 1856 put forward the"Ammonia Theory of Blood Coagulation", propounding that the fluidity of the circulating blood is due to small amounts of free ammonia in the blood which kept the fibrin in solution, and that in shed blood the ammonia is given off, thereby diminishing the alkalinity of the blood, causing the solidification of the fibrin. This theory was discredited by Joseph Lister (1863) who showed conclusively that blood in an open vessel kept in an ammoniacal atmosphere clotted as quickly as blood exposed to air. Lister was also the first to draw attention to the influence of foreign surfaces in hastening blood clotting.

Hammarsten (1879) in 1875 succeeded in isolating a fairly pure fibrinogen preparation from horse plasma by repeated precipitations with 25 per cent saturation with sodium chloride. This fibrinogen preparation was used by Schmidt in further experiments.

Schmidt (1892) confirmed Buchanan's previous findings and observed that the active coagulant, the fibrin ferment, later named thrombin by him, could be extracted by alcohol precipitation from serum, but not from freshly shed unclotted blood. He thus inferred that an active coagulant substance was produced in the blood during the process of clotting. In 1895 he postulated the main features of the classical theory of blood coagulation as occuring in two phases. The first is the union between fibrinogen and a paraglobulin,

a hypothetical substance derived from the colourless corpuscles. The second phase being the action of the fibrin ferment "thrombin" on the product of the first union, producing fibrin. He believed thrombin to be derived also from the colourless corpuscles, and that it was present in all types of living cells; maintaining that clotting would not occur so long as the colourless corpuscles circulate uninjured. A schematic presentation of his theory could be expressed as follows:-

$$\text{Fibrinogen} + \text{Paraglobulin} \xrightarrow[(\text{Thrombin})]{\text{Fibrin ferment}} \text{Fibrin}$$

He also assumed the necessity of calcium for the thrombin-fibrinogen reaction, which was demonstrated experimentally by Arthus and Pagés (1890) who have shown that calcium precipitants prevented clotting, their effect being reversed by the addition of calcium. These workers, however, were more correct in inferring the necessity of calcium for the formation of thrombin from its precursor. Later experiments confirmed this, but only when thrombin formation occurs in a normal physiological system, i.e. plasma. In 1899, Hammarsten proved that calcium is not necessary for the thrombin-fibrinogen reaction.

Conradi (1901) showed that from all types of organs it was possible to extract substances which enhanced blood clotting, and

also those which opposed it. The latter he found to be heat stable, and can be precipitated by alcohol. Now it is known that heparin can be extracted from different tissues.

Morawitz (1905) using Buchanan's washed tissue extracts, found that they had no effect on a solution of fibrinogen, though Schmidt's thrombin from serum produced immediate clotting. Thus it seemed improbable that thrombin originated in the tissues. He therefore suspected the presence of an inactive precursor of thrombin in the plasma, and that this precursor was activated by tissue extracts or thrombokinase. His hypothesis could be represented as follows:-

$$\text{Pro-thrombin} \xrightarrow[\text{Calcium}]{\text{Tissue extract}} \text{Thrombin}$$

$$\text{Fibrinogen} \xrightarrow{\text{Thrombin}} \text{Fibrin}$$

Morawitz believed that the thrombokinase in the tissue extract was the same substance as was liberated from the platelets. He also further elaborated Lister's foreign surface theory, maintaining that shed platelets coming into contact with a water-wettable surface disintegrate releasing thrombokinase, and reported that the endothelial lining of the blood vessels is non-water-wettable. He was also aware that calcium is essential in the initial phase of clotting.

Morawitz's theory deservedly stood unchallenged for nearly forty years, till the application of Quick's one-stage prothrombin time test posed some questions not adequately explained by the classical theory, which though still holds in its main features, has been expanded to encompass the newly recognized factors.

In view of the growing complexity of the blood coagulation mechanism, and the huge amount of information published on the subject thereafter, it is proposed to discuss the modern theory of the human blood coagulation mechanism in the next chapters in terms of the different factors concerned, alluding to interesting historical background when appropriate.

MODERN THEORY OF HUMAN BLOOD COAGULATION MECHANISM

The mechanism of human blood coagulation is a very complex chain of reactions, which is as yet very controversial and not universally agreed upon. However, there is fair agreement on the more salient features, and it is these that are now to be discussed.

The appearance of fibrin in any clotting mixture is usually the last stage in the coagulation mechanism, but being the only phenomenon that can be observed visually with a certain degree of accuracy, it is perhaps more instructive to describe first the factors and variables affecting the thrombin-fibrinogen reaction.

F I B R I N O G E N (Factor I)

Fibrinogen, a soluble protein present in normal blood, is responsible for the change of blood on coagulation from the sol state to that of a gel, due to the action of thrombin on it, transforming it into fibrin, an insoluble protein precipitated as a network of fibres.

Properties of fibrinogen

It is a globulin, with a molecular weight of about 340,000, present in Cohn's fraction I-2. Its isoelectric point is at

pH 5.5 (Seegers, Nieft, and Vandenbelt, 1945). It is precipitated from plasma by 25 per cent saturation with ammonium sulphate, or 50 per cent saturation with sodium chloride (Biggs and Macfarlane, 1957), by 10 per cent (v/v) ethanol at pH 7 (Bailey and Bettelheim, 1955), and by 11 per cent (v/v) ether (Kekwick, Mackay, Nance, and Record, 1955). Fibrinogen is destroyed by heating to 47°C., and precipitates irreversibly at 56°C. The terminal amino acid groups of bovine fibrinogen were shown by Lorand and Middlebrook (1952a) to be two tyrosine groups and one glutamic acid molecule; while human fibrinogen contained one N-alanine molecule instead of the glutamic acid, (Lorand and Middlebrook, 1953).

Role of fibrinogen

Fibrinogen is the natural substrate of thrombin which converts it into fibrin. Laki and Mommaerts (1945) suggested that fibrinogen is transformed into fibrin by a two-step reaction. First, the fibrinogen is transformed under the influence of thrombin to pro-fibrin, which then polymerizes to the fibrin proper. At acid reaction (pH 5.1) only the first step occurs, but the substance formed produces a clot when the mixture is neutralized (Laki and Mihályi, 1949). The act of polymerization is inhibited by the addition of neutral salts, (Mommaerts, 1946).

Lorand (1952, 1954) demonstrated the release of a soluble fibrino-peptide and the appearance of a fibrin monomer during the

clotting of fibrinogen by thrombin. He showed that at optimum pH and ionic strength, polymerization of the monomer occurs independent of the presence of thrombin. He suggested that the linkage of the fibrin molecules forming the solid clot may be due to the effect of electrostatic forces following the removal of the highly charged peptides. Laki and Folk (1958) suggested that the release of peptide A allows end-to-end association (intermediate polymer), while release of peptide B allows side-to-side association.

Van Zandt et al (1947) and Porter et al (1949) studied the formation of fibrin from fibrinogen and thrombin with the aid of the electron microscope. They noted end-to-end linkages between particles of about the same size as the fibrinogen molecules. Later, these aggregates form bundles which have regular cross--striations of different densities. Hall and Slayter (1959) explained this appearance as due to the alignment of the fibrin molecules, the darker bands corresponding to the terminal nodules lying end to end, and the intermediate bands corresponding to the central nodules lying side by side. The sequence of events could thus be represented as follows:-

Fibrinogen $\xrightarrow{\text{Thrombin}}$ Fibrin monomer + Peptides

Fibrin monomers $\xrightarrow{\text{Optimum pH}}$ Fibrin polymer

Fibrin polymers $\longrightarrow$ Fibrin clot (urea soluble).

Preparation of fibrinogen

Many methods for the preparation of fibrinogen were devised by various authors, using different precipitating procedures. Astrup and Darling (1942c) were the first to remove the prothrombin from the plasma by adsorbing agents (tricalcium phosphate) before precipitating the fibrinogen, thereby obtaining a more pure preparation. Jaques' method (1943) of fibrinogen preparation by phosphate precipitation was modified by Biggs and Macfarlane (1957) who adsorbed the citrated bank plasma with alumina before fibrinogen precipitation. This is perhaps the most practical method, and it yields a fairly pure fibrinogen preparation, suitable for use as substrate in the various assay systems of blood coagulation factors. It was this method that was used in this work for the preparation of fibrinogen, and is described in detail in appendix III, 6, A.

It should be noted, however, that fibrinogen prepared by any of the known methods is likely to contain antihaemophilic globulin and plasminogen as impurities.

Measurement of fibrinogen

Since fibrinogen is converted quantitatively into fibrin by thrombin, estimation of the fibrin in a clot is indicative of the fibrinogen content of the original plasma which formed that clot. Two methods were used for the estimation of fibrin previously clotted with thrombin and thoroughly washed to get rid of any

contaminant proteins entrapped in the fibrin network.

1. micro-Kjeldahl method

The fibrin is digested with concentrated sulphuric acid into protein nitrogen in the form of ammonia. The ammonia is driven off from solution by excess sodium hydroxide, and collected volumetrically in standardized sulphuric acid solution. By a series of titrations the amount of protein present is calculated on the assumption that 16 per cent of the dry weight of protein is nitrogen. The result is then multiplied by the factor 6.25 to convert nitrogen into fibrinogen. This method is fully described in appendix IV, 2, I.

2. Modified Greenberg method

After the fibrinogen is clotted with thrombin, its tyrosine content is released by hydrolysis with sodium hydroxide, and a colour reaction made with Folin-Ciocalteu's phenol reagent. The depth of colour produced is measured photometrically and is indicative of the original fibrinogen content, as read from a standard tyrosine calibration curve. It is assumed that each milligram tyrosine is equivalent to 11.7 milligrams of fibrinogen. Detailed procedure is described in appendix IV, 2, II.

A third turbidimetric method for the determination of fibrinogen was occasionally used (appendix IV, 2, III). It depends on precipitating the plasma fibrinogen in a solution of

one per cent sodium chloride, and measuring the degree of turbidity spectrophotometrically. On comparison of the results of animal plasma samples (chicken), estimated simultaneously by the previous classical methods, however, a great difference was observed which may be due to the different precipitation characteristics of the fibrinogens of different species. This method was therefore abandoned in later investigations.

Normal plasma fibrinogen content

Normal human plasma contains 300-500 mg of fibrinogen per 100 mls. of plasma (Biggs and Macfarlane, 1962). The minimal fibrinogen concentration necessary for effective haemostasis is uncertain. Biggs (1955) reported that a fibrinogen concentration of 25 to 40 per cent of the normal level (i.e. 60 to 100 mg per cent) produced effective haemostasis.

Fibrinogen deficiency

Fibrinogen is infrequently reduced below the minimum haemostatic level required, but when it does so it results in a severe haemorrhagic condition. Deficiency may occur as a congenital defect due to a simple recessive character, or as an acquired condition. Acquired fibrinogen deficiency may be acute due to rapid utilization and/or destruction as occurs in the acute defibrination syndrome encountered as a complication of pregnancy, parturition, and surgical operations with massive tissue destruction

or involving the use of extracorporeal circulation. This may be due to the sudden release in the circulation of excess thromboplastic substances, and also to the activation of the fibrinolytic mechanism.

Chronic fibrinogen deficiency due to defective production occurs in patients suffering from severe liver damage.

Species specificity of fibrinogen

Dissimilarity between fibrinogens from different species was shown to occur, as regards its various properties. Hektoen and Welker (1927) have shown that fibrinogens from a series of mammals had antigenic properties more or less in common, and that those from a series of birds, while having precipitinogenic properties common to themselves, were slightly different from mammalian fibrinogens. Kenton (1933) employing the specificity of the in vivo sensitization of animals to different fibrinogens, obtained by multiple precipitations with sodium chloride, demonstrated that antibodies produced to sheep fibrinogen do not react with horse, fowl, or rabbit fibrinogens. Also, that antibodies for bovine fibrinogen do not react with rabbit fibrinogen. His findings were corroborated by desensitization experiments with the homologous fibrinogen.

Davide (1925) using salting-out experiments with sodium chloride solutions found the following concentrations necessary to produce optimum fibrinogen precipitation :-

Species	Percentage of NaCl
Guinea-pig	30 - 40
Dog	30 - 40
Rabbit	35 - 45
Man	40 - 50
Horse	45 - 60
Sheep	60 - 65

Wöhlisch (1929) also used the same method of differentiation. Astrup and Darling (1942c) reported that horse, ox, and chicken fibrinogens were optimally precipitated by 25, 30, and 35–40 per cent saturation of ammonium sulphate, respectively. They also found different pH optima for the action of the same thrombin solution on purified fibrinogens of man, horse, ox, and chicken, stating that the specificity is due to the fibrinogen since they found the same pH optima on using thrombins from different species for the same fibrinogen solution (Astrup and Darling, 1942b).

Differences in solubility studies in ethanol-water mixtures were observed by Morrison et al (1951) for fibrinogens of different species.

The terminal amino groups of bovine fibrinogen were found to contain one glutamic acid molecule instead of the N-alanine molecule present in human fibrinogen (Lorand and Middlebrook, 1952a, and 1953).

Species specificity of fibrinogens was also demonstrated

biologically by observing the differences in the clotting times of different fibrinogens or plasmas clotted with the same thrombin solution (Seegers and Smith, 1942; Fantl and Ebbels, 1953; Burstein and Guinand, 1954). This biological method was the one adopted in this study to investigate the species specificity of fibrinogens.

THROMBIN

Thrombin is the natural enzyme which converts fibrinogen into fibrin. It is derived from prothrombin by various mechanisms which shall be described later.

Properties of thrombin (Biggs and Macfarlane, 1957, and 1962)

Thrombin is a carbohydrate-containing protein which belongs to the albumin group, and whose molecular weight varies from 31,000 to 62,000. Preparations of 15,000 to 20,000 molecular weight have also been described. Various thrombin preparations had iso-electric points of 3.6, 4.1, and 4.7. Thrombin is highly soluble both in water and in saline. In saline solution it is permanently inactivated by acid at pH 3.5, and reversibly inactivated in the zone pH 3.5 - 4.1. Alkali inactivation begins at pH 10 and is marked above pH 11 (Seegers, 1940). In aqueous solutions thrombin shows partial inactivation after heating for

thirty minutes at 40°C. It is completely destroyed at 60°C. Dilute thrombin solutions lose their activity rapidly in ordinary glassware, but not if the surface is lined with paraffin or silicone, (Waugh and Livingstone, 1951). This, however, is not noticed with concentrated solutions, because the relative amount removed from solution is small (Seegers et al, 1952).

Action of thrombin

Thrombin is a proteolytic enzyme which acts on the arginyl-glycine bonds in the fibrinogen molecule (Gladner, Folk, and Laki, 1958), with the splitting off of fibrinopeptide groups (Lorand, 1952; and Bettelheim and Bailey, 1952). The loss of these negatively charged peptides results in a change in the electrostatic forces which allows the polymerization of the newly formed fibrin monomers (Lorand, 1954), provided the pH is optimum.

Thrombin preparations also attack arginine esters (substrates which contain arginine as the specific amino acid residue), but have no activity on lysine esters (Sherry and Troll, 1954). The latter authors further maintain that the ester-splitting action of thrombin is quantitatively related to its clotting activity. Landaburu and Seegers (1959), however, believe that the esterase and clotting activities of thrombin may vary independently, and consider the clotting activity related to the formation of dissociable complexes of sub-units with esterase activity.

Ronwin (1960) regards the esterase activity of thrombin as essentially peptidase activity, and considers it inseparable from the overall clotting activity.

Preparation of thrombin

The earliest preparation of thrombin is that of Schmidt (1892) who precipitated it from fresh serum by alcohol. Mellanby (1933) separated the globulin fraction from diluted plasma by acidification to pH 5.3, dissolved it in saline and allowed thrombin to form spontaneously on recalcification of the solution. This method was modified by Biggs and Macfarlane (1957) by further purification of the thrombin by acetone precipitation. This method is described in detail in appendix III, 7, B, and was used throughout this work.

The purist preparation of thrombin as yet known is that prepared by Seegers (1962) and known as Citrate Thrombin. It was obtained as an electrophoretically and chromatographically homogeneous material, prepared by auto-activation of prothrombin in 25 per cent citrate solution, purifying the re-dissolved precipitate by acetone precipitations, and finally fractionating the product over IRC-50 columns, eluting the thrombin with 0.3 M sodium phosphate buffer at pH 8. The specific activity of this preparation was 3,600 Iowa units per mg dry weight. One Iowa unit is the amount of thrombin required to clot 1 ml. of standardized fibrinogen solution in 15 seconds at 28^{o}C. (Seegers and Smith, 1942). Thrombin prepared

in this manner is referred to as "Citrate resin thrombin".

Measurement of thrombin

Thrombin is measured by the speed of clotting fibrinogen in seconds, and expressed as units. Several specifications for these units have been proposed. The following two are those generally used.

Owren's unit: is defined by him as the amount of thrombin which in a volume of 1 ml. containing 0.1 per cent fresh pro-fibrin-free human fibrinogen, at a temperature of 37°C. and pH 7.3 and sodium chloride concentration of 0.154 M, causes coagulation in 15 seconds, (Owren, 1947).

National Institute of Health (N.I.H.) unit: is that amount of thrombin which will clot 1 ml. of a standard solution of fibrinogen at 28°C. in 15 seconds. It is reported that 1 N.I.H. unit is equivalent to 1.25 Iowa units (Seegers, 1962).

It should be noted that any results hereby expressed in terms of thrombin units are of no absolute value, but were adopted to allow a reliable comparison of all the experiments on the same footing. The original stock thrombin solution used for calibration was Thrombin topical (Parke Davis and Company) taken at its face value of 5000 N.I.H. units per vial, and dissolved in glycerol saline solution to contain 1000 units per ml. From this stock solution the necessary dilutions were made in saline and a

thrombin-fibrinogen dilution curve constructed as described in appendix IV, 7, and illustrated in figure 1 (Volume 2, page 55). From this dilution curve the results of all relevant experiments were read in terms of thrombin units as equivalent to the clotting times obtained in seconds.

New batches of fibrinogen preparations or thrombin solution were tested before use against a reference stock solution, and only those complying with the original thrombin-fibrinogen dilution curve (figure 1) were released for use, so as to maintain a fairly standard unit of activity.

Species specificity of thrombin

The presence of species specificity in the clotting action of thrombin on fibrinogen or plasma was first shown by Quick (1938a, and 1941) who noted that thrombin from different mammals is the same but differs from that of birds or toads. Warner et al (1939a, and 1939b) assumed that thrombin from all vertebrate bloods to be the same, and demonstrated that dogfish thrombin is species specific. Bryce (1954) found that sheep thrombin had the same activity on sheep and bovine plasma, while bovine thrombin had more activity on bovine plasma than sheep thrombin. He was aware of the possibility of specificity in the inactivating mechanisms present in the plasma which cannot be ignored until it can be shown not to exist. Lyttleton (1954), however, thought it is

improbable that antithrombin should be the cause for differences in plasma reactivity to thrombin, since he found antithrombin to be without effect on clotting times shorter than 100 seconds.

Nour-Eldin (1962b) demonstrated differences in the adsorption characteristics of human and bovine thrombins, the latter being adsorbed with calcium phosphate, while the human thrombin was not affected. He noted that both thrombins generally had the same trend in respect of various adsorbing agents, though bovine thrombin was usually more affected.

THROMBIN-FIBRINOGEN REACTION

When the thrombin-fibrinogen reaction is performed using purified solutions of thrombin and fibrinogen, its speed is influenced by the following factors: (Biggs and Macfarlane, 1957)

- Concentration of fibrinogen: the optimum being about 0.1 mg per cent.
- Fibrinogen content of pro-fibrin, a product of intermediate solubility between fibrinogen and fibrin (Apitz, 1937) which reacts more rapidly to thrombin (Owren, 1947). Pro-fibrin is easily removed from fibrinogen solutions by centrifugation in the cold, when it is deposited at the bottom.
- Optimum pH within the range 6.5 to 7.5.

- Optimum temperature is at 37°C.
- Presence of other proteins.
- Ionic strength: excess of neutral salts leads to delayed clotting.
- Species of both fibrinogen and thrombin.

The influence of these factors is largely eliminated when using plasma instead of purified fibrinogen solution, as they are more or less constant in normal plasma. However, the use of plasma is complicated by the presence of other known and possibly unknown factors, such as prothrombin, platelets, and lipids; and also by the variation in plasma constituents from one sample to another.

Though calcium is not needed for the thrombin-fibrinogen reaction, yet its presence shortens the clotting time, this effect being more apparent in plasmas with relatively long thrombin clotting times.

Thrombin converts many times its weight of fibrinogen into fibrin, since it acts enzymatically.

The clotting time of fibrinogen is inversely proportional to the thrombin concentration, when observed in pure solutions. This relation is somewhat more complicated when plasma is used, (Astrup, 1944).

FIBRIN STABILIZING FACTOR (Factor XIII)

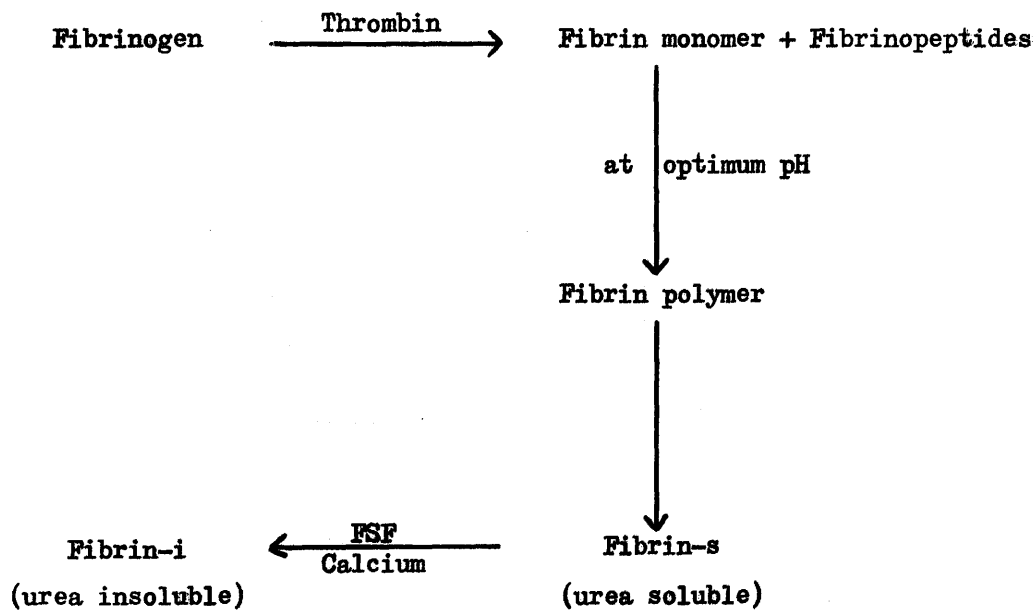
Clots obtained by mixing fibrinogen and thrombin were found to be soluble in weak acids (0.03 per cent HCl) and in weak alkalies (0.5 per cent Na_2CO_3) (Robbins, 1944), and in a 5 molar solution of urea (Laki and Lorand, 1948); while clots formed in plasma are not soluble, signifying that in the latter the particles are bound by stronger cross linkages (Lorand, 1950a; and Ferry et al, 1951). Lorand (1954) suggested this may be due to reinforced linkages of disulphide bonds, a step which involves the fibrin stabilizing factor.

Calcium was found to be necessary for the formation of urea--insoluble clots (Edsall, 1951).

Lorand and Jacobsen (1958) prepared a purified preparation of the fibrin stabilizing factor from human plasma, which was found to be a globulin, thermolabile, and non-dialysable (Lorand et al, 1958). It is stable in frozen plasma, and is present only in traces in serum, as it is used up in the clotting process by being incorporated into the clot.

It is interesting to note that Murray (1959) found antemortem clots to be soluble in urea solutions which fail to dissolve blood clots formed in glass tubes.

The steps involved in the thrombin-fibrinogen reaction can therefore be schematically presented as follows:-



ANTITHROMBIN

Thrombin is inhibited or neutralized by various antithrombins. Schmidt (1892) hypothesized that blood contained a thrombin inactivating substance which Morawitz (1904) named antithrombin. Morawitz (1905) and Mellanby (1909) showed that weak solutions of thrombin clotted fibrinogen more readily than oxalated plasma, stating that the amount of antithrombin present in plasma could inactivate many times the amount of thrombin formed from the same amount of plasma. This was confirmed by Gasser (1917), and later by Quick (1938b) and by Klein and Seegers (1950).

Nature of antithrombin

The antithrombin activity of plasma seems to be due to the presence of several antithrombins which have been designated in Roman numerals as follows:-

Antithrombin I : is the activity removed by the adsorption of thrombin onto fibrin (Klein and Seegers, 1950).

Antithrombin II : is the heparin co-factor which shall be discussed later (Brinkhous, Smith, Warner, and Seegers, 1939).

Antithrombin III: is the main inhibitory activity present in plasma, and is described below.

Antithrombin IV : is the antithrombin activity which appears during

coagulation (Seegers, Johnson, and Fell, 1954).

Antithrombin V : is an antithrombin which appeared in the blood of a patient with rheumatoid arthritis (Loeliger and Herts, 1957).

Antithrombin VI : is a substance derived from fibrinogen lysed by plasmin (Kowalski, 1959).

Properties of antithrombin III

Antithrombin III is separated in the α-globulin fraction which contaminates the crude albumin fraction precipitated by ammonium sulphate or by acidification (Biggs and Macfarlane, 1957).

It is destroyed by ether fractionation, chloroform, and acetone (Astrup and Darling, 1942d), by heating to 56°C., and at pH values above 9.5 or below 6. It is not adsorbed by calcium phosphate, (Astrup and Darling, 1942d).

Action of antithrombin

Mellanby (1909) found that the neutralization of antithrombin in chicken plasma is a slow process, and is complete after 30 minutes at 30°C. This was confirmed by Collingwood and MacMahon (1914) who stressed the necessity of incubation at 37°C. to speed up the process. Astrup and Darling (1942d) state that the neutralization is complete after 15 minutes at 37°C. They also believed the reaction to be of the first order. Biggs and Macfarlane (1957) also agree that thrombin and antithrombin react

together stoichometrically, and suggest that the process of prothrombin activation also activates the antithrombin system in plasma as well.

Measurement of antithrombin

The antithrombin is measured by determining the residual thrombin activity after neutralization with a known concentration of thrombin.

In the first method used (Astrup and Darling, 1942d) a sufficiently high thrombin concentration is achieved by using small amounts of diluted plasma, allowing complete neutralization to occur in 15 minutes at 37°C., then subsampling equal aliquots onto purified fibrinogen to determine the residual thrombin activity. The accuracy of this test is enhanced by varying the amount of plasma (or the antithrombin contained in it) while the amount of thrombin was kept constant; by allowing for thrombin decay in the saline control tube; by measuring the various samples in triplicate; and by reading corrected fibrinogen clotting values from a specially drawn graph. In its authors' hands, the accuracy of the test was ± 5 per cent. Under the conditions of the test they found direct proportionality between the amount of antithrombin added (i.e.plasma) and the amount of thrombin inactivated.

Originally, the plasma under test was first defibrinated, but in my assays whole untreated plasma was used, thus measuring mainly the antithrombin III and to a lesser degree the antithrombin I

content of the plasma. The test is described in detail in appendix IV, 11, B.

The second method used was that of Douglas and Biggs (1953) in which a known amount of thrombin is added to whole plasma and the residual thrombin estimated at one minute intervals by subsampling onto purified fibrinogen solution. A curve is drawn relating the residual thrombin in units to the incubation time in minutes (on semilogarithmic graph paper), and the distance in minutes at which thrombin activity is zero is compared with that of the control normal plasma treated identically. This method also measures the total capacity of plasma antithrombin activities. It is described fully in appendix IV, 11, A.

C A L C I U M (Factor IV)

The removal of calcium from blood by the addition of sodium oxalate was found to inhibit blood coagulation (Arthus and Pagés, 1890). Morawitz (1905) also realised the necessity of calcium for the initial phase of clotting when using tissue extracts.

It is now generally accepted that calcium is essential for both extrinsic and intrinsic systems of prothrombin activation (Biggs and Macfarlane, 1957), though Cekada (1926) showed that in highly purified preparations of prothrombin the formation of thrombin can

occur in the absence of calcium. Seegers (1962) also was able to obtain thrombin from purified prothrombin in a calcium-free system.

As was mentioned previously, calcium is not essential for the thrombin-fibrinogen reaction to occur, though it shortens it when present.

Mellanby (1930) and Loomis and Seegers (1944) found that calcium can be replaced by strontium, magnesium, and barium, which however were much less active than calcium (Seegers et al, 1945).

Large concentrations of calcium are inhibitory on coagulation, similar to the effect of neutral salts at a comparable high concentration (Loomis and Seegers, 1944).

P R O T H R O M B I N (Factor II)

Historical

In 1909 Mellanby prepared a substance from bird plasma which was not coagulant but could be converted into one which caused the formation of fibrin from fibrinogen. He prepared this substance by dilution of the plasma with water and precipitating it by acidification to pH 5.3.

Bordet and Delange (1914) observed that adsorbed plasma would not clot on the addition of tissue factor and calcium. They were able to elute a substance from the tricalcium phosphate precipitate,

which when added to the supernatant adsorbed plasma rendered the latter coagulable on the addition of tissue extract and calcium.

Howell (1914b) precipitated a prothrombin-containing fraction from cat plasma with an equal volume of acetone. Cekada (1926) modified Howell's method by preheating the plasma to 56°C. to remove the fibrinogen before precipitating with acetone.

These findings established the presence of prothrombin as a definite entity, and was corroborated later by various workers, notably Owren (1947).

Properties of prothrombin (Biggs and Macfarlane, 1957, and 1962)

Prothrombin is a protein present in Cohn's fraction III-2, having an electrophoretic mobility of α_2-globulins, an isoelectric point of pH 4.2, and a molecular weight of 68,000. It is water soluble, but precipitates out at pH 3.9 to 5.6. The optimum pH for its activation is 7.2. It is adsorbed from plasma by alumina, barium sulphate, barium carbonate, tricalcium phosphate, etc..., and can be eluted from the precipitate by citrate or phosphate buffers, or carbon dioxide under pressure in the case of magnesium hydroxide precipitates only. Prothrombin withstands heating in aqueous solution at 56°C. for 2 hours, and the preparation obtained from elution of alumina withstands even boiling. It is destroyed at pH values beyond 4.5 or 11 (Seegers et al, 1945), and by proteolytic enzymes (Eagle and Harris, 1937). Prothrombin is

synthetised in the liver, only if sufficient vitamin K is present, (Seegers et al, 1945).

Role of prothrombin

Prothrombin, as its name implies, is the inactive precursor of thrombin, which is present as such in the circulating blood. Under the influence of different substances it is converted quantitatively into thrombin. Prothrombin activation may be effected enzymatically by trypsin, in which case the conversion is incomplete and does not require the presence of calcium or other accessory clotting factors (Kleinfeld and Habif, 1953). Or it can be brought about in purified solutions, spontaneously, in 25 per cent sodium citrate solution, without the addition of other plasma coagulation factors or calcium, the so called citrate activation or auto-activation process of Seegers et al (1950).

The physiological activation of prothrombin is either by the extrinsic tissue thromboplastin which together with factors V, VII, X, and calcium forms the extrinsic active thromboplastin (extrinsic prothrombinase), or by the intrinsic or plasma active thromboplastin (intrinsic prothrombinase) formed by the interaction of the blood platelets with factors VIII, IX, X, V, and calcium, the whole mechanism being triggered by contact activation of factors XII and XI. These two mechanisms shall be described in greater detail in the following chapters.

Preparation of prothrombin

Several methods are used for the preparation of purified prothrombin from plasma. Seegers et al (1945) have prepared the most pure prothrombin as yet known, by a method entailing its isoelectric precipitation, adsorption with magnesium hydroxide, and elution of the prothrombin with carbon dioxide under pressure, followed by further fractionation by ammonium sulphate precipitation in the cold, and finally its separation by isoelectric precipitation. Their preparation was homogeneous in the ultracentrifuge and as judged from solubility studies.

Surgenor et al (1951) adsorbed prothrombin from plasma onto barium sulphate, and eluted it with phosphate buffer; while Quick (1951) adsorbed it with tricalcium phosphate and eluted with sodium citrate solution. Biggs and Macfarlane (1957) used alumina as the adsorbing agent, and eluted the prothrombin with phosphate buffer. This method is described in appendix III, 8; and was used whenever a purified prothrombin preparation was required for this study.

Measurement of prothrombin

Since the amount of thrombin formed is directly proportional to the amount of precursor substance (Eagle, 1935; Herbert, 1940; and Owren, 1947), the clotting time of substrate plasma or purified fibrinogen is indicative of the prothrombin concentration, which is usually expressed in terms of thrombin units.

A unit of prothrombin is defined as that amount capable of forming one unit of thrombin (Owren, 1947; and Ware and Seegers, 1948c).

In one-stage methods for prothrombin estimation, the clotting times are a measure of the prothrombin conversion rates, and therefore are very sensitive to varying concentrations of blood clotting accelerators. When, however, these are present in their optimum amounts, the clotting time of the mixture becomes a fair measure of the prothrombin concentration. On plotting the clotting times obtained with various dilutions against the reciprocal of the prothrombin concentration a straight line is obtained, which does not pass through the origin due to the initial delay in thrombin formation, and depending on the activity of the tissue extract and the reactivity of fibrinogen. The one-stage method of Koller et al (1951) employed in this study, entails the use of adsorbed bovine plasma as substrate to provide factor V and fibrinogen, with the addition of prothrombin-free oxalated human serum to supply factors VII and X. After the addition of the diluted test plasma and the brain extract, the clotting time is measured on recalcification. This method ensures the uniform ample concentration of all other necessary clotting factors, but for the purpose of this study suffers from the interference of species specificity between brain extracts and plasma combinations. It is described in appendix IV, 12, A.

In two-stage methods, thrombin is evolved in a mixture of prothrombin (purified or plasma), brain extract, and calcium. At intervals aliquots from this mixture are removed and added to fibrinogen solution whose clotting time is recorded. The fibrinogen clotting time is a measure of the amount of thrombin evolved. It was shown by Biggs (1951) that when prothrombin is converted into thrombin in the two-stage technique, the amount of thrombin formed, as represented by the minimum clotting time of fibrinogen, is directly proportional to the amount of prothrombin. And since the fibrinogen clotting time is inversely proportional to the thrombin concentration, its relation to prothrombin concentration may thus by expressed as follows:-

$$\text{Clotting time} = \frac{k}{\text{Prothrombin concentration}}$$

In other words, the fibrinogen clotting time is proportional to the reciprocal of the prothrombin concentration, depicting a straight line when plotted on graph paper. This line passes very nearly through the origin.

Two-stage methods are independent of tissue extract activity, as well as of fibrinogen reactivity, provided the latter conforms to the reference thrombin-fibrinogen dilution curve.

Douglas and Biggs' method (1953) comprises the incubation of the euglobulin precipitate (containing the prothrombin) with brain

extract and calcium for 30 minutes, after which time an aliquot is delivered onto fibrinogen solution and the clotting time recorded. This is compared with the result of the control plasma. This system has the advantage of being free from antithrombin. Also, the long incubation time precludes the need for optimum amounts of the accelerating factors, and allows for the complete conversion of the prothrombin present in the mixture to occur. The method is described in appendix IV, 12, B.

The two-stage area method of Biggs and Douglas (1953a) is perhaps the most suitable method for the investigation of prothrombin levels in plasmas of different species. The citrated plasma is incubated with the brain extract and the mixture recalcified, thereafter subsampling at intervals onto fibrinogen solution. When the clotting times are converted into thrombin units and a curve drawn relating the thrombin units to the incubation time, the area enclosed by the curve is a measure of the prothrombin concentration in the plasma, and is compared to that of the control plasma similarly tested. As the clotting times are the net result of thrombin generation and thrombin neutralization by antithrombin, the latter's concentration in both control and test plasmas has to be assessed in order to exclude its variation from vitiating the interpretation of the test results, or to take it into consideration if present. The authors have shown that variation in the potency

of the brain extract does not affect the results. The limitations when using species specific brain extracts shall be discussed when relevant. This method is described in full in appendix IV, 12, C.

Prothrombin deficiency

Normal plasma will generate about 300 units of thrombin per ml. (Seegers et al, 1945). Biggs and Douglas (1953a) have investigated a patient with acquired prothrombin deficiency who had 10 per cent of the normal prothrombin concentration as estimated by their two-stage area method, but whose one-stage prothrombin time was only slightly lengthened. Biggs (1955) reported that the minimum level of prothrombin required for effective haemostasis is about 10 per cent of the normal. Usually, however, higher levels are necessary to achieve normal haemostasis. Prothrombin deficiency occurs rarely as a congenital defect, but usually as an acquired condition in patients under dicoumarin therapy, in cases of vitamin K deficiency, and in patients with severe liver disease.

PROTHROMBIN ACTIVATION

The activation of prothrombin into thrombin can be brought about by several mechanisms, namely:-

1. Extrinsic thromboplastin system.
2. Intrinsic thromboplastin system.
3. Citrate activation, or auto-activation.
4. Trypsin activation.

The first two systems are those physiologically operative in the human body, and shall now be described in turn.

THE EXTRINSIC THROMBOPLASTIN SYSTEM

(Tissue thromboplastin system)

As its name implies, activation of prothrombin by the extrinsic thromboplastin system can only be effected by the presence of the extrinsic or tissue thromboplastin, a factor not normally present in the circulating blood. Blood coagulation factors involved in this reaction are : Tissue thromboplastin (factor III),

Calcium (factor IV),

Factor V,

Factor VII,

Factor X.

It should be noted that fibrinogen was not included, as it is not required for prothrombin activation, but its clotting time is used as an indicator of the rate and yield of thrombin formation, whether originally present in the activation system as in one-stage procedures, or samples from the activation mixture delivered later onto it as in two-stage procedures.

F A C T O R V

Discovery and historical

Nolf (1908, 1928, and 1945) claimed that prothrombin (thrombozyme) isolated from plasma by adsorption onto calcium phosphate would not form thrombin on the addition of tissue thromboplastin alone, but that a substance from the adsorbed plasma was necessary, namely "thrombogen". He thought thrombozyme, thrombogen, and fibrinogen combined to form fibrin. This theory is now known to be incorrect, but his experiments were the first to demonstrate the necessity of factor V in the process of prothrombin activation.

Quick (1943) observed that storage of oxalated plasma lengthened its one-stage prothrombin time, this defect was corrected by the addition of small amounts of normal fresh plasma. Thus he suggested the presence of a "labile factor" that is required for normal prothrombin activation.

In 1947 Owren discovered a patient in whose blood a prolonged one-stage prothrombin time was restored to normal by the addition of prothrombin-free plasma, thus establishing beyond doubt the missing factor's different identity from prothrombin, and called it "factor V". Also, Ware and Seegers (1948a) were able to identify a substance essential for the rapid activation of purified prothrombin, in the presence of tissue thromboplastin. They called it "accelerator globulin".

Properties of factor V

Factor V is a water soluble globulin present in Cohn's fraction III. It is destroyed by heating to 50°C. for 30 minutes, or immediately if heated at 58°C. Factor V deteriorates on storage in oxalated plasma, while it is stable in citrated plasma (Fahey, Ware, and Seegers, 1948; and Quick, 1943, and 1960). Factor V is labile at all pH values, but most stable at pH 5 to 9. It is adsorbed firmly on blood platelets and cannot be washed off them, (Ware, Fahey, and Seegers, 1948; and Hjort, Rapaport, and Owren, 1955). Factor V can be precipitated from plasma by 33 - 50 per cent saturation with ammonium sulphate, after the primary removal of the plasma prothrombin by adsorption. Factor V cannot be adsorbed by any of the adsorbing agents, and is therefore present in the supernatant adsorbed plasma. Also, it is not retained by Seitz filters containing 50 per cent asbestos. Factor V is used

up during the coagulation of the blood, and therefore is not present in normal human serum (Stefanini and Crosby, 1950).

Action of factor V

The presence of factor V is essential for prothrombin activation whether by the extrinsic thromboplastin system or the intrinsic thromboplastin system.

It was found to behave as a substrate whose further addition increases the yield of thrombin in vitro (Straub and Duckert, 1961).

It is supposed that factor V (proaccelerin) is converted by traces of thrombin into a more active form, accelerin, (Ware, Murphy, and Seegers, 1947; Hjort, 1957; Therriault et al, 1957; Rapaport, Ames, and Duval, 1960; and Rapaport et al, 1963). Strong thrombin solutions, however, are known to inactivate accelerin (Hjort, 1957).

Preparation of factor V

The most pure preparation known is that prepared by Ware and Seegers (1948b) which still was said to contain about 50 per cent of substances other than accelerator globulin.

In this work, factor V preparation was made by ammonium sulphate precipitation as described by Douglas and Biggs (1953), a method that entails adsorbing the plasma to get rid of the prothrombin, then the fibrinogen is removed by 33 per cent saturation with ammonium sulphate, and the factor V is obtained by raising the ammonium sulphate concentration to 50 per cent. Factor V is

finally recovered from the precipitate by dissolving it in saline, and the solution dialysed against saline to free it from the ammonium sulphate ions. Solutions prepared as such were used in the relevant experiments immediately after preparation without any storage. Details of the method are described in appendix III, 9.

Measurement of factor V

Factor V activity can be measured either in one-stage or in two-stage methods.

In one-stage methods, the substrate plasma is either from a patient with congenital deficiency of factor V, a rare defect, or plasma made artificially deficient in factor V by storage. The degree of correction of this substrate plasma by the test material is compared with that of a control normal plasma, in a one-stage procedure utilising brain thromboplastin; as described in detail in appendix IV, 14.

In the two-stage method of Ware and Seegers (1948a) the rate at which the thrombin titre develops from purified prothrombin under standardized conditions is proportional to the amount of the factor V present.

Factor V deficiency

Factor V deficiency occurs in the following conditions:-

1. As a congenital abnormality, giving rise to a haemorrhagic diathesis.

2. In diseases involving the liver, e.g. cirrhosis, leukaemias, and advanced carcinoma.

3. As an associated finding in defibrination syndromes.

Factor V is not significantly reduced by dicoumarin therapy. Quick (1960) has shown that factor V has to be reduced at least by 50 per cent before the one-stage prothrombin time is prolonged. He also noted that the concentration of factor V in human serum is apparently higher than in the corresponding plasma, this observed hyperactivity is lost when the serum is adsorbed with calcium phosphate, and factor V then found to be actually very low.

F A C T O R VII

Historical

The possibility of another factor present in the serum was first recognized by Mann et al (1947). Owen and Bollman (1948) also found that serum shortens the prothrombin time of dicoumarin plasma, indicating that the factor supplied could therefore be neither prothrombin nor factor V. Alexander and de Vries (1949 a and b) found that serum of a dicoumarin-treated patient lacked the ability to shorten the one-stage test in a system using plasma diluted with barium sulphate adsorbed plasma as substrate. Owren (1950, 1951, and 1952) observed that Seitz-filtered bovine plasma contains both

factor V and some prothrombin, and that the latter is not readily converted to thrombin unless some serum was added. He called the serum accelerator "proconvertin". Koller et al (1951) confirmed Owren's findings, and called the new factor "factor VII", the term now internationally adopted.

Properties of factor VII

Factor VII is a protein present in the β-globulin fraction of normal plasma and serum (Owen and McKenzie, 1954). It is adsorbed from plasma or serum by alumina, barium sulphate, tricalcium phosphate, etc...; and is also removed from the plasma by Seitz filtration through pads containing 50 per cent asbestos. It is stable for long times when stored at -20°C., and stable for four days at 25-37°C. in serum; but it is heat labile, and is destroyed at pH 3. Factor VII is not consumed during clotting, and therefore is present in normal serum. Its activity is identical in silicone--coated or uncoated glass surfaces, as judged by the clotting of whole blood with brain thromboplastins (Fantl and Osborn, 1962).

Action of factor VII

Factor VII is an enzyme (not consumed) that accelerates the conversion of prothrombin into thrombin in the presence of tissue thromboplastins. The rate of product formation was found to be directly proportional to the enzyme concentration (Straub and Duckert, 1961). Owren (1951 and 1955) suggests that factor VII

reacts with brain extracts to form a specific intermediate product which he called "convertin".

Though factor VII is essential for the extrinsic activation of prothrombin, satisfactory intrinsic thromboplastin generation and activation of prothrombin in the absence of factor VII was reported to occur by Koller (1955).

Preparation of factor VII (Biggs and Macfarlane, 1957)

Normal serum is adsorbed with barium sulphate, the precipitate washed with distilled water, then eluted with 5 per cent sodium citrate in saline. The eluate is dialysed against citrate saline solution, and kept at -20°C. This method is described in appendix III, 10. It should be noted that such a preparation also contains factors IX and X.

Measurement of factor VII

The ideal method is naturally to measure the degree of correction of plasma from a patient with congenital deficiency of factor VII, caused by the addition of various amounts of the test material, as compared with a normal control plasma. Since this defect is rare, the scarce availability of such plasma does not allow for continued use of this method.

Biggs and Macfarlane (1957) noted that factor VII is greatly reduced in the plasma of patients given dicoumarin drugs, especially in the first few days, while other factors are less rapidly reduced

(Douglas, 1962). They made use of this fact by observing the degree of correction of dicoumarin plasma on the addition of the test plasma or serum as compared to a normal control plasma, in the presence of tissue thromboplastin and calcium chloride solution. This method is described in appendix IV, 15, A.

Koller et al (1951) devised a method based on the correction of Seitz-filtered bovine plasma, as described in detail in appendix IV, 15, B. This method, however, is also sensitive to changes in factor X concentration, the substrate plasma being totally deficient in factor X. The observed values are therefore indicative of the combined factor VII and X activities.

Serum was reported to have a greater factor VII activity than the original plasma (Hjort, 1957; Johnston et al, 1959; and Johnston and Hjort, 1961). This may be explained by the presence of residual thromboplastin activity in the serum. Also, there are observations which indicate no difference between plasma and serum factor VII activities (Koller et al, 1951; Hougie, 1959a and 1959b; and Pantl and Osborn, 1962). In view of these conflicting reports, all measurements of factor VII of the different species were made on plasma samples, which reflects the factor's true level as it occurs in the circulating blood.

Factor VII deficiency

Factor VII deficiency occurs in the following conditions:-

1. Congenital defect (Alexander et al, 1951; and Dische, 1958).
2. During dicoumarin drug therapy.
3. Vitamin K deficiency.
4. Diseases of the liver.
5. In the newborn.

FACTOR X

The term "factor X" was first used by Duckert, Flückiger, and Koller (1954) who described a deficiency of a factor in patients treated with dicoumarol which was different from factor VII or factor IX, that produced an abnormal result in the thromboplastin generation test of Biggs and Douglas (1953b).

However, conclusive evidence of the presence of yet another serum factor was presented by Hougie, Barrow, and Graham (1957) who described a case of congenital Stuart factor deficiency, which originally was diagnosed as factor VII deficiency by Lewis, Fresh, and Ferguson (1953). The patient's plasma had a prolonged R.V.V. clotting time, while his serum was defective when tested in the thromboplastin generation test. Both these tests do not require the presence of factor VII. They found that the defect was corrected by plasma or serum from a patient with a known congenital factor VII deficiency. They named the missing factor "Stuart factor"

after the patient's surname.

Properties of factor X

Factor X is a protein that separates with the α-globulin, (Denson, 1958), having a molecular weight of 86,000 (Esnouf and Williams, 1962). It is stable at pH 5 to 9, and withstands storage at 4°C. for two months. It is destroyed by heating to 56°C. in 30 minutes (Bachmann et al, 1957). Factor X can be adsorbed from the plasma by alumina, barium sulphate, or calcium phosphate, and can be eluted by 0.14 molar sodium citrate solution. Also, it can be removed from the plasma by Seitz filtration (Bachmann et al, 1957). It is not affected by glass surfaces (Bachmann et al, 1958).

Action of factor X

The presence of factor X is essential for the extrinsic activation of prothrombin with tissue thromboplastin or with Russell's viper venom, and also for the normal intrinsic generation of thromboplastin (Duckert, Flückiger, Matter, and Koller, 1955; and Rabiner and Spaet, 1959).

In the reaction with brain thromboplastins, a quantitative reaction occurs between the brain thromboplastin and factor X, both being consumed during the process of clotting (Straub and Duckert, 1961). Also in its reaction with R.V.V. it was split as seen in the ultracentrifuge, on electrophoresis, and by the appearance of

a new terminal amino acid (Esnouf and Williams, 1962).

It is interesting to note that during normal blood coagulation by the intrinsic thromboplastin system, factor X is not consumed, and therefore is present in normal serum. In this reaction it acts as an enzyme accelerating the formation of the intrinsic active thromboplastin (Fisch and Duckert, 1959).

Preparation of factor X

Factor X was prepared by Esnouf and Williams (1962) from barium sulphate eluates of bovine plasma, passed through a chromatography column of DEAE resin, and after further purification steps was obtained as a homogeneous protein fraction as judged by its uniform behaviour in the ultra-centrifuge.

For this investigation, the preparation of factor VII as was described before was also used as a source of purified factor X, (appendix III, 10).

Measurement of factor X

The method described by Bachmann, Duckert, and Koller (1958) was used. It measures the ability of the test or normal plasma to correct bovine plasma made artificially factor X deficient by Seitz filtration, using Russell's viper venom - cephalin reagent as the thromboplastic agent. The bovine plasma contained all other necessary factors in optimum concentrations, namely factor V, prothrombin, and fibrinogen. Using various dilutions of the

test plasma rendered factor X the only variable. This method is described in appendix IV, 18.

It should be noted that until the recognition of factor X, results attributed to factor VII by the then available methods may have been partly due to the presence in the serum of factor X.

Factor X deficiency

Deficiency of factor X may occur as a congenital abnormality (Telfer et al, 1956; and Hougie et al, 1957), or as an acquired deficiency in liver disease, dicoumarin therapy, or vitamin K deficiency (Bachmann et al, 1957).

T I S S U E T H R O M B O P L A S T I N (Factor III)

Historical

Buchanan (1879) observed that emulsions of certain body tissues promoted clotting when added to cooled blood. Schmidt (1892) prepared alcoholic extracts of tissues which accelerated blood clotting, and he noted that their activity was resistent to heating. Morawitz (1905) named this substance "thrombokinase", and was the first to suggest its action on a precursor present in the plasma, namely "pro-thrombin", converting it into thrombin in the presence of calcium. He noted that aqueous extracts of tissues had greater activity than alcoholic extracts. Howell in 1912 coined the term

"thromboplastin", and emphasised that alcoholic extracts resist boiling, while aqueous extracts lose their activity on heating.

Properties of tissue thromboplastin

Tissue or extrinsic thromboplastin is an intracellular substance present in muscles, lungs, brain, and most other tissues of the body.

Bordet and Delange (1913) thought the active principle to be a lipoid of the lecithin group, while Howell (1912) believed it to be a lipoid component of cephalin character associated with a protein, which was also the view held by Gratia and Levene (1922), Tocantins (1944 a and b), and Chargaff et al (1944).

Purification of aqueous preparations was reported by various workers by physico-chemical methods. It is the opinion of Fischer and Hecht (1934), Wöhlisch (1940), and Astrup and Darling (1942a) that all attempts to purify tissue thromboplastin by such methods result in decreased activity. The molecule of the active component of tissue thromboplastin is believed to be large, because all its activity could be removed by centrifugation at 10,000 r.p.m. (Biggs and Macfarlane, 1957). The activity of brain extracts on plasma is unaffected by changes in plasma lipids, it also resists boiling, and will not clot prothrombin-free fibrinogen (Biggs and Macfarlane, 1957). A powerful tissue thromboplastin could be prepared from the tissues of a haemophilic patient who died of haemorrhage (Brown, 1952, cited by Biggs and Macfarlane, 1957).

Action of tissue thromboplastin

The activity of tissue thromboplastin is preformed. Tagnon et al (1942) believe it to act as a proteolytic enzyme which accelerates thrombin formation, and is not consumed during the reaction. It was shown by Macfarlane and Pilling (1946) and by Macfarlane (1947) that soya-bean trypsin inhibitor, an anti--proteolytic substance, inhibits the action of tissue extracts. Recently, Straub and Duckert (1961) using purified reagents, found that the tissue factor behaves like a substrate whose further addition to the reaction mixture after maximal activity has been attained increases the yield of the final activator product. They also found it was consumed during the process of coagulation.

The most active tissue extracts are those prepared from the brain, lungs, or placenta. Tissue thromboplastin was found to act most strongly when in optimal concentration, undiluted preparations giving relatively long clotting times, while diluted ones caused shorter clotting times (Quick, 1935; Larsen and Plum, 1941; and Owren, 1949).

Biggs and Macfarlane (1951) and Biggs and Nossel (1961) have drawn attention to the fact that in wounds, even those with severe damage, the concentration of tissue thromboplastin released is unlikely to equal the concentration used in laboratory tests. They concluded that in physiological haemostasis the activity of

of the extrinsic thromboplastin system must therefore be brought about by much more dilute concentrations of tissue thromboplastin. They further noted that blood from patients with factors VIII or IX deficiencies, though reacts normally to strong tissue extracts, its reaction to weak preparations is poor.

Preparation of tissue thromboplastin

After the chosen tissue is cleaned from blood vessels and membranes, it is emulsified in saline in a homogeniser, or ground with sand in a mortar. The supernatant is separated after centrifugation, and its optimum dilution assessed on fresh normal plasma. Finally the whole preparation is thus adjusted and kept at $-20^{\circ}C$. in small bottles.

Brain is usually the best organ for the preparation of tissue thromboplastin as it can be very easily freed from blood vessels and membranes virtually completely. This ensures the least possibility of contamination with the other blood clotting factors. Two methods for the preparation of various tissue thromboplastins are described in appendix III, 13.

Species specificity of tissue thromboplastin

Muraschew (1904) observed that tissue thromboplastin from lower vertebrates was incapable of inactivating mammalian prothrombin. Quick (1935) found differences in the thromboplastic activities of tissue extracts prepared from various common laboratory animals,

namely Guinea-pig, cat, dog, rabbit, and man; as well as differences in the response of the blood of these animals to thromboplastin.

Trevan and Macfarlane (1936) demonstrated that lung preparations from different animal species gave varied one-stage clotting times with the same plasma samples from the different animal species. Warner et al (1939a) were unable to activate chicken prothrombin with mammalian tissue thromboplastin. Mann and Hurn (1952) observed that pre-incubation of the tissue extract with the homologous serum removed its species specificity, inferring that the specificity of a serum co-factor may be the cause of the species specificity, rather than the specificity of the tissue extract itself. This serum co-factor is now known to comprise factors VII and X, and from the available data it is very difficult indeed to ascribe the specificity to one or the other of the components involved in the reaction. It is perhaps safer to say that the reaction between the serum factors and the tissue thromboplastin is species specific.

Stormorken (1957d) also confirmed the species specificity of tissue thromboplastins and of proconvertin (factor VII), in the ox, horse, dog, and man.

Shirakura et al (1959) demonstrated that serum containing antibodies against human brain thromboplastin did not neutralize rabbit brain thromboplastin activity, concluding that human and rabbit brain thromboplastins are antigenically different.

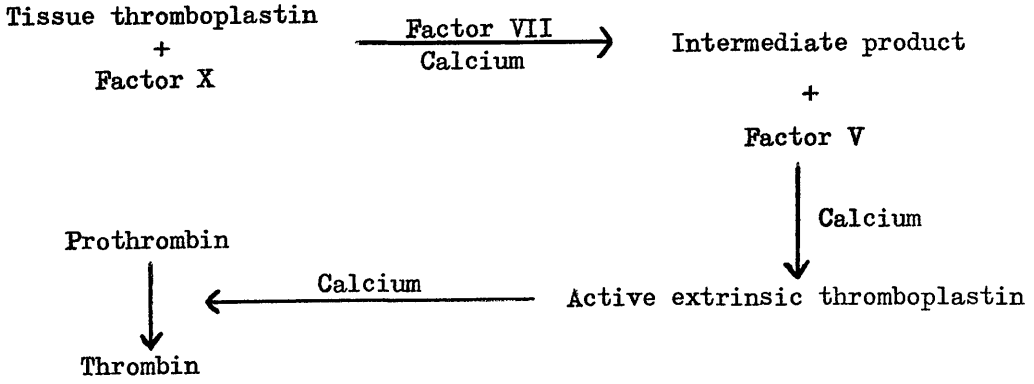
Fantl and Osborn (1962), however, found that "the combination of factors VII and X with the brain precursor showed no absolute species specificity, although a markedly higher activity in the homologous system was apparent". They used purified human and rabbit reagents. They concluded that a higher concentration of these factors is required for prothrombin conversion in a heterologous reaction mixture.

FORMATION OF ACTIVE EXTRINSIC THROMBOPLASTIN

The active extrinsic thromboplastin (extrinsic prothrombin activator, or extrinsic prothrombinase) is the end result of the interaction of tissue thromboplastin, factors V, VII, and X, together with calcium. Since a simultaneous interaction of all factors concerned is highly improbable, it was suggested by Jaques (1954) that such a reaction is a multi-stage one, with factors reacting in pairs and subsequent similar interaction of their products. Mann et al (1950, 1951, and 1952) believed that factor VII (co-thromboplastin) reacts with tissue thromboplastin before prothrombin enters the reaction. Later, Biggs, Douglas, and Macfarlane (1953a) demonstrated that pre-incubation of the brain extract with normal serum and factor V resulted in the formation of a powerful prothrombin converting substance. Straub and

Duckert (1961) using purified reagents proved conclusively that tissue thromboplastin reacts first with factors X and VII in the presence of calcium forming an intermediate product which then reacts with factor V forming the active extrinsic prothrombinase. They showed that factors V, X, and the tissue factor behave like substrates, and are consumed during the reaction, while factor VII acted as an enzyme accelerating the reaction without being consumed. This confirmed the previous observations of Koller et al (1951) that factor VII only affects the speed of the reaction, and of Hardisty (1955) that serum eluate (containing factors VII and X) affects the speed of the formation of the coagulant, while factor V affected its yield. Hougie (1959a) using sera from patients with congenital factor VII and factor X deficiencies also has demonstrated that factor VII affects the speed, while factor X affects the amount of prothrombin converting substance formed.

The sequence of events can therefore be represented as follows:-



RUSSELL'S VIPER VENOM

The venom of the viper snake has a powerful coagulant activity on normal plasma. It is a sort of extrinsic prothrombin activator which, however, differs from tissue thromboplastin in two respects.

Firstly, its activity was found to depend on the presence of lipids (Leathes and Mellanby, 1939), a finding which was confirmed by Macfarlane, Trevan, and Attwood (1941) who demonstrated that if the plasma is freed from the lipid co-factor, the activity of the R.V.V. disappears. Fullerton and Anastasopoulos (1949) noted that increase of the plasma lipid content caused shortening of the clotting times obtained when using R.V.V. as the extrinsic thromboplastin. Lee et al (1955) also found that R.V.V. does not activate purified prothrombin in the presence of factor V and calcium, while if platelets or platelet extracts were added, prothrombin activation was rapid. However, the platelet extract they used was purified from bovine platelets and is reported to be rich in carbohydrates and protein, but presumably little if any phospholipid. It is now generally accepted that phospholipids are essential for the full activity of R.V.V. Poole and Robinson (1956) and O'Brien (1957) identified these phospholipids as phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl

inositide, and diphosphoinositide.

The second property by which R.V.V. differs from tissue thromboplastin is that it acts independently of the proconvertin (factor VII) content of the plasma (Rapaport, Aas, and Owren, 1954). Factor V, however, was found by them to be necessary.

Macfarlane (1961) incubated various combinations of R.V.V., factor V, factor X, phospholipid, and calcium, and neutralized the residual venom activity with a specific antibody for R.V.V. He demonstrated that the initial concentration of factor X affected greatly the amount of active venom product formed, while changes in the R.V.V. concentration affected mainly the speed of the reaction.

This evidence suggests that the venom acts enzymatically on factor X to form the active product, in the presence of factor V, phospholipid, and calcium. This was confirmed by Williams and Esnouf (1962) who further isolated the venom coagulant protein as a physically homogeneous component by chromatography of the crude venom on DEAE-cellulose (diethylaminoethyl-cellulose) columns. Esnouf and Williams (1962) reported the purified coagulant protein to have a molecular weight of about 150,000, and also noted it had an amino-acid esterase activity when TAMe was used as substrate, resembling other proteolytic enzymes, especially thrombin, though it had no coagulant action on fibrinogen. R.V.V. does not seem to possess any species specificity as regards its coagulant action.

THE INTRINSIC THROMBOPLASTIN SYSTEM

(Plasma thromboplastin system)

Blood plasma contains all the necessary factors for prothrombin activation into thrombin, without the addition of any extrinsic reagents. The active intrinsic thromboplastin or intrinsic prothrombinase is formed by the interaction of several factors, namely:-

Factor IV (Calcium),
Factor V (Proaccelerin),
Factor VIII (Antihaemophilic globulin),
Factor IX (Christmas factor),
Factor X (Stuart-Prower factor),
Factor XI (Plasma thromboplastin antecedent),
Factor XII (Hageman factor),
Platelets, or phospholipid.

Gross deficiency of any of these factors halts the sequence of events at the stage where the missing factor is operative, preventing the formation of active intrinsic thromboplastin. This can be corrected only by supplying the missing factor, whether in vitro or in vivo.

Factors not described previously shall now be discussed in the light of present day knowledge.

F A C T O R XII (Hageman factor)

Historical

Lister in 1863 drew attention to the influence of foreign surfaces on blood clotting, which have an accelerating effect. Morawitz (1905) suggested that shed platelets coming into contact with a water-wettable surface disintegrate releasing thrombokinase. He intimated that the endothelial lining of the blood vessels is non-water-wettable. Bordet and Gengou in 1901 observed that the clot promoting effect of glass was exerted partly upon cell-poor plasma (cited by Ratnoff, 1961). Lozner, Taylor, and McDonald (1942) suggested that contact with glass surfaces activates a precursor of blood thrombokinase. This was subsequently confirmed by Hartmann, Conley, and Lalley (1949), Conley et al (1949), Ratnoff and Conley (1951), and by Quick and Eppstein (1952).

Rose and Broida (1954) found that nylon which is non-water--wettable stimulates clotting; also, Hirschboeck (1940) found that collodion (water-wettable) does not stimulate clotting. However, normal vascular endothelium which is presumably the least clot promoting surface is water-wettable.

It was also observed that surfaces which are negatively charged in relation to the blood appear to inhibit clotting (Lovelock and Porterfield, 1951) while the creation of a positive charge will

provoke clotting, even in vivo (Sawyer and Pate, 1953; and Sawyer, Pate, and Weldon, 1953). Later, Sawyer et al (1960 and 1961) demonstrated that thrombus formation is enhanced in vivo by a positive charge, and prevented by the presence of a negative charge, even when the vessel wall has previously been damaged.

The presence in blood of a contact factor responsible for the activation of blood clotting was established by Ratnoff (1954), and Ratnoff and Colopy (1955) who described a patient whose blood showed grossly abnormal laboratory results, and whose anomaly was corrected by the addition of normal plasma or plasma from patients suffering from congenital deficiencies of other known coagulation factors.

Properties of Hageman factor

It is a protein of the β_2-globulin group (Frick and Hagen, 1956), though it is believed to be associated with β and/or γ-globulin fractions (Lewis et al, 1958; and Haanen et al, 1960).

Hageman factor precipitates partly with all concentrations of ammonium sulphate saturation (Frick and Hagen, 1956), but the maximal activity is obtained in the fraction precipitated between 50 and 75 per cent saturation (Haanen et al, 1960). It is not adsorbed by inorganic precipitants but is adsorbed onto glass from which it can be eluted at pH 9 - 11. It resists heating to 56°C. for 30 minutes (Frick and Hagen, 1956), and is activated by storage at -20°C.

Role of Hageman factor

The exact role of Hageman factor is as yet not conclusively revealed. Frick and Hagen (1956) postulated the presence of an inhibitor opposing the activation of Hageman factor in patients thought to have Hageman factor deficiency. This was shown not to be the case by Hardisty and Margolis (1959), Egli et al (1959), and Haanen et al (1960).

Ratnoff (1961) believes that Hageman factor is activated by glass contact through molecular re-arrangement, and enzymatically activates factor XI (P.T.A.). Ratnoff et al (1961) found that variation in the concentration of Hageman factor affected the speed of formation of the active product formed together with factor XI, while variation in the concentration of factor XI affected the amount formed, confirming the enzymic nature of Hageman factor, and the substrate function of factor XI (P.T.A.).

Activated Hageman factor is reported to be extremely labile, being rapidly destroyed by another enzymic principle present in normal decalcified plasma, delipidized plasma, as well as in plasma of patients with the Hageman trait (Margolis, 1957, 1958a, and 1958c; and Margolius and Ratnoff, 1956). Again, an inhibitory activity has been found in Hageman trait plasma, but not in untreated normal plasma; inhibitors which may have this property have also been observed in horse, bovine, and fowl plasma (Ratnoff, 1961).

Niewiarowski et al (1962) found that purified Hageman factor eluates prepared from normal intact plasma exhibited significant arginine esterase activity, an activity that could be inhibited by soya-bean trypsin inhibitor or pancreatic trypsin inhibitor. Similar eluates prepared from Hageman deficient plasma lacked any clotting or arginine esterase activities.

Further addition of Hageman factor to normal plasma accelerates its clotting (Ratnoff, 1961).

Margolis (1957, 1958 a and b) demonstrated that while normal blood possessed a contact factor which on activation by glass contact accelerates clotting, it also triggers a mechanism involving pain and permeability factors.

In contrast to the usual effect of the deficiency of a blood coagulation factor, deficiency of Hageman factor does not give rise to a haemorrhagic diathesis in patients suffering from this defect (Ramot et al, 1956; and Margolius and Ratnoff, 1956), the only demonstrable change in these patients is altered tissue permeability (Margolis, 1959 a and b).

The action of Hageman factor may be conveniently represented as follows:-

Hageman factor (proenzyme) $\xrightarrow{\text{Glass surface}}$ Active Hageman factor (enzyme)

Factor IX (P.T.A.) $\xrightarrow{\text{Active H.F.}}$ Active P.T.A.

Measurement of Hageman factor

The activity of Hageman factor in plasma or plasma preparations is measured by the degree of shortening in the recalcification clotting time of either normal intact plasma, or of plasma from a patient with congenital Hageman factor deficiency, on the addition of the maximally activated test sample. Two such methods are described in appendix IV, 24, I and II. The second method is naturally the ideal one, but due to the scarcity of the Hageman deficient plasma recourse was made to the first method devised by Ratnoff and Rosenblum (1958). It should be noted that, now, it is appreciated that this latter method is mainly reflective of the P.T.A. activity in the test material (Ratnoff, 1962), and slightly indicative of Hageman factor activity.

PLASMA THROMBOPLASTIN ANTECEDENT (Factor XI)

Rosenthal, Dreskin, and Rosenthal (1953) first described P.T.A. as a third thromboplastic factor, through their finding a patient with haemorrhagic diathesis whose plasma was found to contain all other known coagulation factors, by cross-correction experiments.

Properties of P.T.A.

Rosenthal (1955) described P.T.A. as a β_2-globulin occuring in Cohn's fraction VII-I and fraction III, precipitated by 25 - 33

per cent saturation with ammonium sulphate, partially and irregularly adsorbed by barium sulphate, not retained or removed by Seitz filtration, and stable on storage at -20°C. Hyndman et al (1961) found it to be present in barium sulphate adsorbed serum which has been heated at 56°C. for 30 minutes, a procedure which eliminates or inactivates all other known coagulation factors.

Role of P.T.A.

Plasma thromboplastin antecedent is required for the formation of the contact active product necessary for the proper development of the active intrinsic thromboplastin.

Its deficiency results in a defect clinically less severe than that due to A.H.G. or Christmas factor deficiencies. The only overt indication of the defect may be excessive bleeding following tooth extraction. Laboratory tests are also less seriously affected, abnormalities are detected in whole blood clotting time, prothrombin consumption, and in the thromboplastin generation test when both the patient's adsorbed plasma and serum are incubated together. In a thromboplastin generation mixture the defect in P.T.A. deficient serum is poorly corrected by Christmas disease serum (Biggs et al, 1958), though fully corrected by normal serum. This is due to the fact that the lag for the activation of the Christmas factor in the former case is measured at the same time together with the time necessary for the generation of the active

thromboplastin, while normally the diluted test sera are allowed to stand for a while before use, during which time the formation of the contact active product has taken place in a normal serum containing both Hageman factor and P.T.A. and already activated the Christmas factor present. In other words, in case of immediate correction of P.T.A.-deficient serum with Christmas disease serum, we are measuring the total speed of the Christmas factor activation process together with further steps in active thromboplastin generation. But if the mixture of the deficient sera is incubated for a period before testing it, we get full correction with the normal generation of active thromboplastin.

Ratnoff et al (1961) and Biggs and Macfarlane (1962) agree that activated P.T.A. acts as a proteolytic enzyme which activates the precursor of factor IX into active factor IX, expressing the reaction as follows:-

$$\text{Precursor of factor IX} \xrightarrow[\text{(Active P.T.A.)}]{\text{Contact activation product}} \text{Active factor IX}$$

Measurement of P.T.A.

Due to the unavailablity of P.T.A. congenitally deficient plasma, measurement of this factor was not performed on plasmas of the species studied in this investigation.

Gallick et al (1960) devised a test system to assay P.T.A. in

vitro, which did not require the use of P.T.A. deficient plasma or serum, using highly purified reagents. However, they still acknowledged that it is unknown whether Hageman factor contributes any activity to this system which might be mistaken for P.T.A. activity.

CHRISTMAS FACTOR (Factor IX)

Christmas factor was discovered by Biggs et al (1952) who observed mutual correction between blood samples from patients previously thought to be all haemophiliacs. The deficiency was traced to a serum factor whose absence in these patients resulted in the haemorrhagic tendency characteristic of the condition, and in the impaired thromboplastin generation observed when their sera were used instead of the normal serum. These patients were found to have normal levels of A.H.G., the missing factor in classical haemophilia. The same findings were also reported by Aggeler et al (1952a and 1952b) who named the missing factor the "plasma thromboplastin component".

Properties of Christmas factor

Factor IX is a protein present in Cohn's fractions VII and III, which has the mobility of a β_2-globulin (Rosenthal, 1955). Its molecular weight is reported to be 110,000 as estimated by its

sensitivity to inactivation by 7 kilovolt electrons (Aronson et al, 1962). It is not consumed during coagulation, and therefore is present in normal human serum. It is adsorbed by barium sulphate, alumina, and tricalcium phosphate (Biggs and Macfarlane, 1957). It can be precipitated by 33 - 50 per cent saturation with ammonium sulphate, and is completely retained by Seitz filters (Rosenthal, 1955). It is stable on storage, and resists acidity of pH 3 (Biggs and Macfarlane, 1957). It withstands heating at 56°C. for thirty minutes (Stefanini and Dameshek, 1955). Decalcifying agents inactivate it, while its reaction with calcium is accelerated by A.H.G. (Bergsagel, 1955). O'Brien (1958) showed that it can be inactivated by heparin and reactivated by toluidine blue or by protamine sulphate. Biggs and Macfarlane (1962) doubt O'Brien's view, since Biggs et al (1961) were able to prepare an active concentrate of factor IX containing heparin.

Role of Christmas factor

The presence of Christmas factor is essential for the efficient generation of active intrinsic thromboplastin necessary for normal haemostasis. Its deficiency results in a haemorrhagic state very similar to haemophilia, though less frequent. Patients with this defect show abnormalities in the whole blood clotting time, prothrombin consumption test, and thromboplastin generation test. Normal serum corrects all these defects when added to their blood.

Ratnoff et al (1961) and Biggs and Macfarlane (1962) believe that factor IX is present in the circulation in a precursor form which is activated by the contact activation product into an active factor IX, which together with A.H.G. and calcium forms a complex that sets in motion further stages of intrinsic thromboplastin formation.

Preparation of Christmas factor

Factor IX can be prepared either from plasma or from serum; the latter is preferred as the preparation is then free from prothrombin. For use in this study, factor IX was prepared from serum by adsorption onto barium sulphate and elution by trisodium citrate solution, as described in full in appendix III, 12. Most of the serum content of factors VII and X is destroyed by the preliminary step of acidification to pH 2.9 prior to adsorption.

Measurement of Christmas factor

Measurement of factor IX in serum depends on the ability of this serum to correct the defect of a congenitally factor IX deficient serum, in a thromboplastin generation technique, as compared to the corrective ability of different dilutions of normal serum. Details of a method based on Pitney's method for A.H.G. assay (1956) are described in appendix IV, 22.

Haemostatic level of Christmas factor

Biggs (1955) found that the minimum level of factor IX required

for effective haemostasis to be 50 per cent of normal factor IX activity. Johnson et al (1957) observed that the concentration of factor IX in serum and plasma of many healthy individuals was often extremely low. Seegers (1962) states that in some healthy persons factor IX concentration was even lower than in patients diagnosed as having haemophilia B (Christmas disease).

ANTIHAEMOPHILIC GLOBULIN (Factor VIII)

The defect caused by the absence of factor VIII was the earliest haemorrhagic condition described (Hopff, 1828; and Bulloch and Fildes, 1911; cited by Biggs and Macfarlane, 1962). Yet, it was in 1937 that Patek and Taylor demonstrated that haemophilic blood lacks a factor present in normal plasma, which they called "antihaemophilic globulin". The presence of this factor as a single entity was later verified by several authors, (Brinkhous, 1947; Quick, 1947; Soulier and Larrieu, 1953 a and b; Biggs and Douglas, 1953b; Biggs, Douglas, and Macfarlane, 1953 a and b; Cramer et al, 1953; Seegers, 1954; and Koller, 1954).

Properties of factor VIII

Factor VIII is a protein with the mobility of a β_2-globulin (Creveld et al, 1956), present in Cohn's fraction I. The molecular weight of purified A.H.G. was found by Shulman et al (1960) to be

196,000; also Aronson et al (1962) estimated it as 180,000 as measured by its sensitivity to inactivation by 7 kilovolt electrons, assuming that the sensitivity of the macromolecules bombarded by the ionizing radiation is primarily a function of their molecular weight. Factor VIII is not adsorbed by inorganic precipitants as barium sulphate (Biggs and Douglas, 1953b) and therefore factor VIII activity is present in adsorbed plasma. Factor VIII can be precipitated from plasma by 25 per cent saturation with ammonium sulphate. It is partially removed by Seitz filtration (Rosenthal, 1955). In plasma, factor VIII activity is greatly diminished on storage at -4°C. for 24 hours (Biggs and Macfarlane, 1962). Spaet and Garner (1955) state that human factor VIII is considerably more storage-stable in citrated plasma than in oxalated plasma.

Freeze-dried purified preparations were found to lose half their activity if kept at room temperature for two months, while in humid atmosphere half the activity was lost in two weeks (Nour-Eldin, 1961). This author also demonstrated that in sealed ampoules this preparation retained its activity over a period of four months at room temperature, and of six months if kept at 4°C., while at 37°C. deterioration of half the activity occured in 5 to 8 days.

Factor VIII is stable at pH 6.2 - 7 (Biggs and Macfarlane, 1957) and withstands heating to 49°C. for 5 minutes (Bidwell, 1955).

Stefanini and Dameshek (1955) found it was stable at 56°C. for 30 minutes, though this is not generally accepted.

Partially purified factor VIII is destroyed by fibrinolysin, 70 per cent of the activity dissappears after its incubation for one minute with 0.8 Loomis unit per ml. (Wagner, Pate, and Brinkhous, 1954). Strong thrombin solutions cause complete inactivation of factor VIII within five minutes (Penick, 1957; and Fantl, 1958); this explains the apparent increased stability of factor VIII in adsorbed plasma in which there is no incipient thrombin formation, the mechanism that may well be responsible for the storage lability of factor VIII in plasma.

Role of factor VIII

The presence of factor VIII in sufficient amounts is necessary for normal haemostasis to occur through the development of the intrinsic active thromboplastin. The fact that factor VIII is consumed during coagulation suggests its behaviour as a substrate during the process of intrinsic thromboplastin formation, which in fact was demonstrated by Fisch and Duckert (1959).

Therriault et al (1957) working with mixtures of partially purified precursors of the intrinsic thromboplastin presented evidence that traces of thrombin activate factor VIII as well as factor V. Rapaport et al (1963) also demonstrated that minute traces of thrombin (0.1 ml of 0.01 units/ml solution) markedly

augmented factor VIII activity by 5 to 40 times, while increasing factor V activity by twofolds. They suggest that A.H.G. and proaccelerin must be activated by traces of thrombin before they can participate effectively in the generation of active intrinsic thromboplastin.

Factor VIII together with activated factor IX and calcium form a calcium complex which then interacts with factor X and platelets or phospholipid giving rise to an active product. This active product together with factor V, in the presence of calcium, forms the active intrinsic thromboplastin or prothrombin activator (Biggs and Macfarlane, 1962).

Preparation of factor VIII

Various methods were devised for the preparation of factor VIII from plasma, the simplest preparation suitable for the purposes of this study is that described by Biggs and Macfarlane (1957). The factor VIII present in alumina-adsorbed plasma is precipitated by 33 per cent saturation with ammonium sulphate, dissolved in saline, and dialysed against citrate-saline solution overnight. This method is cited fully in appendix III, 11.

More elaborate methods for the preparation of highly purified factor VIII were described by Kekwick and Wolf (1957) and by Blombäck et al (1958).

Measurement of factor VIII

Several different methods are available for factor VIII assay, mostly depending on the ability of the test plasma to correct the defect of a congenitally factor VIII deficient plasma.

Merskey (1950) and Soulier and Larrieu (1953 a and b) used plasma recalcified clotting times as indicative of the degree of correction of the various mixture prepared in haemophilic plasma. This method, however, seldom gives reproducible results due to the great influence of glass contact and platelet concentration on the clotting time of recalcified plasma. Tests based on changes in the clotting time of whole blood or recalcified plasma appear to be less specific (Brinkhous and Wagner, 1958).

The improvement in prothrombin consumption of haemophilic plasma on the addition of different dilutions of test or normal plasma was used as a measure of the A.H.G. content by Graham et al (1951) and by Quick (1951). This method, however, gives normal results at a stage when the coagulation mechanism is still impaired.

So far the most accurate method is that described by Biggs, Eveling, and Richards (1955) which is essentially a thromboplastin generation technique and requires the availability of purified reagents and of a standardized preparation of bovine factor VIII against which the test plasma is compared.

The method used in this study is also based on the thromboplastin

generation test of Biggs and Douglas (1953b), measuring the ability of the test adsorbed plasma to correct the defect of adsorbed haemophilic plasma in a thromboplastin generation mixture containing optimum amounts of all other necessary coagulation factors (Pitney, 1956). It is described in detail in appendix IV, 21.

Haemostatic level of factor VIII

The level of factor VIII in normal plasma samples varies between 50 and 200 per cent of the average (Pitney, Biggs, and Macfarlane, 1957). The minimum level required for effective haemostasis is 35 per cent (Biggs, 1955).

F A C T O R X

Factor X has been discussed in detail earlier as regards its role in the extrinsic prothrombin activation system.

Factor X is yet another factor necessary for the full activation of the intrinsic system. While in the extrinsic system it behaves like a substrate, in the intrinsic system it seems to act as an enzyme, being not consumed, and therefore present in serum obtained from the clotting of whole blood or recalcified plasma (Fisch and Duckert, 1959). It appears to be activated by the product formed from the interaction of factors IX, VIII, and calcium (Biggs and Macfarlane, 1962).

BLOOD PLATELETS

Though the blood platelets were recognized early in the nineteenth century, their implication in the blood coagulation mechanism was first suggested some thirty years later by Hayem in 1878 who believed that the platelets set free a substance into the plasma without which the latter could not solidify. Bizzozero in 1882 described platelets in thrombi in the mesenteric vessels of rabbits and Guinea-pigs. Bürker (1904) observed that when blood coagulation was prevented by any means platelet disintegration was also prevented, and that when coagulation was allowed to occur platelet disintegration also took place. Bordet and Delange (1912) recognized that platelets have thromboplastic activity, and in fact used platelet extracts which they called "cytozyme" as a source of thromboplastin.

Properties of blood platelets

Human blood platelets are non-nucleated, colourless, round or oval cells, and about 2 to 3 μ in size but occasionally larger forms are seen. In stained blood films they appear blue or purple (with Leishman's stain) with a hyaline cytoplasm, the hyalomere, and some granules towards the centre, the granulomere.

The normal number of platelets in the circulation is 250,000 to 500,000 per c.mm. whole blood. The life-span of platelets

varies from four to nine days (Mollison, 1956).

When platelets come in contact with a foreign surface, damaged endothelium, or a water-wettable surface, they exhibit a strong adhesive property and undergo disintegration after passing through a stage of viscous metamorphosis.

Role of blood platelets

That blood platelets are essential for normal blood coagulation is evident from various observations. A significant decrease in their number results in a prolonged bleeding time, and in impaired prothrombin consumption, two facts that denote defective haemostasis. This could also be shown by the administration of heparin, or by the use of siliconed glassware, procedures which prevent platelet disintegration (Biggs and Macfarlane, 1962). Platelet viscous metamorphosis is also inhibited by the absence of calcium (Sharp, 1958), and delayed in cases of P.T.A. deficiency or Hageman factor deficiency (Biggs et al, 1958). Otherwise, it appears to be largely independent of other known coagulation factors.

Schmid et al (1962) demonstrated that thrombin in concentrations which will not cause macroscopic conversion of fibrinogen, will cause platelets to clump; while platelets from which surface proteins have been digested off with trypsin do not clump in the presence of thrombin. It seems therefore that an early change in the plasma involving the contact activation product of factors XI

and XII, and calcium is responsible for their viscous metamorphosis and disintegration.

On disintegration, platelets release among other factors a thromboplastic material, "platelet factor 3", which participates in the formation of the active intrinsic thromboplastin. This material is now known to be mainly composed of phospholipids, namely phosphatidyl ethanolamine, phosphatidyl serine, lecithin, and other less important fractions (Biggs and Macfarlane, 1962). This platelet factor 3 reacts with the active product formed from factors IX, VIII, X, and calcium to form another active product which again reacts with factor V in the presence of calcium giving rise to the active intrinsic thromboplastin or intrinsic prothrombinase.

The blood platelets also contain other factors concerned with blood coagulation, for example platelet factor 1 (resembles factor V activity), platelet factor 2 (accelerates the thrombin-fibrinogen reaction), platelet factor 4 (an anti-heparin), also a fibrinogen--like substance, a fibrin stabilizing factor, a co-thromboplastin, and an antifibrinolysin. Of these, the platelet factor 1, the fibrinogen-like substance, and the antifibrinolysin are believed to be adsorbed by the platelets from the surrounding plasma, and are not actual components of the platelets themselves (Hjort, Rapaport, and Owren, 1955; Salmon et al, 1957; and Stefanini and

Murphy, 1956). The exact identity and role of the remainder constituents ascribed to the platelets is as yet a controversial subject beyond the scope of this work.

Blood platelets also affect clot retraction. Intact viable platelets in sufficient number are necessary for normal clot retraction (Biggs and Macfarlane, 1962). This has been shown to depend on the intact glycolytic system of the platelets involving adenosine triphosphate (Bettex-Galland and Lüscher, 1959).

Born and Gilson (1959) have found that platelets assimilate serotonin (5-hydroxytryptamine) from the plasma through a special transport system involving adenosine triphosphate. This is a vasoconstrictor substance whose exact role in haemostasis is still disputed.

The most important functions of the blood platelets may therefore be ascribed to platelet aggregation forming platelet plugs (white thrombi) which seal any discontinuity of the vascular tree, release of platelet factor 3 allowing efficient generation of active intrinsic thromboplastin, and lastly their ability to cause clot retraction which if occurs in a platelet plug further seals the wound, while if in an intravascular or intra-luminal blood clot will help repair through recanalization.

Preparation of platelet suspensions

Platelets are separated from the plasma by high speed

centrifugation, then washed in saline two or three times, and finally resuspended in saline solution half the original volume of the plasma. Methods for the preparation of the various platelet suspensions from the different species are described in appendix III, 15, A, B, and C.

Occasionally cephalin suspension was used as the phospholipid factor instead of the platelets, as described by Bell and Alton (1954). It was prepared from human brain tissue as described in appendix III, 14.

Platelet counts

Whole blood was diluted immediately after withdrawal in formol citrate solution in the proportion of 1 in 100 respectively, and the platelets counted in a haemocytometer chamber under the microscope. For the nucleated thrombocytes of the fowl, total and differential white blood cells' counts were made, from which the thrombocyte counts were derived. Counts of the lobster explosive cells were derived from total lobster blood cells' counts performed as ordinary total white blood corpuscles' counts, and from the differential counts of lobster blood cells made with the aid of phase contrast microscopy. Such methods for platelet, thrombocyte, or explosive cell counts are described in detail in appendix IV, 1, A, B, and C, respectively.

ACTIVE INTRINSIC THROMBOPLASTIN

(Intrinsic prothrombin activator)

Nolf (1908 and 1909b) always maintained that plasma contains all that is necessary for its own coagulation. This was confirmed by various workers, as discussed earlier. However, plasma also contains factor VII which is not required for the intrinsic system, as satisfactory thromboplastin generation was shown to occur in its absence (Hicks, 1955; Koller, 1955; and Bergsagel and Hougie, 1956).

Nour-Eldin and Wilkinson (1956) were able to isolate an active preparation of plasma thromboplastin by an ether precipitation technique. Spaet and Cintron (1959) also prepared purified active plasma thromboplastin by phosphatide sedimentation at 20,000 r.p.m.

That plasma or intrinsic active thromboplastin is different from tissue or extrinsic thromboplastin was shown by Shirakura et al (1959) who found that anti-sera neutralized the activity of the appropriate thromboplastin with no cross-reaction. This was also confirmed by Spaet and Cintron (1960a) who found that the antithromboplastin activity induced in rabbits by the injection of sedimented human intrinsic thromboplastin failed to affect intrinsic thromboplastin precursors, and had no activity on tissue thromboplastin, establishing their antigenic dissimilarity.

Since all necessary factors are present in optimum amounts in

the circulating blood, it is difficult to explain the blood fluidity only by the effect of inhibitors. Spaet and Kropatkin (1958) proposed the hypothesis that blood fluidity is preserved in vivo largely by cellular clearance of clotting intermediates. Spaet and Cintron (1960b) presented evidence that coagulation intermediate product I dissappeared from the blood of intact rabbits more rapidly than could be explained by the action of circulating anticoagulants alone.

Species specificity of the intrinsic thromboplastin

Nour-Eldin and Wilkinson (1957) observed the specificity of the intrinsic thromboplastin in homologous systems when using platelets as the thromboplastic agent, and found this specificity dependent on the homologous factor V. While on using brain phospholipid as the thromboplastic agent, they found that the serum then becomes the determining factor. Spaet and Cintron (1960a) found that the antithromboplastic activity induced in rabbits by the injection of sedimented human thromboplastin was inhibitory only in a human thromboplastin generation mixture, but not effective in a rabbit thromboplastin generation mixture. Though this reflects an antigenic difference between the thromboplastins of different species, it does not necessarily preclude the possibility of their functional inter-reaction in a thromboplastin generation mixture.

ANTITHROMBOPLASTIN

The presence of antithromboplastin was first postulated by Collingwood and MacMahon in 1912. Later, Tocantins (1943 a and b; 1944 a and b; and 1945) and Tocantins and Carroll (1949) gave evidence supporting the existence of antithromboplastin in the normal blood, and that it is present in excess in haemophiliacs. They have isolated this inhibitory substance from the plasma. Various substances were found to be inhibitory in a thromboplastin generation mixture. Robinson and Poole (1956) found that the inhibitory activity was associated with the content of phosphatidyl ethanolamine; while Barkhan and Silver (1960) have shown that purified phosphatidyl serine from normal human platelets had an antithromboplastin activity in the thromboplastin generation test and inhibited the thromboplastic activity of suspensions of both platelets and phosphatidyl ethanolamine.

Niewiarowski et al (1959) maintain that heating the plasma to 60°C. for 20 minutes destroys the antithrombin activity while leaving the antithromboplastin activity unimpaired.

Artificial antithromboplastin activity has been induced in rabbits by the injection of sedimented human thromboplastin (Spaet and Cintron, 1960a). Shirakura et al (1959) induced different antithromboplastin activities by the intramuscular injection of

tissue or plasma thromboplastins prepared by ether precipitation, and observed that the antithromboplastin activities are species specific with no cross-reaction between tissue and plasma antithromboplastins, or between human and rabbit reagents.

Biggs and Macfarlane (1962) regard antithromboplastin as a "hypothetical substance until further information is available". Their method for the demonstration of the presence of inhibitors of human intrinsic thromboplastin in plasma or serum samples in a thromboplastin generation mixture was utilised in this study as described in appendix IV, 23.

H E P A R I N

Heparin, a sulphate-containing mucopolysaccharide, is an anticoagulant frequently used as a therapeutic agent in thrombo--embolic disease. It is not naturally present in the circulation in measurable amounts (Biggs and Macfarlane, 1962).

Its action is dependent on a fraction of plasma proteins precipitated between one third and one half saturation with ammonium sulphate, which is now known to be identical to antithrombin II, the heparin co-factor (Burstein and Loeb, 1956). Howell and Holt (1918) considered heparin as an inhibitor of thrombin formation. Mellanby (1935) and Quick (1938b) showed that heparin is mainly an

inhibitor of the thrombin-fibrinogen reaction of the plasma. Brinkhous et al (1939) found that heparin in combination with the co-factor in plasma impedes prothrombin conversion into thrombin.

The process by which heparin delays the thrombin-fibrinogen reaction is reversed by toluidine blue (Lyttleton, 1950, cited by Biggs and Macfarlane, 1962), or by hexadimethrine bromide (Douglas, 1962).

Heparin also promotes the adsorption of thrombin by fibrin (Biggs and Macfarlane, 1962). Biggs, Douglas, and Macfarlane (1953b) demonstrated that heparin prevents the formation of the intrinsic thromboplastin. Solandt and Best (1940) observed that heparin stabilizes the blood platelets and inhibits their agglutination when administered in doses greatly in excess of those needed to prevent blood coagulation. Murphy et al (1961) found that heparin in large doses depresses or suppresses platelet deposition, noting that heparin carries a negative charge.

Heparin was found to be adsorbed by alumina (MacMillan and Brown, 1954; and Douglas, 1957) and therefore absent in the supernatant adsorbed plasma used in the thromboplastin generation test.

VITAMIN K

Dam in 1929 (quoted from Seegers, 1962) noted a haemorrhagic condition in chicks fed on a synthetic diet while studying cholesterol metabolism, and from later experiments concluded that some un-identified substance was missing from the diet, which he called "Koagulation vitamin".

Halbrook in 1935 (cited by Biggs and Macfarlane, 1962) showed that 5 per cent alfalfa added to the diet prevented the haemorrhages. In 1936, Dam et al. established the relation between vitamin K and prothrombin formation. This was confirmed by Quick (1937) who found that the prothrombin concentration falls rapidly in chicks given vitamin K deficient diet, and that bleeding occurs when the prothrombin concentration drops to 10 per cent, while it stops on administering alfalfa. Smith et al (1938) reported that prothrombin synthesis was stopped through malabsorption of vitamin K in cases with biliary fistulae or obstructive jaundice, due to the lack of the bile acids necessary for vitamin K absorption from the gut.

Douglas (1958) suggested that vitamin K plays also a part in the synthesis of factor VII and possibly factor IX.

Since this work deals with healthy animals of different species, it is assumed that they did not suffer any deficiency of vitamin K.

CLOT RETRACTION

Clot retraction is the phenomenon of contraction of clotted blood. Its role in haemostasis is not yet clear, and several functions have been ascribed to it none of which are convincing.

Barkhan and Silver (1962) have drawn attention to the fact that since retraction is mainly a function of viable platelets, it may well be that contraction of intravascular platelet thrombi is the biologically important function in haemostasis, while the retraction of the fibrin web may be a secondary and perhaps an incidental phenomenon.

Several factors, physical and biological, affect the degree of clot retraction. Apart from the shape and nature of the surface of the vessel containing the clot, the most important determinant factors are the platelet number and the fibrinogen concentration. Budtz-Olsen (1951) has shown that clot retraction varies directly with the platelet count, and inversely to the fibrinogen concentration. The platelets should be in a viable condition, and more than 100,000 per c.mm., otherwise retraction is proportionately deficient (Macfarlane, 1938). The optimum concentration of fibrinogen is 100 mg per 100 mls of plasma; higher fibrinogen content causes decreased values of retraction.

The packed cell volume also affects clot retraction inversely.

Macfarlane (1939) has shown that plasma clots containing no red cells will shrink until they are only about 10 per cent their original volume. Unfortunately, there is no simple method for correcting such retraction values for the variation in the packed cell volume (Biggs and Macfarlane, 1962).

The optimum pH of plasma for clot retraction ranges between 6 and 8.2 (Ellicot and Conley, 1951, cited by Biggs and Macfarlane, 1962).

Measurement of clot retraction

Macfarlane (1938) estimated the degree of clot retraction in terms of the amount of serum squeezed from the clot in one hour, expressed as a percentage of the total clot volume. This is done simply by carefully removing the contracted clot from a graduated centrifuge tube, draining it completely, and reading the volume of expressed serum and red cells from the scale on the tube, then calculating the percentage value. This method was used in this study, as modified by Aggeler et al (1942) who used a wire spiral around which the clot is allowed to contract instead of the originally devised glass rod with projections. A detailed description of this method is presented in appendix IV, 4.

Normal values obtained with this method are from 48 to 64 per cent serum expressed in one hour (Biggs and Macfarlane, 1962).

CONTROL HUMAN BLOOD SAMPLES

In all the following investigations test blood samples from the various animal species were compared to human blood samples. For qualitative tests, pooled test plasma or serum samples were compared with human plasma or serum samples pooled from at least three individuals. For quantitative tests, the test plasma or serum samples were compared to plasma or serum obtained from the same individual - the author - which were arbitrarily designated the value of 100 per cent to allow valid comparison between the various species on a uniform basis.

It should be mentioned that though this comparison gives a fair idea about the variations present in the different species examined, the results obtained cannot be compared with those previously reported by other workers except in a general way, due to the different control samples used. However, in order to compare such results slightly more critically, the level of the blood coagulation factors in the control plasma and serum used in this work has been assayed against samples pooled from ten normal human individuals. The results are tabulated in appendix V, pages 97 and 98. From these results the truer levels of the coagulation factors in test samples can perhaps be calculated. Still, due to the wide range in levels of normal samples as regards coagulation

factors, also the biological methods of assay used by their very nature having a rather wide latitude of accuracy, one can only rely on gross variations as significant. No attempt therefore has been made to correct the final results as to the variation in the control sample itself.

PART II

FOWL BLOOD COAGULATION MECHANISM

FOWL BLOOD COAGULATION MECHANISM

The following investigations were made on blood samples obtained from young adult domestic fowl of the Light Sussex strain, which were kept in pens and fed on ordinary pellet diet.

All the animals were healthy, none suffered from any bleeding nor showed undue haematomata formation after venipunctures.

The findings shall be presented in the same order as was used in reviewing the human blood coagulation systems.

F I B R I N O G E N

Fibrinogen in chicken plasma was estimated by the micro-Kjeldahl method (appendix IV, 2, I). The average of four experiments on pooled samples was 264.2 mg fibrinogen per 100 mls plasma, with a range from 246 mg to 298.5 mg per 100 mls plasma (Table 1). This is equivalent to low normal human values, and is reasonably sufficient to effect normal haemostasis.

Estimations of chicken fibrinogen using Stirland's turbidimetric method (1956) yielded very low figures, and in one instance (Experiment 3, Table 2) gave the value of 25 mg as compared with 172 mg when estimated by the micro-Kjeldahl method. It seems therefore that chicken fibrinogen is different from the human

fibrinogen in at least its precipitation characteristics. Astrup and Darling (1942c) demonstrated that chicken fibrinogen is optimally precipitated from plasma by 35 - 40 per cent saturation with ammonium sulphate, a concentration that is higher than that required to precipitate human fibrinogen, suggesting a chemical difference between chicken and human fibrinogens.

Hektoen and Welker (1927) and Kyes and Porter (1931) found that mammalian fibrinogen is also antigenically different from birds' fibrinogen.

Conclusions

The fibrinogen concentration in chicken plasma is about 264 mg per 100 mls, which compares with a low normal human fibrinogen concentration. It is sufficient for effective haemostasis. It seems to be chemically different from human fibrinogen.

THROMBIN-FIBRINOGEN REACTION OF CHICKEN PLASMA

When chicken citrated plasma was clotted with various concentrations of bovine thrombin (Parke Davis and Company) it was observed to take a much longer time than the control human plasma. The difference was maintained with all the thrombin concentrations. Concentrations of 25 thrombin units per ml or lower did not clot chicken plasma in three minutes, while human plasma was clotted in

six seconds (Table 3, and Figure 3).

The addition of an aliquot of M/40 calcium chloride solution to the chicken plasma shortened its thrombin clotting times appreciably (Figure 3, curve b_1) though the latter were still longer than the human plasma clotting times.

Toluidine blue, a heparin neutralizer, did not have any shortening effect, in fact it slightly prolonged the clotting times, as seen from curve b_2 as compared to curve b_1 in Figure 3.

Pyrocatechol together with calcium chloride had the maximum effect in shortening the thrombin clotting times of chicken plasma (Figure 3, curve b_3) and was more obvious with the stronger thrombin concentrations, though on the whole the clotting times were still longer than those of similarly treated human control plasma.

Since the normal body temperature of chicken is 41°C., the test was also carried out at this temperature instead of the usual 37°C.; the result was identical in the two cases (Table 3).

Discussion

The fact that chicken plasma thrombin clotting time is longer than that of the human plasma was shown as early as 1942 by Seegers and Smith who found that 1 ml of human oxalated plasma required 2 units of thrombin to clot in 15 seconds at 28°C., while chicken plasma required more than 10 units to clot in the same time. They believed this to be due to an inhibitory reaction rather than to

the species specificity of fibrinogen. Burstein (1950) similarly found that human thrombin clots human plasma in 4 - 7 seconds, while it clots chicken plasma in 50 - 90 seconds, at 20°C. Hawkey (1960) also observed the same phenomenon, and she showed that the addition of calcium chloride to chicken plasma shortens its thrombin clotting time. Wartelle (1957a), however, has presented evidence that the level of ionised calcium in adult chicken serum is equal to that in man, thus ruling out the possibility that calcium deficiency may be the responsible factor.

Fantl and Ebbels (1953) suggest that this delay may be due to the special configuration of the native fibrinogen, which can be altered by chemical treatment with phenolic substances such as pyrocatechol, or by purification of the fibrinogen by salting out methods. Later in 1954, Fantl showed that phenols depress antithrombin activity in serum or plasma, that pyrocatechol had greater antithrombin depressing activity than gum acacia, and that it did not interfere with clotting reactions if used in concentrations between 0.03 and 0.04 molarity. Though this mode of action of pyrocatechol seems very attractive, yet it does not hold in some other experimental conditions. For example, chicken plasma antithrombin activity was found to be nearly the same as the human as will be shown later, yet the effect of pyrocatechol on chicken plasma was significantly stronger than on human plasma. Secondly,

in a system of purified reagents free from antithrombin, pyrocatechol still shortened the fibrinogen clotting times when different thrombin solutions were used (Table 7). It seems more probable therefore that pyrocatechol acts directly on the fibrinogen.

The delay in chicken plasma thrombin clotting time is not due to excess heparin in chicken plasma, since the addition of toluidine blue solution to the plasma prior to the thrombin solution did not alter the clotting times.

Species specificity of chicken thrombin-fibrinogen reaction

Chicken thrombin-fibrinogen reaction was investigated in a purified system using fibrinogen and thrombin reagents prepared free from antithrombin as described in appendix III, 6, A, and appendix III, 7, A and B, respectively. In this system, 0.4 ml amounts of chicken or human fibrinogen solutions were clotted with 0.1 ml amounts of human, chicken, or bovine thrombin solutions. All thrombins were found to clot the human fibrinogen in about the same time, while they clotted the purified chicken fibrinogen in much longer times (Table 7). All thrombins had nearly the same activity, except that chicken thrombin was slightly weaker in clotting human fibrinogen, possibly because of the lower chicken plasma prothrombin content, as shall be discussed later, thus giving a lower yield of thrombin. The addition of 0.1 ml of a 1 per cent solution of pyrocatechol to the chicken fibrinogen

nearly halved its clotting time with the three thrombin solutions used. Again, the activities of all the thrombins were similar on chicken fibrinogen whose clotting time was still longer than that of the human preparation. These findings suggest that the delay in chicken fibrinogen clotting time is an inherent characteristic of the fibrinogen itself, which can be affected favourably by the addition of pyrocatechol solution. No species specificity was observed, since both fibrinogens were approximately equally affected by the three thrombin solutions. It should be mentioned that these thrombin solutions were prepared from equal volumes of the respective plasmas which, however, differ in their original content of prothrombin. The final thrombin yield in terms of units per ml of solution is therefore not identical by any means in the three preparations. The only valid comparison is that between the effect of one thrombin solution on the different fibrinogens which demonstrates significantly the delay in chicken fibrinogen clotting time.

Since chicken thrombin clotted human fibrinogen in 8 seconds, it can be assumed to contain the equivalent of 22 thrombin units per ml, as read from the thrombin-fibrinogen dilution curve (Figure 1, page 55 in volume 2); while the human thrombin contained about 58 units per ml. This agrees with the finding that chicken plasma prothrombin is about 13 - 24 per cent of human plasma prothrombin.

These findings disagree with those of Didisheim et al (1959) who found chicken fibrinogen to be clotted only with chicken thrombin, though they agree that chicken thrombin clots all other fibrinogens. Warner et al (1939a) also have found that chicken thrombin was active on mammalian fibrinogen.

Conclusions

There is a long delay in the thrombin-fibrinogen reaction of chicken plasma, as compared to the human, which can be shortened by the addition of calcium chloride and pyrocatechol solutions.

The delay is also manifest with purified thrombin and fibrinogen preparations. This phenomenon is most probably due to an inherent characteristic of chicken fibrinogen.

Chicken thrombin is active on human fibrinogen. Chicken fibrinogen is clottable by human, chicken, and bovine thrombins equally well, and is therefore not species specific.

CHICKEN PLASMA ANTITHROMBIN ACTIVITY

Antithrombin was measured in chicken plasma by the modified method of Astrup and Darling (1942d) as described in appendix IV, 11, B. This method measures both plasma antithrombin I and antithrombin III activities. The results are presented in Tables 4, 5, and 6, and Figure 4. The average of the three experiments

shows that chicken plasma has an antithrombin activity about equal to that of the human control plasma, with a range of 90 - 116 per cent of the human plasma antithrombin activity.

No measurement of antithrombin activity in chicken plasma could be found in the literature.

Chicken plasma when added to human plasma was found to prolong the latter's thrombin clotting time from 4 seconds to 37 seconds (Table 8). Heating the chicken plasma at 56°C. for 15 minutes prior to its addition to the human plasma prolonged the latter's clotting time only to 19 seconds. Such heating precipitates most of the plasma's fibrinogen content, and probably destroys all the antithrombin activity present. Since the chicken plasma antithrombin activity is similar to that of the human plasma, such an inhibitory effect is probably due to the chicken fibrinogen or fibrin. Burstein and Guinand (1956) demonstrated this effect using purified chicken fibrinogen, an effect which was completely nullified on heating the fibrinogen solution prior to its addition. They concluded that chicken plasma owed this inhibitory effect to its fibrinogen content.

Conclusion

Chicken plasma antithrombin activity as measured by the modified method of Astrup and Darling (1942d) is about equal to that of human plasma. Chicken antithrombin cannot therefore be blamed for the

delayed thrombin-fibrinogen reaction observed in chicken plasma.

FOWL EXTRINSIC THROMBOPLASTIN SYSTEM

The extrinsic thromboplastin system of prothrombin activation is best exemplified by Quick's one-stage prothrombin time test which reflects the combined effects of all factors concerned, namely factors V, VII, X, tissue thromboplastin, prothrombin, fibrinogen, and calcium.

Using this test, chicken brain thromboplastin was found to cause clotting of chicken citrated plasma in the presence of calcium chloride in 14 seconds, the same time as required for normal human plasma to clot on the addition of human brain thromboplastin and calcium chloride. This means that chicken plasma contains all the necessary factors involved in this reaction in a concentration that is sufficient to maintain effective haemostasis, if it is assumed that chicken plasma clots in the same manner as human plasma.

But chicken brain thromboplastin had very weak activity on human plasma, also human brain thromboplastin was hardly active on chicken plasma. Various combinations of plasmas were tested by both types of tissue thromboplastin as presented in Table 9 from which it can be seen that the mixture of human and chicken plasma was clotted in a longer time than when each plasma was equally mixed with saline,

and clotted with the homologous brain preparation. In other words, each plasma was slightly inhibitory on the other homologous system, and this is believed to be due to the added effect of dilution, since the heterologous plasma contributes totally inactive factors, together with that of antithrombin supplied by the heterologous plasma which is potentially active against the thrombin produced. The latter's effect is slight because thrombin production at a certain stage attains such a high concentration as to momentarily overpower the antithrombin present, and produce clotting of the fibrinogen.

Discussion

The species specificity of the reaction between tissue thromboplastin and plasma has been observed as early as 1902 by Fuld who found that bird muscle extract did not clot horse blood. Loeb (1904c) also stressed the specificity of mammalian and avian muscle extracts when acting on blood from these two classes of animals. This was later confirmed with other types of tissue extracts (embryo, brain, and lung) by several authors (Mendeleef, 1934; Quick, 1935; Trevan and Macfarlane, 1936; Dam and Glavind, 1938; Warner et al, 1939a; Copley, 1942; Wartelle, 1957 a and b; Didisheim et al, 1959; and Hawkey, 1960).

Quick (1941) suggested that there is no absolute species specificity though a considerable relative specificity is found

and is greatest between substances from chicken and mammalian bloods, on the assumption that higher concentrations of the factors involved could bring about clotting of the heterologous blood in a relatively shorter time.

Mann and Hurn (1952) have demonstrated that the species specificity can largely be eliminated by the brief treatment of the tissue thromboplastin with the diluted homologous serum, suggesting a co-thromboplastic reaction to occur. The serum factors responsible for such a reaction are now known to be factors VII and X. Thus there is no doubt that tissue thromboplastins are potentially most active on the homologous plasma, but as yet it is not established which factor is mostly responsible for this phenomenon. An attempt is made in the following section to investigate this further.

Conclusions

Chicken and human tissue thromboplastins are only active on chicken and human plasmas respectively, in one-stage tests, while they are very weak in heterologous systems. Tissue thromboplastin has a marked degree of species specificity towards the homologous plasma.

SPECIES SPECIFICITY OF FACTOR V

The role of factor V in the extrinsic thromboplastin system as far as species specificity is concerned was investigated by using a two-stage technique in which brain thromboplastin suitably diluted was incubated with diluted adsorbed plasma and at intervals aliquots were removed and delivered onto normal substrate plasma, and on plasma rendered factor V deficient. The clotting times of these substrate plasmas were recorded. In such an incubation mixture the tissue thromboplastin is allowed to react with the factor V present in the adsorbed plasma, the degree of interaction is reflected in the clotting times of the substrate plasmas which should become shorter rapidly if there has been any such reaction. The partly preformed active tissue thromboplastin in a sense short--circuits one stage which otherwise would have taken place in the substrate plasma itself. This method is described in detail in appendix IV, 16. The reagents were so diluted as to obtain a somewhat long clotting time after the first minute of incubation, in order that further shortening in the clotting times would be easily demonstrable. Also, the use of factor V deficient plasma as substrate further exaggerates any tendency, and eliminates the interference of factor V present in the substrate plasma from complicating the issue.

The results presented in Table 10 show that while human brain incubated with human adsorbed plasma clotted the substrate plasmas very rapidly, namely in $13\frac{1}{2}$ and 15 seconds after six minutes' incubation, when incubated with chicken adsorbed plasma the human brain had very weak thromboplastic activity (34 and 65 seconds), and was similar to the saline control (34 and 65 seconds). Thus, we can infer that human brain thromboplastin and chicken factor V did not react together under the conditions of this test.

Also, chicken brain incubated with chicken adsorbed plasma clotted the substrate chicken, human, and factor V deficient human plasmas in the shortest times which were 12, 24, and 80 seconds respectively, for the six minute reading; while its incubation with human adsorbed plasma gave similar clotting times as the saline control, namely 24, 135, and more than 180 seconds respectively. This means that chicken brain thromboplastin also did not react with the human factor V under the conditions of the test.

But the combination of chicken brain and chicken adsorbed plasma did have an appreciable thromboplastic activity on the human substrate plasma which clotted in 22 seconds. Such treatment of the brain extract with the homologous adsorbed plasma has therefore suppressed its species specificity towards the heterologous substrate plasma. Because factor VII is also species specific as shall be shown later, it is my belief that such potent clotting

activity of the brain extract - adsorbed plasma combination is partly due to the presence of traces of the homologous factor VII which may have been incompletely adsorbed from the chicken plasma.

Conclusions

Human brain and chicken brain extracts are each species specific towards the homologous factor V. Preincubation of the homologous reagents possibly partly suppresses their species specificity towards the heterologous substrate plasma.

ASSAY OF CHICKEN PLASMA FACTOR V

Chicken plasma factor V was measured by the ability of fresh chicken plasma to correct the defect of plasma made factor V deficient by storage as described in appendix IV, 14. Since chicken factor V cannot be estimated in a human system because of its species specificity, it was measured in fresh chicken plasma by the ability of this plasma to correct chicken plasma made factor V deficient by storage, using chicken brain as the thromboplastic agent. Various dilutions were made ranging from 100 to 0 per cent to enable the drawing of a dilution curve (Figure 5, curve B). If it is feasible to compare this curve with a human homologous factor V dilution curve similarly constructed (Figure 5, curve A) we find that the slope of both curves very similar. It may

therefore be assumed that chicken plasma contains factor V activity in a concentration similar to that of the human control plasma. If its concentration was initially low, one would expect its activity to wane rapidly with dilution, with leveling of the curve slope, which in fact did not occur in any of the three experiments presented in Table 11.

Discussion

From the previous results chicken fresh plasma was found to have factor V activity that corrects the deficiency produced artificially in plasma by storage, in a concentration sufficient to maintain efficient haemostasis. Murphy and Seegers (1948) estimated chicken factor V (Ac-globulin) in plasma by a two-stage technique, utilising purified prothrombin. They found factor V activity in chicken plasma to be about one quarter that present in human plasma. This rather low value could be attributed to the species specificity of the reagents used. The brain extract used in their experiments is presumed to be human, as no mention of its species was noted. Wartelle (1957b) also demonstrated the presence of a labile factor in chicken plasma, which deteriorated on storage for five hours at room temperature, while if kept in siliconed tubes at $0^{o}C$. it was stable for 10 hours, with partial deterioration after 24 hours and complete destruction after 72 hours. The long clotting time of such stored plasma was

completely corrected by the addition of fresh chicken plasma. She also observed that such stored plasma was not corrected by the addition of adsorbed bovine serum (which should contain factor V in ample amount), stressing the species specificity of the factor V - brain thromboplastin reaction. Since this species specificity interferes with the proper comparison with a human control, and in view of the normal values of prothrombin time of chicken plasma and its short stypven clotting time, Wartelle assumed that chicken plasma factor V activity is equal to that of human plasma. Hawkey (1960) investigated White Leghorn chicken, and reported that their plasma and serum contain 3 to 4 per cent of the human control plasma factor V activity. However, in her test system, she used human brain thromboplastin and human factor V deficient plasma, reagents that do not react with chicken factor V, a fact that explains her low results.

Conclusions

Due to the species specificity of chicken factor V - brain thromboplastin reaction, it is impossible to compare chicken factor V activity in plasma with that of the human with any degree of precision. However, in view of the similar factor V homologous dilution curves of chicken and human plasmas, together with the normal prothrombin time of chicken plasma, it is believed that chicken plasma contains factor V in a concentration similar to

that present in human plasma. Chicken factor V is present in an amount sufficient to maintain efficient haemostasis in chicken blood.

SPECIES SPECIFICITY OF CHICKEN FACTORS VII AND X

The species specificity of chicken factors VII and X - brain thromboplastin reaction was investigated by a two-stage technique similar to that used for the investigation of factor specificity. This method entails the incubation of the diluted brain extract with the diluted serum in the presence of calcium chloride solution. Aliquots from the incubation mixture are removed at minute intervals and delivered together with calcium chloride solution onto previously distributed aliquots of normal substrate plasma, both human and chicken, and of phenindione human plasma. The clotting times are then recorded. Various combinations of serum and brain extracts were thus tested. The method is described in detail in appendix IV, 17. The results are tabulated in Table 12.

The factors concerned in this extrinsic thromboplastin system and supplied by the test serum are factors VII and X whose combined action is reflected in the results. This was inevitable, since even in purified fractions from serum, both factors separate together, unless each is prepared from serum or plasma from a

patient with congenital deficiency of the other factor, which being a rare condition was unavailable. Moreover, chicken plasma or serum presumably contained both factors.

While human brain incubated with human serum clotted normal and phenindione human plasmas in a short time (18 and 17 seconds), the mixture of human brain with chicken serum behaved as the saline control, clotting the substrate plasmas in appreciably longer times (38 and 60 seconds).

Similarly, chicken brain extract incubated with chicken serum was highly active on chicken plasma (13 seconds), while with human serum it was much weaker, in fact identical to the saline control (23 seconds). The same difference was observed on using human normal and phenindione plasmas as substrate, except that the clotting times were much longer due to the species specificity of factor V as discussed before. The combined chicken brain and chicken serum mixture had very feeble activity on human substrate plasma, though still demonstrable, clotting it in 34-57 seconds, an activity equivalent to that of chicken brain thromboplastin diluted 1 in 40 in saline.

Discussion

Mann and Hurn in 1952 suggested that the species specificity of brain extracts resides in its interaction with a serum co-thromboplastin (factor VII) and have shown that brief treatment

of the brain extract with the diluted homologous serum largely eliminates this specificity.

That serum and plasma contain factors which are species specific towards the homologous brain extract has also been confirmed by Wartelle (1957b) in one-stage tests.

This work again confirms such findings, and by the two-stage technique traces the specificity to factors present in the serum, which presumably are factors VII and X since they are the only factors that take part in such an extrinsic system and supplied by the serum.

Conclusion

The interaction of chicken brain extract with chicken serum factors, most probably factors VII and X, is species specific.

ASSAY OF CHICKEN PLASMA FACTOR VII

Due to the previously demonstrated species specificity of factor VII towards the homologous brain extract, it was impossible to assay factor VII with any certainty using the degree of correction of human phenindione plasma as a guide, since the use of human brain would be insensitive to chicken factor VII, while chicken brain would be insensitive to the human factor VII in the control plasma.

The preparation of chicken phenindione plasma has been tried by

feeding the chicken phenindione tablets. Unfortunately I was unable to obtain chicken phenindione plasma of sufficiently long one-stage prothrombin time suitable for such a quantitative assay, although huge doses of phenindione were administered orally in the order of 50 mg twice daily per bird (equivalent to 66 mg/Kgm/day) over a period of forty days. Such therapy only prolonged the one-stage test from the normal 17 seconds to 25 seconds which does not give enough spread suitable for the construction of a dilution curve. This is now thought to have been due to the presence of vitamin K in the pellet diet on which the chicken were kept.

Discussion

Since chicken plasma clots in a very short time on the addition of chicken brain extract and calcium chloride solution, it is safe to assume that chicken plasma contains sufficient concentration of factor VII to maintain adequate haemostasis. The difficulty of factor VII assay was also encountered by several workers. Its species specificity accounts for the low values reported by Didisheim et al (1959), and for the absence of correction of human congenitally factor VII deficient plasma reported by Hawkey (1960) who moreover has used human brain extract. Wartelle (1957b) was also unable to correct the defect in bovine Seitz-filtered plasma, and attributed this to the species specificity of this reaction.

Conclusion

As yet there is no satisfactory method for comparing chicken factor VII activity quantitatively with human factor VII activity, due to the species specificity between factor VII, substrate plasma, and brain extract used.

ASSAY OF CHICKEN FACTOR X

Chicken factor X was measured by the method of Bachmann et al (1958) as described in appendix IV, 18. This method measures the degree of correction of Seitz-filtered bovine plasma on the addition of Russell's viper venom - cephalin reagent and calcium chloride solution, caused by the various dilutions of test plasma or serum. The substrate plasma is virtually free from factors VII and X which are retained completely retained by the Seitz asbestos pads. Since the action of R.V.V. is independent of factor VII, the only variant in this system is the concentration of factor X.

Table 13 shows the results of two experiments on chicken plasma and one experiment on chicken serum. All chicken samples were found to have very feeble factor X activity as compared to the human control plasma or serum, invariably less than 1 per cent.

Discussion

Hawkey (1960) using the same method obtained similar results,

namely 3 per cent for chicken plasma and 1 per cent for chicken serum factor X activities.

Judging from the previous results there are three possibilities that can explain the apparent negligible factor X activity in both chicken plasma or serum.

Firstly, that factor X is altogether absent from chicken plasma or serum, a supposition difficult to accept without further evidence. Also against it is the short one-stage prothrombin time of chicken plasma which must be partly contributed to by the presence of a normal level of chicken factor X in the plasma, that is if we assume that chicken blood clots by the same mechanism as human blood.

The second possibility is that chicken factor X is species specific and does not react with R.V.V. Again, this is refuted by the fact that chicken citrated plasma is clotted by R.V.V., though in a longer time than human plasma, which is mainly due to the lower concentration of chicken plasma prothrombin as shall be discussed later. The results of R.V.V.-cephalin clotting times of chicken plasma are presented in Table 14.

The third possibility is that the intermediate product formed by the interaction of R.V.V. and chicken factor X is itself different and does not react with bovine factor V or prothrombin. This is perhaps the most plausible theory. Indeed, it is hard to visualise that such a test system containing reagents from three widely

differing species (mammalian, avian, and reptile) would yield results that can be interpreted in the usual manner as when using a homologous system.

Until factor X can be prepared and identified as a chemically pure substance that can be estimated by some biochemical method, the question of the presence and concentration of chicken factor X cannot be settled with any degree of certainty.

Conclusion

As yet, there is no satisfactory method for the quantitative assay of chicken factor X activity, due to its species specificity.

ASSAY OF CHICKEN PLASMA PROTHROMBIN

Chicken plasma prothrombin was estimated quantitatively by two methods, the two-stage area method of Biggs and Douglas (1953a), and the globulin fraction method of Douglas and Biggs (1953).

The use of the first method as described in appendix IV, 12, C, was complicated by the species specificity of the brain extract in relation to the plasma assayed. Therefore, each of the control and test plasmas was tested using the homologous brain extract. Apart from this, the test systems were identical. By comparison of the amounts of thrombin produced, chicken plasma was found to have about 13 per cent of the human plasma prothrombin content.

Details of the results are presented in Table 15 and illustrated in Figure 6.

The globulin fraction method of Douglas and Biggs (1953) is also a two-stage technique measuring the thrombin produced from a purified euglobulin fraction which contains the prothrombin. Both control and test plasmas were tested with human brain extract, as described in appendix IV, 12, B. By this method, chicken plasma prothrombin amounted to about 23 per cent of the human control plasma prothrombin content (Table 16).

Discussion

The choice of two-stage techniques was made as they are fairly accurate in reflecting the amount of prothrombin in the plasma investigated. They are minimally affected by variation in the concentration of other accelerating factors, or by variation in the thromboplastin extract activity; while one-stage methods are greatly affected by the amount of accelerators present, and at the same time measure only the amount of thrombin formed in the initial explosive stage of prothrombin conversion.

Though the area method is affected by the amount of antithrombin present in the mixture, here this factor can be ignored since it was shown that chicken plasma antithrombin activity is about the same as that of the human control plasma. Such a low prothrombin content as found in chicken plasma is by no means paradoxical to

the finding of very short clotting times in the one-stage prothrombin time test which depends on the initial concentration of thrombin only. Also, Biggs and Douglas (1953a) have described a patient with acquired prothrombin deficiency whose prothrombin was 10 per cent, yet his prothrombin clotting time was only very slightly prolonged (18 seconds instead of the normal 15 seconds).

In the globulin fraction method the incubation mixture is free from antithrombin. Also the long incubation time of 30 minutes renders the effect of variation in accelerator factors very negligible. Still it shows that chicken plasma prothrombin is very low compared to that of human plasma. It is assumed that the acid precipitation characteristics of both human and chicken prothrombins are similar, though they may well be different.

Warner et al (1939a) measured the prothrombin content of White Leghorn chicken by a two-stage method and report it to be 50 per cent as compared to 84 per cent for the human plasma which was compared to dog plasma that was designated the 100 per cent value. Murphy and Seegers (1948) also utilising a two-stage method found that chicken plasma contained 93-110 units of prothrombin per ml., while human plasma prothrombin was 290 units per ml. (i.e. chicken plasma prothrombin nearly one third the concentration of human plasma prothrombin). The differences in these reported values of chicken prothrombin are to be expected since the test systems

were not identical, also the control plasmas may have had differing prothrombin concentrations, and finally each group of workers tested plasmas of different strains of chicken. But there is general agreement that chicken plasma prothrombin is much lower than human plasma prothrombin. This low concentration of chicken prothrombin explains the long R.V.V. clotting times of chicken plasma previously discussed.

Conclusion

Chicken plasma contained less than one third the prothrombin content of human plasma.

EFFICIENCY OF CHICKEN EXTRINSIC THROMBOPLASTIN SYSTEM

The previous in vitro observations demonstrate that chicken have an efficient extrinsic system for prothrombin activation, which is best exemplified in the one-stage prothrombin time. All the factors concerned in this reaction seem to be present in sufficient amounts as demonstrated by the individual assays, except in the case of prothrombin which is present in rather a borderline concentration in chicken plasma, and is only reflected in the Russell's viper venom clotting time of chicken plasma.

In vivo observations also support the fact that the chicken extrinsic thromboplastin system is efficient. During aspiration

of blood samples from the wing veins, the slightest traumatization to the vein walls by the needle resulted in the clotting of the freshly withdrawn blood inside the syringe within few seconds, inspite of the use of siliconed ware. Also any haematomata that were caused were found to clot quickly.

CHICKEN INTRINSIC THROMBOPLASTIN SYSTEM

The active intrinsic thromboplastin is formed by the interaction of factors derived wholly from the plasma, without the addition of any extraneous reagents. The factors concerned in the intrinsic system shall now be described in turn.

CHICKEN THROMBOCYTES

Chicken thrombocytes are the formed elements in blood analogous to the human blood platelets. They adhere together, as well as to foreign surfaces. Their removal from chicken plasma greatly retards the latter's coagulability, as they possess thromboplastic properties as shall be discussed later. They differ from human platelets in being nucleated cells very similar to the lymphocytes. They are usually oval cells, the size of a small or intermediate lymphocyte, the nucleus is oval or rounded, with a heavy chromatin

network, while the cytoplasm is pale violet and appears vacuolated. The thrombocytes occur in the ordinary blood films either singly or in clumps of two, three, or four cells.

Being nucleated it is very difficult to distinguish them in unstained preparations from the other blood cells of chicken blood. Therefore, an ordinary total white blood cells' count was first made, then a differential count carried out on a blood film stained with Leishman's stain. From these data the total thrombocyte count was calculated. The average of five estimations was 19,650 per c.mm. of whole blood (Table 17), with a range of 16,800 to 21,060 per c.mm. The thrombocytes constituted nearly 50 per cent of the total white blood cells' count.

Thrombocyte counts reported in the literature varied widely. Albertoni and Mazzoni (1891) cited by Forkner (1929) reported 45,566 thrombocytes per c.mm. blood, while Klieneberger and Carl in 1912 found them to vary from 22,900 to 130,000 per c.mm. (cited by Forkner, 1929). Forkner (1929) studied White Leghorn, Plymouth Rock, and Black Jersey Giant Rooster strains. From 29 estimations on 11 animals he found the average thrombocyte count of 34,990 with a range from 5,408 to 142,048 per c.mm. of whole blood. In 1937, Olson has made the most comprehensive survey on the subject. From 408 observations on 89 domestic fowl, he found a variation from 5,400 to 120,000 thrombocyte per c.mm. His average for adult fowl

was 25,700 ± 400 with a standard deviation of 125. He found no daily, weekly, or seasonal variation in the thrombocyte number, nor differences between the sexes, or between birds kept indoors and those kept outdoors.

The thromboplastic characters of chicken thrombocytes will be discussed later in the section on thromboplastin generation.

CHICKEN WHOLE BLOOD CLOTTING TIME

The whole blood clotting time is a measure of the overall efficiency of the intrinsic thromboplastin system of prothrombin activation. Decrease of any of the factors concerned results in the prolongation of the clotting time. It was measured by the method of Lee and White (1913) as described in appendix IV, 3, both in ordinary glass tubes and in siliconed glass tubes under identical conditions. The average clotting time in ordinary glass was about 24 minutes, while in siliconed glass it was about 28 minutes (Table 18). This difference is not significant. These results suggest that chicken blood lacks the coagulation factors concerned in the initial phases of surface contact activation, namely P.T.A. and Hageman factors.

Discussion

Whole blood clotting times obtained ranged from 11 minutes to

45 minutes. This wide difference is believed to be due to the contamination of the fast clotting samples with traces of tissue thromboplastin released from the site of injection. With more experience in obtaining the sample from the wing veins, which are very thin and narrow, with the least possible traumatization, the clotting times were invariably very long. This confirms Bigland and Triantaphyllopoulos (1960) findings that even the fluttering of the vein wall against the needle caused damage to the endothelium and consequently the release of tissue thromboplastin. This can be obviated by the very slow withdrawal of the blood sample. These workers also found that whole blood clotting times varied from wing to wing, and from tube to tube. Their results ranged from 13 to 180 minutes, with an average of 69.2 ± 36.6 minutes.

Wartelle (1957b) reported clotting times of 15 to 20 minutes, with no difference when clotted in glass or siliconed vessels, which was also confirmed by Didisheim et al (1959).

Apart from the lack of contact factors which will be discussed shortly, such long whole blood clotting times may well be due to the lesser number of thrombocytes present, as well as to their greater stability since single thrombocytes were often encountered in blood films indicating an adhesive or clumping tendency much less than that of human platelets.

Conclusions

Chicken whole blood clotting time is much longer than that of human whole blood, and is similar in ordinary glass or siliconed tubes.

CHICKEN PLASMA RECALCIFICATION TIME

Similar to the whole blood clotting time, the recalcification time of citrated plasma also reflects the overall efficiency or potency of the intrinsic thromboplastin system of prothrombin conversion. It is prolonged if any of the concerned factors is deficient.

Chicken plasma recalcification times varied from 480 to 1157 seconds (Table 19), as compared with 160 seconds for the human plasma. It should be noted, however, that while both plasmas were treated identically, chicken plasma was virtually rendered free from the thrombocytes as these sediment easily, while the human plasma was platelet-rich. This lack of thrombocytes naturally accentuated the defect in chicken intrinsic thromboplastin generation, resulting in a more prolonged clotting time as compared to human plasma recalcification time.

CHICKEN PLASMA CONTACT FACTORS

Investigation of chicken plasma contact activation factors concerned in the initial stages of intrinsic thromboplastin generation was carried out utilising the method of Ratnoff and Rosenblum (1958) as described in appendix IV, 24, I.

The addition of chicken intact or glass activated plasma to the intact platelet-poor citrated plasma gave the same result of more than 120 minutes clotting time after the addition of the calcium chloride solution, irrespective of the species of the substrate plasma, whether human or avian. In other words, chicken glass activated plasma did not have any thromboplastic activity, neither in a homologous system, nor in a heterologous one. But human activated plasma shortened the clotting time of intact human plasma from 66 minutes to 13 minutes, and that of chicken intact plasma from 120+ minutes to 38 minutes (Table 20).

Discussion

It is obvious from the above results that chicken plasma lacks completely the factors concerned in the contact activation phase, namely Hageman factor and the plasma thromboplastin antecedent (PTA), to which this test system is sensitive, though lately it has been acknowledged that this method is mainly a measure of the P.T.A., the Hageman factor playing a minor role in it (Ratnoff, 1962).

However, the absolute lack of any activity, together with the fact that whole blood clotting times are similar in ordinary glass and in siliconed glass, strongly suggest that both factors are absent from chicken plasma. It is unfortunate that Hageman factor deficient plasma or P.T.A. deficient plasma were unavailable so that the position could be further clarified. It is highly improbable that these factors are present but species specific, firstly because of the lack of any difference between clotting times in glass and siliconed tubes, and secondly because glass activated human plasma did actually shorten the recalcification time of intact chicken plasma appreciably.

The addition of intact or glass activated chicken plasma had some inhibitory effect on the intact human substrate plasma, when compared to human plasma (Table 20). Though this may be due to dilution effects, it is more probable that it is due to an inhibitor in chicken plasma. The presence of such an inhibitor in chicken plasma was observed by Ratnoff (1961) similar to the inhibitory activity present in plasma of patients with the Hageman trait. Tait and Green (1926) observed that birds' blood or plasma has less tendency to undergo spontaneous coagulation on contact with glass. The absence of Hageman factor in duck plasma was demonstrated by Ratnoff and Margolius (1957), and in chicken plasma by Wartelle (1957 a and b), Thompson et al (1960), and Fantl (1961).

Their experiments were based on the correction of the defect of plasma from patients with congenital Hageman factor deficiency. Wartelle (1957b) also demonstrated that chicken plasma cannot correct the defect of plasma from a patient with congenital P.T.A. deficiency, in a recalcification test system.

Conclusion

Chicken plasma lacks both Hageman factor and plasma thromboplastin antecedent (PTA) activities.

CHICKEN PLASMA ANTIHAEMOPHILIC GLOBULIN ACTIVITY

Antihaemophilic globulin activity was tested for in chicken adsorbed plasma in the thromboplastin generation test of Biggs and Douglas (1953b) as described in appendix IV, 20. The incubation mixture contained human serum diluted 1 in 10 in saline, human platelets' suspension, and M/40 calcium chloride solution, with the adsorbed plasma diluted 1 in 5 in saline as the variable. The substrate was human high-spun plasma. Curve a in Figure 8, I, shows 100 per cent thromboplastin generation from the normal human adsorbed plasma. This activity is due to the A.H.G. content of the adsorbed plasma, as well as its factor V content. Curve b was produced when chicken adsorbed plasma was used instead of the human adsorbed plasma, hardly any active thromboplastin was formed.

In fact the activity of chicken plasma was very similar to that of the saline control (curve c), and to that of the human haemophilic plasma (curve d). Details of the experimental results are presented in Tables 21 and 22.

The test was modified to see the degree of correction of human adsorbed haemophilic plasma by the adsorbed chicken plasma. Apart from the varying dilutions of the chicken plasma in the haemophilic plasma, all the other reagents were kept constant. In Figure 8, II, curves a_1, a_2, a_3, and a_4 represent the thromboplastin generation of mixtures containing human adsorbed plasma diluted 1/2, 1/4, 1/8, and 1/20 in adsorbed haemophilic plasma, respectively. Curve b_1 was obtained for chicken adsorbed plasma diluted 1/2 in haemophilic adsorbed plasma. It has less than 5 per centof the activity of the human control plasma, and very slightly higher activity than the saline control. The actual clotting times in seconds as obtained are presented in Table 21.

These findings suggest that either the chicken plasma does not contain antihaemophilic globulin, or if it does, its activity is species specific. The same battery of tests were therefore repeated using human cephalin as the thromboplastic agent instead of the human platelets. The results when using cephalin are graphically illustrated in Figure 9 (actual findings in Table 23) whose general pattern is nearly a replica of Figure 8, thus confirming the previous

results. Again, the whole procedure was repeated using chicken thrombocyte suspension instead of the human platelets or cephalin, and Figure 10 was compiled from the data obtained and presented in Table 24. It should be noted, however, that for the conversion of clotting times as measured in seconds into percentage values of active thromboplastin, a special thromboplastin dilution curve was made in which the incubation mixture for the production of the active thromboplastin consisted of equal amounts of chicken adsorbed plasma diluted 1/5 in saline, human normal serum diluted 1/10 in saline, chicken thrombocyte suspension, and M/40 calcium chloride solution. Subsampling was onto human high-spun normal plasma. Thus Figure 7 was constructed, and was used to convert the measured clotting times from seconds into percentage of chicken active thromboplastin. Though this curve was made in a similar manner to the human thromboplastin dilution curve, in the latter the 10 seconds clotting time was arbitrarily designated the 100 per cent value since it was the shortest time obtained. In the chicken incubation mixture the shortest clotting time obtained was 20 seconds and this was therefore arbitrarily designated the 100 per cent value for chicken active intrinsic thromboplastin. Therefore, no direct comparison can be made between the curves in Figure 10 and those in Figures 8 and 9.

Analysis of the data presented in Figure 10 shows that chicken

adsorbed plasma when incubated with human serum and chicken thrombocytes produced the greatest amount of active thromboplastin (curve b), while human adsorbed plasma had less than half the activity of the chicken adsorbed plasma (curve a). This means that species specificity occurs in the reaction between the adsorbed plasma and the platelet or thrombocyte suspension. Homologous mixtures generate active thromboplastin optimally, while heterologous mixtures hardly generate any active thromboplastin at all.

Figure 10, II, represents the values obtained by correction of human haemophilic plasma with adsorbed chicken plasma or with adsorbed human plasma, using chicken thrombocytes as the lipid thromboplastic agent. Though this figure is difficult to interpret yet it is seen that curve b_1 which represents chicken adsorbed plasma diluted 1/2 in haemophilic adsorbed plasma, shows better activity than curve a_1 obtained with human normal adsorbed plasma diluted similarly in haemophilic plasma. Both curves show activity much less than would be expected because factor V which is supplied in the adsorbed plasma was also found to be species specific in the thromboplastin generation mixture, as shall be discussed later. Thus mixtures containing both chicken and human haemophilic plasma suffer from decrease of the potentially active factor V by half in the 1/2 dilution, and by one quarter in the 1/4 dilution (curve b_2). This fact accounts for the rapid decline in activity observed when

chicken plasma is diluted in human haemophilic plasma. Also, curve a_1 cannot be favourably compared with curve b_1 because the factor V content of the human mixture used is all human and virtually inactive with chicken thrombocytes, thus impeding the proper generation of active thromboplastin, apart from the species specificity intervening between the chicken thrombocytes and the human A.H.G. activity in the adsorbed plasma.

Assay of the A.H.G. content of chicken adsorbed plasma by the method of Pitney (1956) using human platelets, and measuring the ability of chicken adsorbed plasma to correct the defect of human haemophilic adsorbed plasma, showed that chicken plasma had no A.H.G. activity as compared to the human control plasma (Table 28). This again reflects the species specificity of the chicken A.H.G. towards the human platelets. Since antihaemophilic globulin activity is defined as that activity in adsorbed plasma that corrects the defect in human haemophilic plasma in a thromboplastin generation mixture using human platelets, it is concluded that chicken adsorbed plasma has no such activity. But in a thromboplastin generation mixture using chicken thrombocytes, chicken adsorbed plasma showed an appreciable activity which can only be presumed to be due to its content of a factor analogous to human antihaemophilic globulin.

The species specificity of chicken A.H.G. activity was investigated in a purified thromboplastin generation mixture

consisting of equal amounts of human cephalin diluted 1/100 in saline, human serum diluted 1/10 in saline, purified human factor V prepared by ammonium sulphate precipitation (appendix III, 9) in a concentration equivalent to that present in adsorbed plasma diluted 1/5 in saline, purified human or chicken A.H.G. prepared by ammonium sulphate precipitation (appendix III, 11) in a concentration equivalent to adsorbed plasma diluted 1/5 in saline, and M/40 calcium chloride solution. The results in Table 29 show that in mixture number 71 containing human A.H.G. potent active thromboplastin was produced, while in mixture 71b containing chicken purified A.H.G. no active thromboplastin was produced, signifying that chicken A.H.G. is species specific towards human cephalin, as revealed previously using adsorbed chicken plasma with human platelets or human cephalin (Table 21, mixture 1, Table 23, mixture 17, and curves b in Figures 8 and 9 respectively). This lack of thromboplastin generation cannot be blamed on the heterogeneity of A.H.G. - factor V, since also the combination of chicken purified A.H.G. with chicken purified factor V did not yield any active thromboplastin in the presence of human cephalin (Table 29, mixture 71c).

Discussion

Wartelle (1957b) reported 100 per cent for chicken plasma A.H.G. activity when measuring the degree of correction of human

haemophilic plasma in a recalcification time test using human cephalin as the thromboplastic lipid. The clotting time of the mixture containing chicken plasma diluted 1 in 20 was 3' 50", similar to that obtained for the mixture containing human plasma similarly diluted. I was unable to reproduce her findings on using the same technique, namely the method of Soulier and Larrieu (1953 a and b). The recalcification time of the mixture which contained chicken plasma diluted 1 in 20 was 4' 34", while the mixture containing human plasma diluted 1 in 160 clotted in 4' 3", which gives a value of less than 12 per cent of A.H.G. activity for the chicken plasma A.H.G. activity. I believe this test is not suitable for the measurement of chicken plasma A.H.G. activity as the use of human cephalin, as well as the human factor V content of the haemophilic plasma interfere with the proper formation of active thromboplastin due to their species specificity, as previously demonstrated.

Didisheim et al (1959) also observed that chicken antihaemophilic factor (A.H.F.) does not shorten the recalcification time of human haemophilic plasma. Hawkey (1960) maintains complete correction of haemophilic plasma by 1 in 10 dilution of chicken adsorbed plasma in a thromboplastin generation test using human cephalin, while she reported in another section of her thesis that chicken plasma thromboplastin generation was very poor whether human or chicken

cephalin were used, the actual clotting times varying from 90 to more than 180 seconds. Her findings are contradictory and difficult to analyse, more so because she used chicken high-spun plasma as the substrate which as shall be shown later imposes yet another interfering factor, namely that of delayed chicken substrate reactivity.

Conclusions

In a homologous human thromboplastin generation mixture, chicken adsorbed plasma does not have any A.H.G. activity, nor does it correct the defect of haemophilic plasma. But on using chicken thrombocyte suspension instead of human platelets, the chicken adsorbed plasma together with human serum produced an appreciable amount of active intrinsic thromboplastin, suggesting that chicken plasma contains a factor or factors analogous to human A.H.G. and factor V, which specifically react with chicken thrombocytes. Chicken purified A.H.G. is species specific and does not react with human cephalin.

SPECIFICITY OF CHICKEN FACTOR V IN THE T.G.T.

The specificity of the reaction of chicken purified factor V with human reagents in a modified thromboplastin generation mixture was investigated by incubating equal amounts of purified human A.H.G., human serum diluted 1/10 in saline, human cephalin diluted

1/100 in saline, M/40 calcium chloride solution, and purified chicken or human factor V as the variable. Table 30 and Figure 11 show that while human factor V yielded 80 per cent active intrinsic thromboplastin (curve a), chicken factor V yielded 14 per cent activity (curve b), and the saline control yielded 7 per cent activity (curve c).

Conclusion

Chicken purified factor V is species specific and does not react with the active product formed from human factors IX, VIII, X, and cephalin, in the presence of calcium chloride solution.

CHRISTMAS FACTOR ACTIVITIY OF CHICKEN SERUM

Chicken Christmas factor activity in serum was tested by substituting chicken serum diluted 1/10 for the human serum, in a thromboplastin generation mixture containing all the other necessary factors. Since factor VII does not contribute any activity in the intrinsic system, and factors XII and XI are supplied by the adsorbed plasma as well, the only variables introduced would be factors X and IX.

In Figure 12, curve a represents human serum activity when incubated with human adsorbed plasma, human platelets or cephalin (Figures 12, I and II respectively), and M/40 calcium chloride.

The chicken serum activity was nearly nil, similar to the saline control, and to Christmas disease serum (curves b, c, and d). This was confirmed by correction experiments of human Christmas disease serum, using a method based on that of Pitney (1956) as described in appendix IV, 22. Chicken serum was found to have no ability at all to correct the defect of Christmas disease serum (Table 31). Again we can deduce that chicken serum has no activity either because of the absence of factor IX, or to its species specificity. In view of the previous species specificity observed with human lipid reagents, further investigation for Christmas factor was carried out in a modified thromboplastin generation mixture in which the incubation mixture consisted of equal parts of chicken adsorbed plasma diluted 1/5 in saline, chicken thrombocyte suspension, chicken or human serum diluted 1/10 in saline, and M/40 calcium chloride solution. In Figure 13, I, curve a shows that human serum reacted with chicken adsorbed plasma and chicken thrombocytes yielding a fairly active thromboplastin, and suggesting that human Christmas factor and human factor X are not species specific in such a system. Curve b for chicken serum still had very little activity, though slightly higher than the saline control or the Christmas disease serum (curves c and d). In a similar system, dilutions of human serum in Christmas serum ranging from 1/2 to 1/20 yielded 100 per cent activity (Figure 13, II, curves a_1,

a_2, a_3, and a_4). Chicken serum diluted 1/2 in Christmas serum (curve b_1) yielded about half the activity of the human serum diluted 1/20, proving that chicken serum lacks completely Christmas factor activity. Its species specificity cannot be blamed since the system contains chicken thromboplastic reagents. The factor X content of the chicken serum is of no importance since the human Christmas disease serum already contains optimal concentration of factor X. The exact concentration and/or specificity of chicken factor X in the serum could not be estimated in such a system due to the unavailability of human serum congenitally deficient in factor X.

Discussion

Wartelle (1957b) found that chicken serum lacks Christmas factor which she estimated as 1 per cent of the human Christmas factor concentration, using the recalcification time technique of Soulier and Larrieu (1953 a and b) by correction of Christmas disease plasma. Didisheim et al (1959) also reported similar findings. Hawkey (1960) found that chicken serum diluted 1 in 10 had some corrective activity on Christmas serum in a thromboplastin generation mixture using human cephalin. Her six minute clotting time for chicken serum was 16 seconds as compared to 10 seconds for the human serum; thus chicken serum possessed about one third the activity of the human serum when converted in terms of percentage

active thromboplastin.

Conclusions

Chicken serum has no Christmas factor activity. Human serum factors are not species specific and react with chicken adsorbed plasma and chicken thrombocytes to produce an intrinsic thromboplastin of moderate activity when compared to human active thromboplastin.

THROMBOPLASTIC ACTIVITY OF CHICKEN THROMBOCYTES

The haemostatic function of chicken thrombocytes seems to be much less than that of human platelets. Firstly, this is reflected in the very long whole blood clotting time, though this is affected greatly by the absence of other clotting factors, namely Hageman factor, P.T.A., and factor IX. Secondly, it is evident from the inefficient clot retraction of chicken whole blood as estimated by the method of Macfarlane (1938) described in appendix IV, 4. The average of five estimations on chicken blood was 19.8 per cent of serum expressed in one hour (Table 34), as compared to 48-64 per cent for human normal blood. The chicken blood clots were soft and friable. This deficiency may also be due to the much lower number of chicken thrombocytes per unit volume of blood, though on the other hand this is counteracted by the low haematocrit value of chicken blood which was usually about 30 per cent; since

Macfarlane (1939) has shown that the packed cell volume affects the clot retraction inversely. Thirdly, the weak thromboplastic activity of chicken thrombocytes is manifest in the thromboplastin generation test when substituting them for the human platelets. The shortest clotting times of the substrate were about 20 seconds as compared to 10 seconds when using human platelets or cephalin.

Finally it should be recollected that their adhesive power is less than that of human platelets, since chicken thrombocytes were frequently observed in blood films to occur singly.

Bracco et al (1956) separated from extracts of chicken thrombocytes a material which corresponded perfectly to 5-hydroxytryptamine in its chemical, spectrophotometric, and biological properties. They concluded that as far as the vasoconstrictor factor is concerned, bird thrombocytes are physiologically identifiable with mammalian platelets.

HOMOLOGOUS CHICKEN INTRINSIC THROMBOPLASTIN GENERATION

Because of the species specificity between human platelets or cephalin and chicken adsorbed plasma factors, together with the lack of factors XII, XI, and IX in chicken adsorbed plasma and serum, it was expected to find no generation of active thromboplastin in mixtures of chicken adsorbed plasma and serum alone. Figure 14

depicts virtually no activity for the mixture of chicken adsorbed plasma and serum, whether with human platelets (curve A) or with human cephalin (curve B). On using a completely homologous system containing chicken thrombocytes, chicken adsorbed plasma, and chicken serum, no activity at all was produced when subsampling onto chicken high-spun plasma (Figure 16, IV, curve b), while a negligible amount of active thromboplastin was produced when subsampling onto human high-spun plasma (Figure 16, IV, curve a), the minimum clotting times were 30 seconds for the homologous chicken mixture (Table 24, mixture 34), compared to 10 seconds for a homologous human mixture (Table 21, mixture 4)

Discussion

The poor thromboplastin generation obtained with chicken blood is generally agreed on by the various workers who investigated the subject. Wartelle (1957b) reported that chicken thromboplastin generation was weak compared to that of the human, but she concluded that it was more active on fresh chicken substrate plasma than on human high-spun plasma, contrary to my findings. But if we examine her actual clotting times we find that the homologous chicken mixture clotted the human substrate plasma in about 80 seconds, and clotted the chicken fresh substrate plasma in about 45 seconds, after 11 minutes incubation with human cephalin cephalin. From the active thromboplastin dilution curves it can be seen that such

long clotting times actually denote an active thromboplastin in the region of 0.75 - 3 per cent of the human active thromboplastin, which is very poor indeed. Didisheim et al (1959) and Hawkey (1960) also observed that chicken thromboplastin generation was very poor, even in homologous systems and not improved by using chicken cephalin (Hawkey, 1960).

The inefficient formation of chicken intrinsic active thromboplastin due to the various deficiencies or species specificity previously demonstrated, is responsible for the long chicken whole blood clotting times obtained, also for the long recalcification times of chicken plasma.

Conclusion

The generation of intrinsic thromboplastin from homologous chicken reagents is very poor and virtually nil when compared to human thromboplastin generation.

CHICKEN PLASMA SUBSTRATE REACTIVITY IN THE T.G.T.

The use of chicken high-spun plasma as the substrate in the thromboplastin generation test instead of the human high-spun plasma was found to give much longer clotting times than previously obtained with the human plasma substrate. For example, the human incubation mixture which had 100 per cent thromboplastin activity

on the human substrate plasma yielded only 10 per cent thromboplastin activity on chicken substrate plasma (Figure 15, I, curves a and b respectively). The same findings were found on using human cephalin instead of the human platelets (Figure 15, II, curves a and b). Again, incubation of chicken adsorbed plasma with human serum in the presence of chicken thrombocytes had an activity of 100 per cent on the human substrate, but only 38 per cent on the chicken substrate plasma (Figure 16, III, curves a and b respectively). This observation is exaggerated in a completely homologous chicken thromboplastin generation mixture (Figure 16, IV, curves a and b).

Discussion

Though the delayed reactivity of the chicken substrate plasma may be attributed to the low prothrombin content of chicken plasma, it is my belief that the main factor responsible for this delay is the property of chicken fibrinogen whose reactivity even to preformed thrombin is delayed, as observed in the thrombin--fibrinogen reaction (Table 3 and Figure 3).

Conclusion

The reactivity of chicken substrate plasma to a thromboplastin generation mixture is delayed and is due to its content of chicken fibrinogen.

INHIBITORS AGAINST HUMAN THROMBOPLASTIN IN CHICKEN BLOOD

The presence of inhibitors in chicken plasma and serum against human intrinsic thromboplastin formation was investigated by the method of Biggs and Macfarlane (1957) described in appendix IV, 23.

Chicken adsorbed plasma when added to human adsorbed plasma did not have any immediate inhibitory effect and gave clotting times similar to those obtained with the saline control (Table 32, A, mixtures 75 and 74; illustrated in Figure 17, A, curves b and a_1). But after incubation of the same mixture for two hours at 37°C. the chicken plasma was definitely inhibitory when compared to the saline control (Table 32, B, mixtures 78 and 77; Figure 17, B, curves b and a_1), or when compared to the activity of the same mixture before incubation (Figure 17, A, curve b). When the chicken adsorbed plasma was heated at 56°C. for 15 minutes before mixing it with the human adsorbed plasma its inhibitory activity was found to be much less than the unheated chicken adsorbed plasma, being very similar to the saline control (Figure 17, B, curve c).

On the other hand, testing chicken serum in the same manner for inhibitors against human intrinsic thromboplastin did not reveal any such activity. The serum gave values very much the same as the saline control (Table 33, A and B; Figure 18, A and B).

Discussion

The inhibitory effect of chicken adsorbed plasma on human intrinsic thromboplastin formation cannot be ascribed to its content of chicken thrombocytes or extracts therefrom, as these are removed completely by adsorption. The removal of the observed inhibitory activity by heating the adsorbed plasma at 56°C. for 15 minutes suggests that the main factor responsible for such activity is the fibrinogen, since it is the only factor present in a great amount and removable by heat precipitation. Though not much is known about antithromboplastin, according to Niewiarowski et al (1959) antithromboplastin withstands heating to 60°C. for 20 minutes, a procedure which inactivates the antithrombin. So, if it were an antithromboplastin activity, the heated adsorbed plasma would have had the same inhibitory effect as the unheated plasma. It is unlikely to be due to chicken antithrombin, because the latter was found to be present in nearly the same concentration as the human plasma antithrombin. Wartelle (1957b) has observed that defibrination of the chicken adsorbed plasma by heating at 64°C. for 3 minutes before its addition to the human thromboplastin generation mixture gave a better yield of thromboplastin and concluded that chicken fibrinogen in the substrate or adsorbed plasma inhibits thromboplastin formation.

It may well be that chicken plasma thromboplastic factors react

with human reagents (cephalin) as it were by competetive interference, rendering it unavailable for the human plasma factors, thus producing an inactive thromboplastin. This is suggested by the fact that such plasma factors are also destroyed by heating and may have therefore been the responsible agents. This is unlikely, because if chicken plasma factors other than fibrinogen did react with human cephalin their inhibitory effect would have also been obvious in mixtures of human and chicken plasmas without incubation, since such a reaction occurs in a short time and does not require prolonged incubation.

Conclusions

Chicken adsorbed plasma has an inhibitory effect when added to a human thromboplastin generation mixture for a sufficiently long time, and can be removed by heating the adsorbed plasma to precipitate the fibrinogen, which is believed to be the main factor responsible for such anti-human thromboplastin activity.

Chicken serum does not possess any inhibitory activity against human intrinsic thromboplastin.

EFFICIENCY OF THE CHICKEN COAGULATION MECHANISM

In view of the demonstrated deficiency of various coagulation factors in chicken blood, namely Hageman factor, plasma thromboplastin factor, Christmas factor, and the low prothrombin content, together with the delayed reaction of chicken fibrinogen, it may seem that chicken are severely handicapped by a haemorrhagic diathesis. This in fact is not the case. Firstly, it is quite obvious that chicken survive the stresses they are subjected to quite admirably and flourish extremely well without showing any sign of a haemorrhagic condition. Secondly, the adequacy of the chicken intrinsic thromboplastin system is partly reflected in the complete prothrombin utilisation as assessed from the very low prothrombin consumption index obtained for chicken blood, whose average was 7.3 per cent (Table 35), a value that is perfectly normal when compared to human prothrombin consumption index values, though it may be argued that chicken prothrombin consumption -and hence thrombin generation- does not necessarily occur at the same rapid rate as in human blood. Indeed, this may well be the case, judging from the prolonged clotting times observed for chicken whole blood and recalcified plasma.

Though chicken have an efficient extrinsic thromboplastin system, by itself it is not sufficient for a normal clotting defence mechanism since both the intrinsic and extrinsic thromboplastin systems are

known to complement each other in the intact individual; deficiency in one or the other might lead to a life handicapped with haemorrhages or haematomata from the least of injuries.

It should be remembered, however, that the anatomy and physiology of chicken is different from those of the human. A system known to be required for the well-being of human individuals need not be necessary at all for chicken. Furthermore, chicken may well have their own particular, as yet unknown, clotting defence mechanism.

The striking anatomical differences from the human are the anatomy of their legs which have very tight and strong integument in the exposed parts, a feature that perhaps prevents the formation of haematomata as well as shields the underlying structures from external stresses. Also, such a wealth of feathers as they possess again seems to protect their bodies from external injuries. To my mind, it seems fair to expect that any injury in chicken would be detrimental mainly because of the interference with the proper functioning of vital organs, rather than to the ensuing haemorrhage.

SUMMARY OF CHICKEN BLOOD COAGULATION MECHANISM

Chicken blood contains many coagulation factors in concentrations sufficient for effective haemostasis, namely fibrinogen, prothrombin, factor V, and probably factors VII, VIII, and X.

It completely lacks the Hageman factor (factor XII), the Plasma Thromboplastin Antecedent (factor XI), and the Christmas factor (IX).

Chicken platelets are nucleated cells with a weak thromboplastic activity. Chicken fibrinogen has a characteristic delayed reactivity to thrombin, whether homologous or heterologous. Chicken adsorbed plasma possesses an inhibitory activity against human intrinsic thromboplastin, possibly due to its content of fibrinogen. The antithrombin activity of chicken plasma is similar to that of human plasma.

Several coagulation factors show absolute species specificity, namely tissue extracts, factor V, factor VII, factor VIII, the thrombocyte factor, and possibly factor X.

They possess an efficient extrinsic thromboplastin system, while their intrinsic system suffers from multiple deficiencies that render it very inefficient to sustain normal haemostasis, judging by the accepted normal human standards.

PART III

BOVINE BLOOD COAGULATION MECHANISM

BOVINE BLOOD COAGULATION MECHANISM

Data presented in this section are based on the results of experiments conducted on blood samples obtained from domesticated cattle at the slaughter-house, which were of several breeds. All the animals were healthy and were killed for human consumption purposes only. None of them suffered any disease detectable by the medical authorities of the slaughter-house.

BOVINE PLASMA FIBRINOGEN

Estimation of bovine plasma fibrinogen by the micro-Kjeldahl method of Cullen and van Slyke (1920) and by Stirland's method (1956) gave similar results ranging from 620 to 750 mg fibrinogen per 100 mls plasma, with an average of 697 mg per cent, (Table 36).

This suggests that bovine fibrinogen has the same heat precipitation characteristics as the human fibrinogen.

Discussion

Fibrinogen is present in bovine plasma in a much higher concentration than human plasma fibrinogen, nearly twice the latter. Stormorken (1957b) also using Kjeldahl's method obtained values of 450 - 750 mg with an average of 560 mg per 100 mls of plasma.

The observed similarity in heat precipitation characteristics

of bovine and human fibrinogens agrees with the fact that both have a molecular weight of about 340,000 (Caspary and Kekwick, 1954).

Conclusion

Bovine plasma fibrinogen concentration is higher than the human, with an average of about 700 mg per 100 mls of plasma.

THROMBIN - FIBRINOGEN REACTION OF BOVINE PLASMA

Bovine citrated plasma gave slightly prolonged clotting times when clotted with various concentrations of bovine thrombin, as compared to human citrated plasma similarly treated (Table 37 and Figure 19). This prolongation was more obvious with the lower thrombin concentrations as expected, since the longer clotting times exaggerate the difference. The addition of a 1 per cent solution of pyrocatechol (final concentration in the clotting mixture is 0.3 per cent) shortens both the bovine and human plasma clotting times similarly, though the former was still slightly longer (Figure 19, curves b_1 and a_1 respectively).

Discussion

The longer clotting times observed with bovine plasma is believed to be mainly due to its higher content of fibrinogen, since the clotting time is directly proportional to the fibrinogen concentration provided the thrombin concentration is constant.

There can be no question of species specificity of the thrombin fibrinogen reaction since bovine thrombin was used in clotting both plasmas; if any such specificity existed the bovine plasma clotting times should have been the shorter.

The delay is also not due to antithrombin which was found to be present in the same concentration in bovine plasma as in the human plasma as shall be demonstrated later.

Seegers and Smith (1942) observed that 1 ml of human oxalated plasma required 2 units of bovine thrombin to clot in 15 seconds at 28°C., while bovine oxalated plasma required 2.2 - 2.5 units, again reflecting the delayed reactivity of bovine plasma when clotted with the homologous thrombin solution. Astrup (1950) found that the optimum pH for bovine thrombin in phosphate buffer of 0.2 ionic strength to be 7.5 for human fibrinogen and 7 for bovine fibrinogen suggesting their species specificity. Stormorken (1957b), however, found that the pH optima of purified fibrinogens were less distinct than those of the respective plasmas and were nearly the same between pH 7.05 and 7.8, but that the lower limits varied, that for human fibrinogen was 7 while that for bovine fibrinogen was 6.9 which is a very slight difference. He also observed that ox plasma was less reactive than human plasma to bovine thrombin, being 93 per cent and 100 per cent respectively.

Conclusion

Bovine plasma thrombin-fibrinogen reaction is slightly more delayed than that of human plasma when clotted with bovine thrombin. This is thought to be due to its higher fibrinogen content, and not to species specificity or antithrombin interference.

BOVINE PLASMA ANTITHROMBIN ASSAY

Antithrombin was estimated in bovine plasma by the modified method of Astrup and Darling (1942d). The average of two such experiments was 100.7 per cent of the human plasma antithrombin content (Table 38, and Figure 20). However, using the method of Douglas and Biggs (1953)the average of three experiments was 130 per cent of the human plasma antithrombin activity (Table 39, and Figure 21). This discrepancy can perhaps be explained by the assumption that the latter method is more sensitive to the fibrinogen content of the plasma tested as it is present in an appreciable amount in the incubation mixture and therefore its adsorptive effect on thrombin would be more likely to affect the amount of residual thrombin. While in the method of Astrup and Darling (1942d) the highest plasma volume present in the last incubation mixture is 0.06 ml mixed with 1 ml of thrombin solution, thus rendering the adsorptive effect of fibrinogen very negligible due to the minute

amount of fibrinogen present.

Conclusion

Antithrombin III concentration in bovine plasma is equal to that present in human plasma. The total antithrombin activity of bovine plasma is about 130 per cent that of human plasma, most probably due to its higher content of fibrinogen.

BOVINE EXTRINSIC THROMBOPLASTIN SYSTEM

Using Quick's one-stage prothrombin time test, the bovine brain extract was found to clot bovine plasma in as short a time as the human brain and human plasma mixture, namely 14.8 and 15.8 seconds respectively (Table 40). The use of the heterologous brain resulted in a prolonged clotting time in either case, 31 and 36 seconds respectively. A mixture of human and bovine plasma was clotted with bovine brain in a slightly longer time than when bovine plasma was mixed with saline, which means that human plasma had an inhibitory effect on the bovine extrinsic system. No such inhibitory activity was observed for bovine plasma when added to a human system, in fact the bovine plasma shortened the clotting time slightly presumably due to its much higher factor V content as shall be seen later.

Discussion

The previous findings suggest a certain degree of species specificity in the reaction between bovine brain extracts and bovine plasma. Similar findings have been reported by Stormorken (1957d), Goldstein (1958), and Quick et al (1961).

Conclusion

Bovine brain extract is species specific towards bovine plasma. A homologous bovine extrinsic system clots in as short a time as a homologous human extrinsic system. The bovine extrinsic system is therefore assumed to be haemostatically as efficient as the human system.

SPECIES SPECIFICITY OF BOVINE FACTOR V

The species specificity of purified human or bovine factor V was investigated in a two-stage extrinsic thromboplastin system by incubating the purified factor V with the diluted human or bovine brain extract and testing the formed thromboplastin activity of the mixture by subsampling onto human substrate fresh plasma and observing the latter's clotting time on simultaneous recalcification as described in appendix IV, 16.

Table 41 shows that human brain extract produced more activity when incubated with the bovine factor V than with the human factor V.

It seems therefore that bovine factor V does not exhibit any species specificity, or if any this is masked by the higher concentration of the bovine factor V. The species specificity of the bovine brain extract towards the human substrate plasma was counteracted by the previous inclusion of human serum diluted 1 in 10 in saline to the incubation mixture allowing the serum factors to react with the bovine brain, as they seem to be the main factors responsible for such species specificity. Prolonged incubation was not necessary since the complete reaction seemed to occur within one minute of incubation.

From the way Stormorken (1957c) used bovine and human purified factor V preparations interchangeably in his two-stage methods for prothrombin estimations it seems that he believed bovine factor V not to be species specific towards human brain thromboplastin.

Conclusions

Bovine factor V is not species specific towards human brain extracts. Similarly, human factor V is not species specific towards bovine brain extracts.

BOVINE PLASMA FACTOR V ASSAY

Bovine plasma factor V content was estimated by the degree of correction of human stored plasma, utilising human brain extract.

The average of three estimations was 268 per cent of the human plasma factor V activity (Table 42 and Figure 22).

Owren (1947) first observed the high bovine plasma factor V content. Murphy and Seegers (1948) using a two-stage technique reported about a tenfold concentration of bovine factor V as compared to the human factor V concentration in plasma (120-140 units per ml and 12-17 units per ml of plasma respectively). Using one-stage techniques Wartelle (1957b) found it to be four times the human, Stormorken (1957a) reported it to be five times the human, Nour-Eldin and Wilkinson (1957) found it three to four times the human, while Quick et al (1961) reported it as eight times the human factor V concentration. Stormorken (1957a) also demonstrated the increased stability of bovine factor V, a fact confirmed by Wartelle (1957b). This stability accounts for the presence of factor V in the bovine serum as observed by Alexander, Goldstein and Landwehr (1951). Bergna (1960) reported a value of 200 per cent for the factor V activity in bovine serum as compared to human plasma factor V content.

Conclusion

Factor V concentration in bovine plasma is much higher than its concentration in human plasma, at least by threefolds.

SPECIES SPECIFICITY OF BOVINE SERUM FACTORS

A purified preparation containing factors VII and X (appendix III, 10) was incubated with the brain extract and calcium chloride solution, and at minute intervals samples were removed and pipetted onto fresh human plasma or onto phenindione plasma together with an aliquot of calcium chloride solution. The clotting time of the substrate is indicative of the thromboplastic activity produced in the incubation mixture, this being dependent on the proper interaction of the reagents concerned.

The incubation of human brain extract and human purified factor VII and X preparation resulted in clotting of the substrate normal plasma and phenindione plasma after one half minute of incubation in 11 and 15 seconds respectively (Table 43), while if incubated with the bovine serum factors preparation the clotting times were 20 and 27 seconds respectively. The six minute clotting times were similar. Thus there was a certain degree of species specificity between the bovine serum factors and the human brain extract which was only demonstrable within the first minute of incubation, being surmounted with prolonged incubation.

Similarly, incubation of the human serum factors with bovine brain extract resulted in longer clotting times within the first minute of incubation than those obtained with the homologous bovine

mixture. Again, further incubation eliminated this delay. Furthermore, the bovine brain extract activated with the bovine serum factors clotted the human substrate plasma in a shorter time than that obtained with the human homologous mixture. This is difficult to explain satisfactorily, but it should be remembered that both the brain preparations were not necessarily of identical concentration in terms of active material per unit volume.

Discussion

Stormorken (1957d) has emphasised the species specificity of the bovine factor VII in one-stage assay systems. This is confirmed by the findings reported here in the two-stage system. The delay observed in the half and one minute readings is that measured in a one-stage test which is the sum total of the time required for the brain extract activation (incubation time in the above system) and that required for prothrombin activation and fibrin formation. The dissappearance of this delay on further incubation of the heterologous reagents suggests that the species specificity observed is not absolute as for instance in the case of chicken-human mixtures.

As yet no information has been published regarding the species specificity of bovine factor X. Since the purified preparation of factor VII also contained factor X, it is assumed that partial species specificity of both these factors is responsible for the observed delay. Until methods are devised for the preparation of

factors VII and X in pure form free from each other, the exact specificity of each factor cannot be known with certainty.

Conclusions

Human and bovine serum factors are partly species specific towards the homologous brain extract. Prolonged incubation of heterologous mixtures eliminates their species specificity.

BOVINE PLASMA FACTOR VII ASSAY

Utilising the one-stage methods of Biggs and Macfarlane (1957) and Koller et al (1951) it was found that the concentration of factor VII in the test plasma was dependent on the type (species) of the brain extract used. The use of human brain in the first method showed that bovine plasma contained about one fifth of the human plasma factor VII content (Table 44, experiments 1 and 2; and Figure 23, curve A). Its use in the second method gave a value of 13 per cent of the human factor VII concentration (Table 44, method B with human brain; and Figure 23, curve B). Assay of the same plasma pools with bovine brain extract instead showed that the factor VII content of bovine plasma was about four times that of human plasma. In other words, the human plasma factor VII content was calculated as 23 per cent of the bovine plasma factor VII activity (Table 44, method B with bovine brain; and Figure 23, curve C).

Discussion

Firstly it should be noted that the factor VII deficient plasmas used in both the above assay systems were also deficient in factor X to a certain extent in the phenindione plasma and completely in the Seitz-filtered bovine plasma. Therefore the measured corrective activity of the test or control plasmas was actually due to both factors VII and X. However, in view of the presence of factor X in bovine plasma in a similar concentration to human plasma as shall be shown shortly, the differences observed in these assay systems may be assumed to be largely due to changes in factor VII content. Still, the issue is further complicated by whatever effect the species specificity of factor X may have towards the type of brain extract used.

Judging from the short one-stage prothrombin time of bovine plasma, together with the reciprocal findings observed in the factor VII assays according to the species of brain used, it can be safely presumed that bovine plasma contains factor VII in a concentration sufficient to effect normal haemostasis.

The low values of bovine plasma factor VII content reported by Koller et al (1951), Wartelle (1957b), and Didisheim et al (1959) can be ascribed to the use of human brain extracts in their assay systems. Stormorken (1957d) tried measuring the factor VII content by correction of human plasma congenitally deficient in factor VII,

supplying excess factor V of bovine origin, and clotting the mixture with the homologous brain thromboplastin. He stressed that the quantitative comparison between the species cannot be done with present day methods. Quick et al (1961) also realised this difficulty while investigating variations in the prothrombin complex in various species, and did not attempt to study the quantitative differences of factors VII and X in the species surveyed. Strangely enough, Hawkey (1960) reported a value of 86 per cent for bovine plasma factor VII activity, and 90 per cent for bovine serum factor VII activity, though she measured the degree of correction of human factor VII deficient plasma of congenital origin, using human brain extract as the lipid thromboplastin agent.

Conclusion

No valid quantitative measurement of bovine factor VII as compared to human factor VII in plasma could be made with present day methods with any degree of precision. Bovine plasma, however, contains sufficient factor VII to give a normal one-stage prothrombin time in a homologous system.

BOVINE PLASMA FACTOR X ASSAY

Bovine plasma factor X content was estimated by the method of Bachmann, Duckert, and Koller (1958) which measures the degree of correction of Seitz-filtered bovine plasma when clotted with Russell's viper venom and cephalin in the presence of calcium chloride solution. The action of R.V.V. is independent of factor VII and therefore the only variant in the test mixture will be the factor X concentration, all the other necessary factors being present in optimum amounts.

Bovine plasma was found to contain 101 per cent of the human plasma factor X content, while bovine serum contained 90 per cent of the human serum factor X activity (Table 45). No species specificity towards the R.V.V. was observed.

Hawkey (1960) using the same method reported that bovine reagents contained comparable amounts of factor X as in human serum.

Conclusion

Bovine plasma and serum contain about the same concentration of factor X activity as present in the human control reagents.

MEASUREMENT OF BOVINE PLASMA PROTHROMBIN

The bovine plasma prothrombin content was estimated by three different methods. Firstly, by the one-stage technique of Koller et al (1951) which measures the degree of correction of bovine plasma rendered prothrombin-free by adsorption, adding prothrombin-free human serum to supply the serum factors necessary for the extrinsic prothrombin activation, and clotting the mixture with brain extract in the presence of calcium chloride solution. Using human brain extract, the bovine plasma prothrombin was estimated as 45 per cent of the human plasma prothrombin (Table 46, experiment 1; and Figure 24, curve A). When bovine brain extract was used, the bovine plasma prothrombin content was found to be about twice the amount present in the human plasma (Table 46, experiments 2 and 3; and Figure 24, curve B).

The second method used was the two-stage area method of Biggs and Douglas (1953a). Bovine plasma prothrombin values when estimated using human brain were 116 per cent and on another occassion 233 per cent of the human plasma prothrombin (Table 47, experiments 8 and 7 respectively). The use of bovine brain extract, however, gave higher results for the same plasmas, namely 162 and 299 per cent respectively (Table 47, experiments 5 and 6). A third bovine plasma pool had 101 per cent of human plasma prothrombin,

even when estimated with bovine brain extract.

The third method employed was the globulin fraction technique of Douglas and Biggs (1953) which is also a two-stage system. In spite of the use of human brain extract only, the bovine plasma prothrombin content was about the same as that of human plasma, namely 96 and 89 per cent of the human prothrombin activity (Table 48, experiments 1 and 2).

Discussion

The low results obtained by the one-stage method when using human brain were expected in view of the previous experiences of Koller et al (1951) who reported bovine prothrombin as 40 per cent of the human prothrombin. Didisheim et al (1959) also reported low bovine plasma prothrombin content. Quick et al (1961) found that in one-stage methods the results varied greatly according to the type of brain extract used. This is only natural since in such methods the delayed reaction between the heterologous brain-plasma mixture causes an initial lag until enough active extrinsic thromboplastin is formed, then this converts the prothrombin into thrombin thereby clotting the fibrinogen in the mixture. All these steps contribute to the final reading which is therefore very misleading as far as the concentration of the prothrombin is concerned. For quantitative comparative purposes, the presence of a heterologous brain-plasma mixture is unavoidable in any one-stage test system,

which is therefore rendered inadequate for such estimations.

Two-stage procedures are less amenable to the interfering effect of heterologous mixtures since incubation is over longer periods which though largely eliminates the species specificity does not completely nullify it, since the use of the homologous brain (Figure 25, curve B) gave a final higher value of prothrombin than when the same plasma was assayed with a heterologous brain extract (Figure 25, curve C). The latter curve illustrates the slow prothrombin conversion due to the delay in active thromboplastin formation caused by the use of a heterologous brain-plasma mixture.

However, if it is feasible to compare the values obtained for bovine and human plasmas when each was assayed with the use of the homologous brain extract, on the assumption that we are measuring the maximum amount of prothrombin that could be converted optimally into thrombin, we find that bovine prothrombin concentration in the plasma varied from 101 to 299 per cent of the human control plasma prothrombin content. Plasma antithrombin levels were about the same and therefore without effect on these results.

Murphy and Seeger (1948) using a two-stage method reported 250-285 prothrombin units per ml of bovine plasma as compared to 290 units per ml of human plasma. Quick et al (1961) obtained similar findings for both the bovine and the human plasmas. Stormorken (1957c) stressed the need for homologous factor VII -

brain combinations in one- and two-stage methods for prothrombin assays. He further used a modified two-stage method which is in fact a three-stage method. In the first stage the activated extrinsic thromboplastin is formed by the incubation of the brain extract with the homologous purified factor VII preparation (which also contains factor X), bovine factor V, and calcium chloride solution for five minutes at 37°C. An aliquot from this mixture added to equal aliquots of the test plasma and calcium chloride solution constitutes the second stage. The third stage comprises subsampling from the latter solution onto purified fibrinogen solution. He had to use a combination of brain - factor VII - plasma that was homologous. With this method he found bovine prothrombin in plasma to be 86 per cent that in human plasma.

Perhaps the most suitable method for the comparative estimation of bovine prothrombin is the globulin fraction method of Douglas and Biggs (1953). Firstly, this system is free from antithrombin. Secondly, the prolonged incubation over a thirty minutes' period with the brain extract largely eliminates the effect of species specificities and allows for the maximal formation of thrombin, which is proportional to the prothrombin content of the mixture. The average concentration of prothrombin in two bovine plasma pools estimated by this method is 92.6 per cent of the human control plasma prothrombin content.

Conclusions

The bovine plasma prothrombin content is at least equal to the human plasma prothrombin content. One-stage methods are unsuitable for its estimation. The globulin fraction method of Douglas and Biggs (1953) is believed to be the most suited method for its measurement.

EFFICIENCY OF THE BOVINE EXTRINSIC THROMBOPLASTIN SYSTEM

All the factors concerned in the extrinsic system of prothrombin activation seem to be present in the bovine plasma in optimum amounts when measured by specific assays.

In vitro tests also show that extrinsic prothrombin conversion can be brought about in bovine plasma as quickly and efficiently as in human plasma, either by the use of the homologous brain extract, or of Russell's viper venom (Table 50).

BOVINE INTRINSIC THROMBOPLASTIN SYSTEM

Bovine whole blood clotted in ordinary glass tubes in 6' 41" while in siliconed glass tubes it clotted in 13' 15" (Table 52). This rapid clotting in ordinary glass tubes is within the normal limits for human whole blood clotting times, and indicates the efficient generation of intrinsic thromboplastin in bovine whole blood, a fact which is also substantiated by the finding of an average clotting time of the recalcified bovine citrated plasma of 145 seconds (Table 53) which again is within the normal limits for human plasma recalcified clotting time.

The delayed clotting of bovine whole blood in siliconed tubes suggests the presence of the contact factors in bovine blood whose action is interfered with by the use of the siliconed ware.

Conclusions

The whole blood clotting time and the recalcification time of bovine reagents are within the normal range obtained with human reagents.

BOVINE CONTACT FACTORS

The activity of the bovine contact factors was measured by the method of Ratnoff and Rosenblum (1958). The glass activated bovine

preparation behaved in a virtually identical manner as the human preparation, clotting the intact platelet-deficient human substrate plasma in 15 minutes after recalcification (Table 54).

Discussion

The similar activities of bovine and human plasma preparations suggest that bovine plasma contains both Hageman factor and P.T.A. in a concentration sufficient for the proper production of intrinsic thromboplastin. This is substantiated by the normal behaviour of bovine reagents in the thromboplastin generation mixtures as shall be shown later. Wartelle (1957b) by correction of congenitally deficient plasma with bovine plasma in a recalcified clotting time technique reported that bovine plasma had 20-30 per cent of the human plasma Hageman factor activity, while its content of P.T.A. was much higher than that of human plasma.

Conclusions

Bovine plasma contains both the contact factors in a concentration sufficient for the production of normal amounts of intrinsic prothrombin activator. They are active in a human system and therefore are not species specific.

BOVINE PLASMA ANTIHAEMOPHILIC GLOBULIN ACTIVITY

The thromboplastic activity of bovine adsorbed plasma when incubated with human serum in the thromboplastin generation test of Biggs and Douglas (1953b) using human platelets was found to be very similar to that of human adsorbed plasma (Figure 26, A, curves a and b). However, active thromboplastin was rapidly formed in appreciable amounts within the first minute of incubation in the case of the bovine adsorbed plasma (curve b). This is believed to be due to the higher factor V content of the bovine plasma. Essentially similar results were obtained when bovine platelets were used instead of the human platelets (Figure 26, B).

The ability of bovine adsorbed plasma to correct the deficiency in haemophilic adsorbed plasma was tested in a similar thromboplastin generation technique. Various dilutions of the adsorbed bovine plasma in the adsorbed haemophilic plasma were tested as compared to dilutions of human adsorbed plasma in the haemophilic adsorbed plasma. All other reagents in the incubation mixture were constant and of human origin. Figure 27 shows the activity produced by the human plasma dilutions (Graph A) together with that produced by the bovine plasma dilutions (Graph B). The latter were found to possess much higher A.H.G. activity, about five times the human plasma A.H.G. activity.

A.H.G. measurement in bovine plasma by the method of Pitney (1956) gave A.H.G. values ranging from 590 to 1107 per cent of the human control plasma A.H.G. content, with an average of 816 per cent (Table 57). Figure 28 illustrates how the A.H.G. content of each plasma pool was calculated from the human plasma dilution curve.

Discussion

The high A.H.G. content of bovine plasma has been reported by several groups of investigators. Macfarlane, Biggs, and Bidwell (1954) found it to be 16 times the content of human plasma, as tested in a modified thromboplastin generation test. Biggs, Eveling, and Richards (1955) using perhaps the most accurate method as yet devised for the measurement of A.H.G. found that bovine plasma had an average of 3.5 units per ml, while human plasma contained an average of 0.28 units per ml, when both plasmas were compared to the activity of a standard preparation of purified bovine A.H.G. in a modified thromboplastin generation test. In other words, the bovine plasma A.H.G. activity was about 12.8 times that of the human plasma. Wartelle (1957b) reported a value of 15 to 20 times the content of human plasma, when correcting the recalcification time of haemophilic plasma in a one-stage technique that could have been easily affected by the higher bovine plasma factor V content. Didisheim et al (1959) also found very high values of A.H.G. in bovine plasma, while Hawkey (1960) reported complete correction of haemophilic plasma in the

thromboplastin generation test by 1 in 10 dilution of bovine plasma.

The variation in A.H.G. concentrations reported is by no means strange in view of the different methods used, the different standards of comparison assumed, especially if human plasma was chosen as the standard since its A.H.G. concentration normally varies from 50-200 per cent (Pitney, 1958) or 30-200 per cent (Biggs, Eveling, and Richards, 1955). Also because of the differences in methods of obtaining the blood samples, as well as the differences in methods used to kill the animals from one slaughter-house to another, which as Bidwell (1958) suggested would account for much of the variation in results on plasmas not obtained by venipuncture. Finally, because A.H.G. levels in the bovine plasma are expected to vary at least to a certain extent if not as much as human levels do.

The variation between the values obtained for bovine plasma observed in the six experiments conducted (Table 57) was only of twofolds magnitude. This may be due to the use of pooled plasma for each experiment which would greatly minimise the effect of individual differences. If the average value obtained for bovine plasma is corrected for the actual A.H.G. concentration of the human plasma used as standard which is 150 per cent of the A.H.G. activity of plasma pooled from ten normal individuals (Table i) it would read 12 times the content of average normal human plasma.

Spaet and Kinsell (1953) observed that bovine plasma showed no

loss of A.H.G. activity on storage for 24 hours at both 4°C. and 20°C., while human plasma showed significant deterioration within 4 hours at 20°C. Such increased stability of the bovine A.H.G. activity was also confirmed by Wartelle (1957b) both in citrated and oxalated bovine plasma.

Conclusions

The antihaemophilic globulin concentration in bovine plasma is at least eight times that of the control human plasma. Bovine antihaemophilic globulin is not species specific.

BOVINE SERUM CHRISTMAS FACTOR ACTIVITY

The activity of bovine serum in the thromboplastin generation test using human platelets and human adsorbed plasma to supply the other factors was found to be initially higher than the activity of human serum but the final yield of active thromboplastin was slightly lower (Figure 29, A). Similar findings were obtained with bovine platelets (Figure 29, B). The initial increased activity is believed to be due to the high content of factor V in the bovine serum which is said to be 200 per cent of the human plasma factor V content (Bergna, 1960). Correction of Christmas serum by bovine serum in a thromboplastin generation mixture revealed that bovine serum has about one quarter of the corrective

ability of the human control serum (Figure 30, A and B). This was confirmed by the exact measurement of Christmas factor in bovine serum by a modified method based on that of Pitney (1958) depending on its ability to correct the defect of Christmas disease serum in a thromboplastin generation mixture. The concentration of Christmas factor in bovine serum ranged from 10.5 to 33.5 per cent of the human control serum content (Table 58). The average of the six experiments was 20.8 per cent of human serum Christmas factor activity. Figure 31 illustrates experiment 4 and the method of calculating the Christmas factor values for each dilution of serum tested as read from the human serum dilution curve.

Discussion

In view of the high Christmas factor content of the standard human serum used in the above assays which is about 200 per cent of the average normal human (Table i) it can be assumed that the bovine serum contained 40 per cent of the average human serum Christmas factor concentration. Wartelle (1957b) using the cephalin recalcification clotting time method of Soulier and Larrieu (1953b) found that bovine plasma contained 40-80 per cent of the human plasma Christmas factor, by correction of Christmas disease plasma. Didisheim et al (1959) reported uniform levels of Christmas factor in all mammals, while Hawkey (1960) found that a 1 in 10 dilution of bovine plasma or serum caused complete correction of Christmas

serum in the thromboplastin generation test. Again this variation in the reported levels can be ascribed to the different methods and different standards used.

Conclusions

Bovine serum contains Christmas factor in a concentration about 20 per cent of the human control serum used. Bovine Christmas factor is not species specific.

THROMBOPLASTIC ACTIVITY OF BOVINE PLATELETS

Bovine whole blood contains an average of 312,350 platelets per cubic millimetre (Table 51) which is similar to the average number present in normal human blood. Bovine platelets are also morphologically similar to human platelets. However, it is much more difficult to separate the bovine platelets adequately from bovine blood for the preparation of platelet suspensions for use in the thromboplastin generation test. This is due to the very low sedimentation rate of bovine blood (0.5 mm per hour), therefore comparatively high centrifugation speeds have to be used to effect adequate separation of the bovine blood cells from the plasma, speeds which result also in the sedimentation of most of the platelets present. The lack of platelets in the supernatant plasma separated by the conventional one time centrifugation of blood perhaps accounts

for the poor platelet suspensions obtained by Didisheim et al (1959) which resulted in poor homologous thromboplastin generation. This difficulty was circumvented by the separation of the bovine plasma in stages after centrifugation for 5 minutes' intervals at increasing speeds, each time pipetting off the uppermost layer of relatively clear plasma which usually amounted to not more than 0.5 ml each time. The method is described in detail in appendix III, 1, ii and appendix III, 15, C. Such prepared plasma yielded an appreciable amount of platelet sediment on centrifugation at 15,000 r.p.m.

The thromboplastic activity of bovine platelets was compared to that of human platelets in the thromboplastin generation test of Biggs and Douglas (1953b) using identical incubation mixtures as far as the other factors are concerned. The bovine platelets had a weaker activity than that of the human platelet suspension, whether the incubation mixture contained homologous bovine or human reagents, (Figure 32, A and B). This cannot be due to any species specificity, but is believed to be due to the fewer number of platelets per unit volume in the bovine preparation. In fact, stronger preparations could have been made, but the comparison would not have been valid since bovine and human platelet suspensions are not prepared in an identical manner anyway. The main feature is that bovine platelets are active in the thromboplastin generation test even when using human adsorbed plasma and serum, yielding over

90 per cent active thromboplastin after 6 minutes of incubation (Figure 32, B, curve b). Their less obvious activity when using bovine adsorbed plasma and bovine serum (Figure 32, A, curve b) is due to the Christmas defect in the bovine serum; the use of bovine adsorbed plasma with human normal serum yielded 103 per cent active thromboplastin (Table 56, mixture 106; and Figure 26, B, curve b).

Conclusion

Bovine platelets possess similar thromboplastic activity to human platelets in the thromboplastin generation test.

BOVINE PLASMA SUBSTRATE REACTIVITY IN THE T.G.T.

The use of bovine high-spun plasma as the substrate in the thromboplastin generation test instead of the human high-spun plasma resulted in a consistent prolongation of the clotting times obtained, irrespective of the incubation mixture reagents being homologous or heterologous or combinations therefrom (Figures 33,A and B, and 34, A and B, constructed from Tables 55, 56, 59, and 60).

This delayed reactivity of bovine plasma is reminiscent of its delayed thrombin-fibrinogen reaction and may perhaps be also due to its higher content of fibrinogen.

Conclusion

Bovine plasma is less reactive than human plasma to active

thromboplastin, whether homologous or heterologous.

HOMOLOGOUS BOVINE INTRINSIC THROMBOPLASTIN GENERATION

The production of homologous bovine active thromboplastin by the incubation of bovine adsorbed plasma, bovine serum, and bovine platelets, and subsampling onto human high-spun plasma resulted in an activity of 60-70 per cent of thromboplastin (Figure 34, B, curve a), while subsampling onto bovine high-spun plasma greatly diminished it to about 30 per cent (Figure 34, B, curve b). In other words, in a completely homologous bovine system the production of active intrinsic thromboplastin was much weaker than in a homologous human system. This is partly due to the Christmas factor deficiency in the bovine serum and partly due to the delayed reactivity of the bovine substrate plasma.

EFFICIENCY OF BOVINE INTRINSIC THROMBOPLASTIN SYSTEM

All the factors concerned in the intrinsic thromboplastin system for prothrombin activation are present in bovine plasma and serum in amounts adequate for effective haemostasis, with the exception of Christmas factor which is moderately deficient in bovine serum and is therefore also assumed to be moderately deficient in bovine plasma,

though this may not be the case. However, since normal clotting times were obtained for whole bovine blood and for the recalcified citrated plasma, and since the bovine blood had a prothrombin consumption index of 15-39 per cent (Table 49), it seems that the amount of Christmas factor present is sufficient for the production of physiologically effective concentrations of intrinsic active thromboplastin. This is further substantiated by the fact that a concentration of 50 per cent of Christmas factor is sufficient for effective haemostasis in the human (Biggs, 1955), and also by the findings of Johnson et al (1957) and Seegers (1962) that low concentrations of Christmas factor have been frequently observed in sera of perfectly normal individuals.

It should be remembered that the formation of active thromboplastin in the thromboplastin generation test occurs in an artificial system and though it reflects the general fundamental steps of reaction, the physiological formation of intrinsic active thromboplastin in vivo may not necessarily proceed in the same speed. Therefore, in spite of the delayed reactivity of bovine plasma in the thromboplastin generation test to a homologous incubation mixture, which is only delayed several seconds, it is believed that the bovine species have an efficient intrinsic thromboplastin system.

SUMMARY OF BOVINE BLOOD COAGULATION MECHANISM

Bovine blood contains all coagulation factors known to take part in human blood coagulation. Compared to the human control plasma the bovine fibrinogen measured approximately 200 per cent, factor V about 300 per cent, and factor VIII about 800 per cent. Prothrombin, factor X, factor XI, and factor XII occured in comparable amounts to the human reagents. Factor VII was present in sufficient amount for normal haemostasis, while factor IX was about 20 per cent of the human. Bovine platelets and tissue extracts were as active as their human analogues.

The bovine plasma exhibited a delayed reactivity to thrombin, most probably due to its higher content of fibrinogen.

Species specificity was not observed except for factors VII and X when tested for in the presence of brain extracts; their specificity, however, was not absolute.

Both the extrinsic and the intrinsic systems of prothrombin activation were found to be efficient.

PART IV

HORSE BLOOD COAGULATION MECHANISM

HORSE BLOOD COAGULATION MECHANISM

Data presented in this section were based on the results of experiments conducted on blood samples obtained from horses, mainly English Thoroughbred racing horses and Clydesdale farm working horses. None of the animals suffered from any known haemorrhagic condition and were under the care of the staff of Glasgow University Veterinary Hospital for minor surgical conditions.

HORSE PLASMA FIBRINOGEN

The fibrinogen in horse plasma was estimated by the modified Greenberg method described in appendix IV, 2, II. The average of four experiments was 436.5 mg of fibrinogen per 100 mls of plasma, with a range of 325-567 (Table 61).

Discussion

The observed horse plasma fibrinogen level approximates a high normal human plasma fibrinogen content. Stormorken (1957b) using the Kjeldahl method obtained the value of 300 mg per 100 mls of horse plasma. Also Fantl and Marr (1958) using the same method reported it to be 200-350 mg per 100 mls of oxalated horse plasma, though they used a conversion factor of 5.92 instead of the usual factor of 6.25 for fibrinogen calculation.

Conclusion

Horse plasma fibrinogen is about equal to a high normal human fibrinogen content, with an average of 436.5 mg per 100 mls of plasma.

THROMBIN - FIBRINOGEN REACTION OF HORSE PLASMA

Horse citrated plasma gave prolonged clotting times when clotted with various concentrations of bovine thrombin, nearly twice the clotting times obtained with the control human citrated plasma (Table 62, and Figure 35). The addition of a 1 per cent of pyrocatechol solution (final concentration in the clotting mixture was 0.3 per cent) greatly shortened the clotting times of horse plasma which were still slightly longer than those obtained for human plasma similarly treated (Figure 35, curves b_2 and a_2). The addition of 1 per cent of pyrocatechol solution together with calcium chloride solution further reduced the clotting times of both horse and human plasmas, but the former still gave slightly longer clotting times, the difference being more obvious with the low thrombin concentrations (curves b_3 and a_3 respectively).

Discussion

The longer clotting times observed with horse plasma seem to be mainly due to the property of horse fibrinogen which has a delayed reaction when clotted with various types of thrombin as

shall be shown shortly in a purified system. The delay may also be partly due to the high antithrombin content of horse plasma which was found to be about 150 per cent of the human plasma antithrombin content.

The delayed thrombin-fibrinogen reaction of horse plasma has been previously demonstrated by various workers. Wartelle (1957b) observed that horse thrombin clotted horse plasma in a longer time than it did human plasma. Stormorken (1957b) has shown that the horse plasma had 65 per cent of the reactivity of human plasma when clotted with bovine thrombin, and that the addition of calcium chloride solution which cancels the influence of changes in the pH did not alter the proportional differences previously reported. He further observed that the same pattern of reactivity holds true for purified fibrinogens, and that horse thrombin was less reactive than either ox or human thrombins. Such findings were again confirmed by Fantl and Marr (1958) who obtained prolonged clotting times of horse plasma when clotted with horse, bovine, and human thrombins. They attributed this phenomenon to the characteristic structure of the horse fibrinogen. Astrup and Darling (1942c) believed that horse fibrinogen is chemically different from other fibrinogens since it was maximally precipitated by 25 per cent saturation with ammonium sulphate as compared to 30 per cent and 35-40 per cent for ox and chicken fibrinogens respectively.

Astrup (1950) has also shown that the optimum pH for horse fibrinogen when clotted with bovine thrombin in phosphate buffer of 0.2 ionic strength was 7 as compared to 7.5 for human fibrinogen.

Species specificity of the horse thrombin-fibrinogen reaction

The horse thrombin-fibrinogen reaction was investigated in a purified system using thrombin and fibrinogen reagents prepared free from antithrombin as described in appendix III, 7, A and B, and appendix III, 6, A respectively. When 0.4 ml amounts of horse and human fibrinogen solutions were clotted with 0.1 ml amounts of human, bovine, and horse thrombins, it was observed that the horse fibrinogen gave consistently longer clotting times than the human fibrinogen (Table 63). Also, horse thrombin exhibited a weaker action than human thrombin, both on human and horse fibrinogen substrate solutions. The addition of 0.1 ml of 1 per cent pyrocatechol solution to the fibrinogen substrates shortened their clotting times appreciably regardless of the type of thrombin used, though horse fibrinogen still had longer clotting times than human fibrinogen.

Conclusions

Horse plasma thrombin-fibrinogen reaction is more prolonged than that of human plasma. The same delay was also observed with purified thrombin and fibrinogen solutions. This is due to the inherent property of horse fibrinogen. In the plasma the delay

may also be partly due to the high antithrombin content of the horse plasma. Horse fibrinogen is not species specific since it is more actively clotted with human and bovine thrombins.

HORSE PLASMA ANTITHROMBIN ASSAY

Horse plasma antithrombin content when estimated by the method of Douglas and Biggs (1953) was found to be about 134 per cent that of human plasma antithrombin content (Table 64, and Figure 36).

Its estimation by the modified method of Astrup and Darling (1942d) gave values of 131-167 per cent of human plasma antithrombin content with an average of 150.7 per cent (Table 65, and Figure 37).

Conclusion

Horse plasma antithrombin content is about 150 per cent that of human plasma. It may therefore be responsible for some of the delay observed in the horse thrombin-fibrinogen reaction of the plasma, especially with the high dilutions of thrombin.

HORSE EXTRINSIC THROMBOPLASTIN SYSTEM

Using Quick's one-stage prothrombin time test, horse plasma was found to clot in the shortest time with horse brain extract, namely 13 seconds, while with human brain extract it clotted in 18 seconds (Table 66). Similarly, human plasma gave much longer clotting times with the horse brain extract. Mixtures of equal amounts of horse and human plasma gave similar clotting times with both horse and human brain extracts, suggesting the absence of demonstrable inhibitory activity in either plasma against the heterologous brain thromboplastin.

Discussion

The above findings suggest that there is a certain degree of species specificity in the reaction between horse brain extract and the horse plasma. Similar findings have been reported by Trevan and Macfarlane (1936), Stormorken (1957d), Barkhan, Tomlin, and Archer (1957), and Fantl and Marr (1958). The latter authors further observed that this species specificity can be abolished by the previous addition of the homologous serum to the brain extract.

Conclusions

Horse brain extract is species specific towards horse plasma. A homologous horse extrinsic system clots in as short a time as a homologous human system. The horse extrinsic thromboplastin system

is therefore assumed to be haemostatically as efficient as the human system.

HORSE PLASMA FACTOR V ASSAY

Horse plasma factor V content was measured by the degree of correction of human stored plasma, using human brain extract, as described in appendix IV, 14. The results obtained from three experiments ranged between 397 and 864 per cent of the factor V activity of human plasma, with an average of 643 per cent (Table 67, and Figure 38). In one experiment factor V was also estimated in horse serum and was found to be 100 per cent of the human plasma factor V activity (Table 67, experiment 2).

Using the same method Stormorken (1957a) found that horse plasma had 300 per cent of human plasma factor V activity, and noted that horse proaccelerin was easily adsorbed onto barium sulphate. Wartelle (1957b) found factor V to be five times as much as human plasma factor V; Fantl and Marr (1958) reported a twofolds increase; while Hawkey (1960) found it to be more than three times in horse plasma and that horse serum had about the same activity as present in normal human plasma. These differences can be explained by the different human control plasmas used by each investigator.

Conclusions

The factor V concentration in horse plasma is much higher than in human plasma, with an average of sixfolds increase.

Horse serum contains factor V in a concentration equal to that present in human normal plasma.

SPECIES SPECIFICITY OF HORSE SERUM FACTORS

A purified preparation containing factors VII and X (appendix III, 10) was incubated with the brain extract and calcium chloride solution and samples were removed at minute intervals and pipetted onto fresh human plasma, fresh horse plasma, or onto phenindione human plasma simultaneously with an aliquot of calcium chloride solution. The clotting times of the substrates were recorded and are indicative of the thromboplastic activity produced in the incubation mixture which is dependent on the proper interaction of the reagents concerned. The method is described in detail in appendix IV, 17.

The horse brain extract when incubated with the horse purified serum factors gave uniformly shorter clotting times of the substrates than when incubated with the human purified serum factors, indicating their species specificity (Table 68). Similarly, the human brain extract gave the shortest clotting times when incubated with human

serum factors, again indicating their species specificity. It should be noted however that the serum factors prepared from the horse and human sera were not completely species specific, since the heterologous mixtures gave substrate clotting times shorter than when the brain extracts were incubated with saline.

Conclusion

Human and horse serum factors are species specific towards the homologous brain extracts.

HORSE PLASMA FACTOR VII ASSAY

The ability of horse plasma to correct Seitz-filtered bovine plasma - deficient in factors VII and X - when clotted with various brain extracts was taken as indicative of its factor VII content (method of Koller et al, 1951). Since the factor X content of the horse plasma has been found to average 85 per cent of the human plasma factor X (see later), the differences observed in the factor VII assays to be discussed may be assumed to be largely due to changes in factor VII, though it should be borne in mind that the species specificity of factor X towards the brain extract in use may affect the clotting times. Using human brain extract, the horse plasma was found to contain about 71 per cent of the human plasma factor VII activity, with a range of 63.3 to 80 per cent; while

with the horse brain extract the human plasma had about 6 per cent of the horse plasma factor VII activity (Table 69, and Figure 39).

Discussion

The above observed discrepancy in the factor VII content of horse plasma as compared to human plasma is explained by the species specificity of the reaction between the brain extracts and the serum factors. The fact that horse plasma has a short one-stage clotting time, that it was found to contain about 70 per cent of factor VII when human brain was used, and even a much higher content when horse brain was used would suggest that horse plasma contains ample amounts of factor VII sufficient to maintain normal haemostasis.

Stormorken (1957d) stressed that quantitative comparison of the horse factor VII with other species cannot be done due to its species specificity. Fantl and Marr (1958) observed that horse serum is as active as human serum when acting in a homologous system. Hawkey (1960) estimated horse plasma factor VII activity by its corrective ability on human plasma from a patient with congenital factor VII deficiency, clotting the mixture with human brain extract and calcium chloride solution. She reported the value of 86 per cent for the horse plasma factor VII and 100 per cent for the horse serum factor VII as compared to human normal plasma factor VII content. Her results for the horse plasma agree fairly well with those reported in this study.

Conclusions

No valid quantitative comparison of horse plasma factor VII could be made with present day methods with any degree of precision. Horse plasma, however, contains sufficient factor VII to give a normal one-stage prothrombin time in a homologous system, and about 70 per cent of human plasma factor VII content in a human system.

HORSE PLASMA FACTOR X ASSAY

The horse plasma factor X content was estimated by the method of Bachmann et al (1958) which measures the degree of correction of Seitz-filtered bovine plasma when clotted with Russell's viper venom and cephalin in the presence of calcium chloride solution.

The horse plasma was found to contain 64.7-106 per cent of the human plasma factor X activity, with an average of 85.5 per cent (Table 70). No species specificity towards the R.V.V.-cephalin reagent was observed. Wartelle (1957b) found that the R.V.V.-cephalin clotting time of horse plasma was somewhat longer than that of human plasma, and inferred that horse factor X was slightly inferior to human factor X. Hawkey (1960) who used the method of Bachmann et al (1958) reported that horse plasma contained 85 per cent of human plasma factor X, while horse serum contained 94 per cent that in human plasma.

Conclusion

Horse plasma contains an average of about 85 per cent of the human plasma factor X activity.

HORSE PLASMA PROTHROMBIN ASSAY

Due to the species specificity between factor VII and brain extracts, attempts to measure the horse plasma prothrombin were made utilizing two-stage methods which are much less affected by the available factor VII content in the reacting mixture.

The first method used was the two-stage area method of Biggs and Douglas (1953a) by which the horse plasma prothrombin values when estimated using human brain ranged between 117.4 and 193 per cent of the human plasma prothrombin content, with an average of 160 per cent (Table 71, and Figure 40). The use of horse brain gave slightly higher values ranging between 124.8 and 247.8 per cent, with an average of 180 per cent of the human plasma prothrombin.

The second method measures the amount of prothrombin in the globulin fraction obtained from the plasma by acid precipitation as described by Douglas and Biggs (1953). With this method the prothrombin was found to be much less in the horse plasma globulin fraction than in the human fraction, namely 32.8 and 29.2 per cent of the human prothrombin with human and horse brain respectively (Table 72).

Discussion

The results obtained with the two-stage area method seem to reflect the true levels of the horse plasma prothrombin content, since the use of both human and horse brain extracts gave roughly similar values. For example in experiment 2C (Table 71, and Figure 40, curve C) the human plasma prothrombin estimated with horse brain was 106.3 per cent that estimated with human brain (curve A). Similarly, experiments 1C and 3C gave values of 88.6 and 116.2 per cent respectively when each human plasma sample was tested with horse brain extract instead of the human brain extract, with an average variation of 11.3 per cent, a variation that is acceptable for such biological assays. It should be stressed, however, that in spite of the above correlation between results obtained with the two different brain extracts, quantitative comparisons were only made between plasma samples tested with the same brain extract.

Since this method is affected by the antithrombin content of the plasma tested as compared to that of the standard plasma, and since horse plasma was found to contain about 150 per cent of the human antithrombin, it is probable that the horse plasma contains even higher prothrombin concentrations than those reported here.

Stormorken (1957c) estimated horse plasma prothrombin activity as 99 per cent that of human plasma, in a modified two-stage method

using homologous reagents except for factor V and fibrinogen which were of bovine origin. While Barkhan, Tomlin, and Archer (1957) did not attempt quantitative assays because of the species specificity of the brain thromboplastins, thay noted that in a homologous system horse reagents were more active than human reagents, and inferred that horse plasma therefore contained a normal amount of prothrombin. Fantl and Marr (1958) reported that the prothrombin activity of the horse plasma was in the same range as has been observed previously for human plasma by Fantl (1954). They used the pyrocatechol two--stage method of Fantl (1954) which measures the amount of thrombin formed in a mixture containing the diluted horse plasma, pyrocatechol, brain extract, and calcium chloride solution. At intervals aliquots are delivered onto barium sulphate adsorbed human plasma which served as the source of fibrinogen. They noted that similar results were obtained with either human or horse brain extract.

It is difficult to explain the low results obtained with the globulin fraction method, especially that this system is free from antithrombin and should have given perhaps truer values for the horse plasma prothrombin. This finding calls for further investigation as regards the precipitation characteristics of the horse prothrombin and its activation in the absence of accelerator factors.

Conclusions

Horse plasma prothrombin concentration was found to be at least 160 per cent of the human plasma prothrombin, as estimated by the two-stage area method. One-stage methods are unsuitable for the estimation of prothrombin in horse plasma because of the species specificity of the brain extract - factor VII reaction.

EFFICIENCY OF THE HORSE EXTRINSIC THROMBOPLASTIN SYSTEM

Horse plasma contains all the blood coagulation factors required for the extrinsic thromboplastin system of prothrombin activation in sufficient concentrations that would ensure effective normal haemostasis.

In vitro tests proved that the extrinsic prothrombin conversion can be brought about in horse plasma as quickly and efficiently as in human plasma, by the use of the homologous brain extract.

The delayed thrombin-fibrinogen reaction of horse plasma could hardly be said to hamper the efficiency of the extrinsic system, since the delay only amounts to fractions of a minute, a finding more of an academic importance rather than of any practical consequence.

HORSE INTRINSIC THROMBOPLASTIN SYSTEM

Horse blood clotted in ordinary glass tubes in 7' 55", while in siliconed glass tubes it clotted in 15' 29" (Table 75). The rapid clotting in ordinary glass tubes indicates the efficient generation of intrinsic thromboplastin in horse blood. Fantl and Marr (1958) reported whole blood clotting times for horse blood to be 13-19 minutes as compared with 14-19 minutes for the human control. They, however, demonstrated a delayed thrombin generation and ascribed it to the increased stability of the horse platelets, since they found the delay to be readily corrected by the addition of phospholipids, a finding previously reported by Sjolin (1957). This may be the explanation for the longer recalcified clotting times of horse plasma as compared to those of human plasma, namely 368 and 147 seconds respectively (Table 76). The preliminary separation of the plasma by centrifugation may have rendered the horse plasma relatively less platelet-rich than the human plasma, especially if we take into consideration the originally lower platelet content of horse blood which averages 117,000 platelets per cubic millimetre (Table 74).

The prolongation of the clotting time of horse whole blood in siliconed ware suggests the presence of some contact factor in the horse blood whose action is interfered with by the use of the

siliconed glass tubes.

Conclusion

Horse whole blood clots in ordinary glass tubes in as short a time as human whole blood.

HORSE PLASMA HAGEMAN FACTOR ACTIVITY

The activity of Hageman factor in horse plasma was measured by the latter's ability to correct the prolonged recalcification time of plasma obtained from a patient with congenital Hageman factor deficiency. Both horse and human control plasmas were maximally glass-activated before being tested. The method is described in full in appendix IV, 24, II. Due to the scarcity of the Hageman deficient plasma only one experiment was made, testing two dilutions of horse plasma. The average Hageman factor activity in horse plasma was found to be 118.5 per cent that in the control human plasma (Table 77, and Figure 41). Wartelle (1957b) using a similar technique reported similar levels of Hageman factor in horse and human plasmas.

Conclusion

Horse plasma contains Hageman factor in comparable amount to that present in human plasma. Horse Hageman factor is not species specific.

HORSE PLASMA THROMBOPLASTIN ANTECEDENT ACTIVITY

Because of the unavailability plasma thromboplastin antecedent congenitally deficient plasma no specific assay for this factor was made. But in view of the shorter clotting time of horse whole blood in ordinary glass tubes than that in siliconed tubes, and since Hageman factor alone could not bring about such shortening in the clotting times, it is presumed that plasma thromboplastin antecedent is present in horse plasma in sufficient concentration to allow normal active intrinsic thromboplastin formation.

HORSE PLASMA ANTIHAEMOPHILIC GLOBULIN ACTIVITY

The antihaemophilic globulin activity of horse plasma was measured by the method of Pitney (1956). It was found to range from 137 to 310 per cent with an average of 209 per cent of the human plasma A.H.G. activity (Table 80, and Figure 43).

Discussion

The presence of A.H.G. activity in horse plasma has been the main controversial subject in the literature on horse blood coagulation. Bell, Archer, and Tomlin (1955) first reported that horses suffer from A.H.G. deficiency which occurs in both sexes, and can be corrected by normal human plasma. In 1957

Barkhan, Tomlin, and Archer again observed a defect in the thromboplastin activity of horse alumina treated plasma which they attributed to be most likely due to deficiency of A.H.G. or P.T.A. factors. Wartelle (1957b) also observed that horse plasma gave longer clotting times in a thromboplastin generation test system than those obtained with human plasma, while horse and human plasma mixtures gave even longer clotting times of the substrate. She thought that perhaps a deficiency of factor V may be a contributing factor since horse factor V is easily adsorbed on barium sulphate from the oxalated plasma. She later ruled out this contention as a possible cause since she found that in a one-stage recalcification time test a human-horse plasma mixture had less corrective ability on the clotting time of haemophilic plasma than a corresponding human-saline mixture. She attributed her results to the presence of an inhibitor in horse plasma identical with that responsible for the delayed thrombin clotting time of human-horse plasma mixtures.

Fantl and Marr (1958) reported that horse oxalated barium sulphate adsorbed plasma had a similar A.H.G. activity to human plasma by correction of haemophilic plasma in a thromboplastin generation mixture using cephalin instead of the platelets. They further observed that thromboplastin formation occured at the same rate as it did in the human system.

In this investigation, it was also observed that horse alumina

adsorbed plasma had about half the thromboplastic activity of the human plasma when tested with human serum in the thromboplastin generation test using human platelets (Table 78, mixtures 118 and 116 respectively, and Figure 42, A, curves b and a). This does not represent a deficiency but is due to the inhibitory activity of horse plasma as shall be discussed in detail later. Such inhibitory activity was not apparent in the A.H.G. assays made by Pitney's method because of the use of higher plasma dilutions than those normally present in an ordinary thromboplastin generation test. Values obtained by Pitney's method are therefore a true estimate of the horse plasma A.H.G. content, which however may not correspond with the actual horse plasma A.H.G. activity in the circulating blood.

Conclusions

The concentration of antihaemophilic globulin in horse plasma is about twice that in human plasma, with a range of 137 to 310 per cent. Horse antihaemophilic globulin is not species specific.

HORSE SERUM CHRISTMAS FACTOR ACTIVITY

The thromboplastic activity of horse serum in the ordinary thromboplastin generation test when incubated with human adsorbed plasma was similar to the activity of human serum (Table 78, mixtures 117 and 116, and Figure 44, A, curves b and a).

Specific measurement of Christmas factor in horse serum by the latter's ability to correct Christmas disease serum in a modified thromboplastin generation technique based on Pitney's method (as described in detail in appendix IV, 22) gave values ranging from 168 to 240 per cent of the human serum Christmas factor concentration. The average of the four experiments was 203 per cent (Table 81, and Figure 45).

Discussion

In 1956 Sjolin reported that horse plasma has an abnormal (delayed) thrombin generation test which was not corrected by Christmas disease plasma or adsorbed normal plasma, but was corrected by haemophilic plasma, normal plasma, and by purified Christmas factor. He concluded that horse blood lacks Christmas factor. However, in 1957 he revised this conclusion in view of his later findings that the delayed thrombin generation of horse plasma could also be corrected by several other ways, namely by the addition of adsorbed normal human serum previously heated at 56°C. for 30 minutes, by the addition of washed human or horse platelets, and by freezing and thawing the horse plasma with its normal content of platelets. He therefore concluded that the clotting defect of horse plasma was caused by the lack of a factor similar to Hageman factor. Also, Barkhan, Tomlin, and Archer (1957) have found that the delayed thrombin generation of horse plasma

was corrected by the addition of Christmas disease serum, and that horse serum was as active as human serum in the thromboplastin generation test. Fantl and Marr (1958) and Hawkey (1960) found that horse serum completely corrected Christmas disease serum in the thromboplastin generation test, having a similar activity to human serum. Wartelle (1957b) was not able to conclude if there existed any Christmas factor deficiency in horse plasma by the recalcification clotting time of horse plasma and Christmas disease plasma mixtures, again because of the inhibitory activity present in the horse plasma.

Conclusions

Horse serum contains Christmas factor activity in at least a similar concentration if not more to that present in human serum. Horse Christmas factor is not species specific.

HORSE PLATELETS

Horse whole blood contains an average of 117,000 platelets per cubic millimetre (Table 74) which is about half the average platelet count of human whole blood. Horse platelets are morphologically similar to human platelets. Barkhan, Tomlin, and Archer (1957) reported platelet counts of 114,000-160,000/c.mm. whole blood of Thoroughbred horses, and of 184,000-312,000/c.mm. for blood from

Shetland ponies. Fantl and Marr (1958) also observed the lower horse platelet count (60,000-100,000/c.mm.) and believed them to be more stable than human platelets.

The thromboplastic activity of horse platelets was investigated by substituting them instead of the human platelets in the thromboplastin generation test and comparing the results. Horse platelet suspension had very slightly less activity than the human platelet preparation when mixed with human adsorbed plasma and human serum (Table 78, mixture 116 compared with mixture 120 in Table 79, as illustrated in Figure 46, curves a and b). Their lower activity was also manifest in all the other mixtures (Tables 78 and 79, and Figure 46). It should be remembered, however, that because of the initially lower number of horse platelets in whole blood and since platelet suspensions were prepared from identical volumes of human and horse blood to make up similar volumes of platelet preparations, the horse platelet suspension must have contained about half the platelet concentration present in the human suspension. This may possibly explain the weaker action of the horse platelet suspensions. Barkhan, Tomlin, and Archer (1957) using platelet concentrations of 400,000-550,000/c.mm. in the thromboplastin generation mixture still observed that human platelets were more active than the horse platelets, while horse cephalin was as active as human cephalin. They further found that

horse platelets yielded more active thromboplastin than the human platelets when horse serum was used instead of the human serum. I was not able to confirm this observation, possibly because of the low concentration of my platelet preparations. But it could very well be explained by the higher Christmas factor content of horse serum as well as to the presence of factor V in horse serum, and not necessarily to species predilection between horse serum and horse platelets.

The activity of the platelets can also be judged by the degree of clot retraction of whole blood. Horse whole blood, while containing similar fibrinogen concentration and with a haematocrit value similar to human whole blood, had an average of 37.6 per cent of clot retraction in one hour (Table 82). This poor clot retraction of horse whole blood was also the experience of Bell, Archer, and Tomlin (1955). Again, it can be explained by the lower platelet count in horse blood.

Conclusions

Horse blood has a total platelet count about half that present in human blood. The thromboplastic activity of horse platelets in the thromboplastin generation test was slightly weaker than the activity of similarly prepared human platelets. Clot retraction of horse blood is poor.

HORSE PLASMA SUBSTRATE REACTIVITY IN THE T.G.T.

The use of horse high-spun plasma as the substrate in the thromboplastin generation test instead of the human high-spun plasma resulted in a consistent prolongation of the clotting times obtained, which in terms of active thromboplastin production depressed the latter to about half the values obtained with the human substrate plasma. This phenomenon was demonstrable with all the incubation mixtures tested (Tables 78, 79, 83, and 84, and illustrated in Figures 47 and 48).

This delayed reactivity of the horse plasma in the thromboplastin generation test is due to the delayed thrombin-fibrinogen reaction of horse plasma as demonstrated previously, and most probably caused by the inherent property of horse fibrinogen; also partly contributed to by the higher antithrombin content of horse plasma.

Conclusion

Horse plasma is less reactive than the human plasma to active thromboplastin, whether homologous or heterologous.

INHIBITORS AGAINST HUMAN THROMBOPLASTIN IN HORSE BLOOD

The presence of inhibitors in horse plasma or serum against human intrinsic thromboplastin formation was investigated by the method of Biggs and Macfarlane (1957) described in appendix IV, 23.

The addition of adsorbed horse plasma to a human thromboplastin generation mixture had an immediate inhibitory activity on the latter (Table 85, A, mixtures 132, and 133; and Figure 49, A, curves a and b). This cannot be simply attributed to dilution effect since the horse adsorbed plasma has been shown to contain all the necessary clotting factors in more than the optimum amounts, except perhaps the plasma thromboplastin antecedent which Wartelle (1957b) believed to be present in a low concentration as tested for in one-stage recalcification time tests that are easily affected by inhibitory substances. Heating the adsorbed horse plasma for 15 minutes at 56°C. before its addition to the incubation mixture nearly completely removed the inhibitory activity (Table 85, A, mixture 135, and Figure 49, A, curve c). Incubation of all the above mixtures for two hours at 37°C. before testing gave a similar pattern. The inhibition produced by the adsorbed horse plasma did not increase but was still apparent, while the heated horse adsorbed plasma mixed with the human adsorbed plasma produced less activity than before incubation presumably because of the lower

content of plasma coagulation factors (Table 85, B, mixtures 136, 137, and 138; and Figure 49, B).

The addition of horse serum did not produce any appreciable inhibitory effect, neither immediate nor after incubation (Figure 50, A and B).

Discussion

The removal of the inhibitory effect of horse adsorbed plasma on human intrinsic thromboplastin generation by heating the adsorbed plasma for 15 minutes at 56°C. suggests that the main factor responsible for such activity is the horse fibrinogen. It may also be due to the high antithrombin concentration in horse plasma, since antithrombin is known to be destroyed by heating at 56°C. (Biggs and Macfarlane, 1957). However, this is unlikely since horse serum which presumably contained the same amount of antithrombin did not have an inhibitory effect. The presence of antithromboplastin as such in the horse adsorbed plasma is doubtful. Though antithromboplastin withstands heating to 60°C. (Niewiarowski et al, 1959), the heated adsorbed horse plasma had no immediate inhibitory effect, while the low production of active thromboplastin obtained after its incubation with human plasma may have been due to the decay of plasma coagulation factors similar to that observed in the in the homologous human system, especially that heating totally destroys the A.H.G. and factor V otherwise supplied by the horse

adsorbed plasma.

Conclusions

The horse adsorbed plasma has an inhibitory effect when added to a human thromboplastin generation mixture, an effect that can be removed by heating the adsorbed plasma to precipitate the fibrinogen which is believed to be the main agent responsible for such inhibitory activity against human intrinsic thromboplastin formation. The horse serum does not possess any inhibitory activity against human intrinsic thromboplastin.

HOMOLOGOUS HORSE INTRINSIC THROMBOPLASTIN GENERATION

The generation of homologous active horse thromboplastin by the incubation of horse adsorbed plasma, horse serum, and horse platelets, but subsampling onto human high-spun plasma resulted in an activity amounting to 23.5 per cent of active thromboplastin (Figure 48, B, curve a); while subsampling onto horse high-spun plasma further diminished it to 12 per cent (Figure 48, B, curve b).

Therefore, in a completely homologous horse system the production of active intrinsic thromboplastin is much weaker than in a homologous human system. In the incubation mixture itself this is contributed to by the weaker action of the horse platelets as well as by the inhibitory effect of adsorbed horse plasma on

the thromboplastin generation. While with the use of horse plasma as substrate, the high antithrombin content and the delayed reaction of horse fibrinogen render the apparently deficient horse intrinsic thromboplastin generation more obvious.

EFFICIENCY OF HORSE INTRINSIC THROMBOPLASTIN SYSTEM

All the coagulation factors cencerned in the intrinsic thromboplastin system for prothrombin activation are present in horse plasma and serum in amounts adequate for effective normal haemostasis. In spite of the poor homologous horse thromboplastin generation demonstrated in vitro, the normal clotting times obtained for whole blood together with the virtually complete prothrombin utilisation during clotting of horse blood (judged from the very low prothrombin consumption indices obtained by either of the two methods used for its estimation, irrespective of the species of brain extract utilised, Table 73) would suggest that the formation of thrombin in ample amounts can easily be brought about by means of the intrinsic thromboplastin system in horse blood, indicating the likelihood of evolution of sufficient active intrinsic thromboplastin that maintains normal haemostasis in vivo.

SUMMARY OF HORSE BLOOD COAGULATION MECHANISM

Horse blood contains all the coagulation factors known to take part in the human blood coagulation. Compared to the human, the horse blood contained an equal concentration of fibrinogen, factor IX, and factor XII, while prothrombin was at least 160 per cent, factor V about 643 per cent, factor VII at least 70 per cent, factor VIII about 209 per cent, factor X about 85 per cent, and the platelets about half as much as in the human blood.

The horse blood had normal whole blood clotting times, normal prothrombin consumption indices, but the clot retraction was somewhat poor.

The horse plasma exhibited a delayed reactivity to thrombin, most probably due to the property of the horse fibrinogen and partly due to the high antithrombin content of the horse plasma.

Species specificity was only observed in the reaction of factors VII and X with the brain tissue extracts.

Both extrinsic and intrinsic thromboplastin systems of prothrombin activation in horse blood are believed to be efficient.

PART V

RABBIT BLOOD COAGULATION MECHANISM

RABBIT BLOOD COAGULATION MECHANISM

The data presented in this section are based on the results of experiments made on blood samples obtained from adult domestic rabbits of the Californian strain kept on ordinary pellet diet supplemented with fresh green vegetables.

All the animals were healthy, none of them suffered from any bleeding tendency.

RABBIT PLASMA FIBRINOGEN

The fibrinogen in rabbit plasma was estimated by the modified method of Greenberg as described in appendix IV, 2, II. The values obtained from seven experiments ranged from 190 to 281 mg of fibrinogen per 100 mls. of plasma, with an average of 228 mg. This compares with a low normal human plasma fibrinogen level.

Spaet and Cintron (1960b) have found rabbit plasma to contain about 240 mg of fibrinogen per 100 mls of plasma.

Conclusion

Rabbit plasma fibrinogen is about equal to a low normal human fibrinogen content, with an average of 228 mg per 100 mls of plasma.

RABBIT THROMBIN-FIBRINOGEN REACTION

Thrombin-fibrinogen reaction of the plasma

Rabbit citrated plasma gave similar clotting times as the human citrated plasma when clotted with various concentrations of bovine thrombin. Also, the addition of a 1 per cent solution of pyrocatechol to the plasma had the same shortening effect on the clotting times of both rabbit and human plasmas (Table 87, and Figure 51).

Species specificity of the thrombin-fibrinogen reaction

The thrombin-fibrinogen reaction was further investigated in a purified system using fibrinogen and thrombin solutions prepared free from antithrombin as described in appendix III, 6, A, and appendix III, 7, A and B. It was observed that the rabbit fibrinogen gave slightly longer clotting times than the human fibrinogen when clotted with human or bovine thrombin, while when clotted with rabbit thrombin it gave a shorter clotting time than the human fibrinogen solution, (Table 88). It should be noted that the human fibrinogen solution contained about double the concentration of fibrinogen present in the rabbit preparation. The use of human fibrinogen solution diluted 1 in 2 with saline as to be comparable with the rabbit preparation made the difference more obvious when clotting with human thrombin. The human

solution clotted in 9 seconds compared to $11\frac{1}{2}$ seconds obtained for the rabbit fibrinogen containing the same fibrinogen concentration. The addition of pyrocatechol solution shortened the clotting times of both fibrinogen solutions, while the differences observed with human thrombin were nearly suppressed, those obtained with rabbit thrombin were still apparent, the rabbit fibrinogen being more rapidly clotted than the human preparation.

Discussion

Seegers and Smith (1942) found that 1 ml of human oxalated plasma required 2.0 units of purified bovine thrombin to clot in 15 seconds at 28°C., while 1 ml of rabbit oxalated plasma required 2.2 units of the same thrombin solution. Fantl and Ebbels (1953) also reported that rabbit plasma had a delayed thrombin clotting time compared to the normal human plasma. They believed that the difference was not due to species specificity or to antithrombin, but to the specific configuration of the native fibrinogen.

Though my results for the thrombin-fibrinogen reaction of citrated plasmas do not agree with the above cited findings, those obtained in the purified system have some agreement and show that each fibrinogen solution clots faster with the homologous thrombin preparation, while bovine thrombin was more active on human fibrinogen than on rabbit fibrinogen. Why such differences were not observed when clotting citrated plasmas is difficult to explain.

However, it should be remembered that the latter system is complicated by the presence of all the other coagulation factors and inhibitors, other than the role played by the physical characters of each plasma that may well be different. The fact that the pyrocatechol shortened the clotting times in the purified thrombin-fibrinogen system which is presumably free from antithrombin suggests its direct action on the fibrinogen rather than its having an anti-antithrombin action. Kenton (1933) found that antibodies produced against sheep or bovine fibrinogens did not react with rabbit fibrinogen as concluded from the lack of anaphylactic shock which should have occured in case an in vivo antibody-antigen reaction has taken place.

Conclusions

The thrombin-fibrinogen reaction of rabbit citrated plasma occurs with the same rate as the human reaction. Purified fibrinogen solutions from rabbit and human plasma, however, were more actively clotted with the homologous thrombin solution, suggesting a certain degree of species specificity.

The delayed clotting time of rabbit fibrinogen with bovine thrombin suggests its difference from human fibrinogen, at least as regards its reactivity to bovine thrombin.

RABBIT PLASMA ANTITHROMBIN ASSAY

The antithrombin content of rabbit plasma was estimated by the method of Douglas and Biggs (1953) and found to be about 92 per cent that of the human plasma antithrombin content (Table 89, and Figure 52). Its estimation by the modified method of Astrup and Darling (1942d) gave values of 100-117.4 per cent of the human plasma antithrombin content, with an average for the three experiments of 105.8 per cent (Table 90, and Figure 53).

Astrup and Darling (1942d) reported that rabbit citrated plasma contained 148 antithrombin units per ml., but did not make a direct comparison with human plasma. Their result cannot possibly be compared with the average value obtained using their method, namely 301.7 antithrombin units per ml., because of the difference in the original thrombin unit terminology. The Astrup and Darling unit is the amount of thrombin required to clot 1 ml of ice-cooled oxalated plasma -immediately transferred to 37°C.- in 30 seconds (Astrup and Darling, 1941).

Spaet and Cintron (1960a) during an investigation on blood thromboplastin specificity casually commented that control rabbits' sera had high antithrombin activity without actual citation of the levels in terms of concentration or percentages.

Conclusion

The rabbit plasma antithrombin content is about equal to that of human plasma.

RABBIT EXTRINSIC THROMBOPLASTIN SYSTEM

Using Quick's one-stage prothrombin time test, rabbit plasma was found to clot in shorter times than the control human plasma, with both human and rabbit brain extracts (Table 91). The human brain preparations used seemed to be somewhat more active than the rabbit ones. The clotting time of human plasma with rabbit brain was appreciably longer (28 seconds) than that with human brain (17 seconds). Mixtures of equal amounts of rabbit and human plasma gave shorter clotting times than the saline controls when clotted with either type of brain extract, suggesting the absence of any inhibitory activity in either plasma against the heterologous brain thromboplastin.

Discussion

The previous findings suggest the absence of any species specificity in the reaction between rabbit and human reagents in the one-stage extrinsic system. The shorter clotting times of the rabbit plasma are believed to be due to its higher content of factor V as shall be shown later. Quick (1935) found no

appreciable difference between the thromboplastic activity of brain, thymus, or lung of the rabbit. Copley (1942) observed that the age of rabbits up to six weeks had a slight influence upon the thromboplastic activity of their brains upon human plasma, while over three months the age had no significant effect. Didisheim et al (1959) reported that most plasmas clotted fastest with rabbit brain extract. Also, Quick et al (1961) reported that rabbit brain was highly active on human, dog, rabbit, and cat plasmas, and that it was more active than the human brain extract. They used acetone-dehydrated brain extracts. In this work, both the human and rabbit brains were extracted with saline. A volume of saline in millilitres equal to the wet weight of the brain in grams was found to give the shortest clotting times for both human and rabbit preparations. From the shorter clotting times obtained with the human brain extract thus prepared as compared to those obtained with the rabbit brain, it can be inferred that it is more active than the similarly prepared rabbit brain extract.

Conclusions

The rabbit brain extract does not exhibit any species specificity in the one-stage prothrombin time test. Rabbit plasma responds to human brain extract in a similar time to human plasma.

The homologous rabbit extrinsic thromboplastin system is as efficient as the human counterpart.

SPECIES SPECIFICITY OF RABBIT FACTOR V

No differences were observed in the clotting times of substrate plasmas when rabbit or human purified factor V preparations were incubated with either rabbit or human brain extracts (Table 92), indicating that rabbit factor V is not species specific. Due to the very high concentration of factor V in rabbit plasma (as will be discussed shortly) the purified rabbit factor V preparation is also presumed to contain much more factor V than is present in the human preparation. Such a high factor V concentration may have masked a slight degree of species specificity, though this is very unlikely since rabbit plasma diluted 1 in 1000 to contain an equivalent concentration of factor V as present in human plasma diluted 1 in 10 still had as much corrective ability on stored human plasma (factor V deficient) when clotted with human brain as that of human plasma. During factor V assays, Wartelle (1957b) noted that the use of either human or rabbit brain extracts gave similar results.

Conclusion

The rabbit factor V is not species specific.

ASSAY OF RABBIT PLASMA FACTOR V

The rabbit plasma factor V content was measured by the degree of correction of human stored plasma using human brain extract as described in appendix IV, 14. It was found to contain factor V in a concentration ranging from 9696-11975 per cent that in human plasma with an average of 10558 per cent, i.e. approximately one hundredfold's increase (Table 93, and Figure 54).

Discussion

The high concentration of factor V in rabbit plasma was generally recognised by various workers. Murphy and Seegers (1948) utilising a two-stage technique found rabbit factor V to be about 12-20 times more than the human. Quick and Stefanini (1948) using their one-stage method reported a value of 50 times for rabbit factor V. Later investigations by the same group confirmed their previous result (Quick, 1960; and Quick et al, 1961). Wartelle (1957b) obtained the value of 40 times for rabbit factor V as compared to the human factor V. The variation of the latter results from those reported in this work is not so great as might be thought at first sight, since the measurement of so high a concentration entails extremely high dilutions which magnify the slightest error. Furthermore, the control samples used may not necessarily have had the same content of factor V.

Conclusion

The rabbit plasma contains factor V in great excess. Its factor V concentration was about one hundred times that in the control human plasma.

SPECIES SPECIFICITY OF RABBIT SERUM FACTORS

The species specificity of the rabbit factors VII and X was investigated by the two-stage extrinsic system described in appendix IV, 17.

The preincubation of either the rabbit or the human purified serum factors with rabbit brain extract resulted in the same clotting times of the substrate plasmas whether normal human or rabbit plasma or phenindione human plasma (Table 94). Also, similar activities were observed when human brain extract was used instead of the rabbit brain. This signifies that the rabbit factors VII and X are not species specific but are interchangeable with the human factors in extrinsic thromboplastin systems containing human or rabbit brain extracts. It is interesting to note that the activities of both human and rabbit serum factors in the previous test were very much the same. Since they have been prepared in parallel from the same amounts of sera, and in view of the later findings that rabbit factor VII is about 90 per

cent of the human, while rabbit factor X is about four times the human factor X concentration, it may well be that such a high concentration of factor X has to some extent masked the effect of a slight degree of species specificity. This is unlikely because factor X is present in the mixture in optimum concentration, and were a higher concentration effective its effect would have been obvious in the mixture containing rabbit brain and rabbit serum factors, being then more active than the mixture containing the human serum factors. The long incubation period seems to cancel any interferring effects due to varying concentrations of the serum factors since it allows ample time for the formation of maximum activity which was observed to occur within the first two minutes of incubation. However, the issue is further complicated by the concentration of the brain extract itself which is known to behave as a substrate (Straub and Duckert, 1961) and may have been a limiting factor to the further formation of active extrinsic thromboplastin. It is my feeling that brain precursor substance was present in the incubation mixture in excess in view of the very short clotting times obtained. Mann and Hurn (1952) believed rabbit brain thromboplastin to be species specific and that such specificity could largely be eliminated by the brief treatment of the thromboplastin with the dilute homologous serum. Fantl and Marr (1957) reported that rabbit factor VII was species specific,

though later Fantl and Osborn (1962) observed that the combination of purified rabbit or human factors VII and X with the brain precursor showed no absolute species specificity, but noted a higher activity in the homologous systems, concluding that a higher concentration of these factors is required for the prothrombin conversion in a heterologous reaction mixture.

Conclusion

Human and rabbit serum factors reacted similarly with human and rabbit brain extracts and are therefore not species specific.

RABBIT PLASMA FACTOR VII ASSAY

The concentration of factor VII in rabbit plasma was first estimated by the degree of correction of human phenindione plasma. Thus it was calculated to be 820 per cent of the human plasma factor VII activity (Table 95, experiment 1). Such a high concentration is believed to be not true, the result being inflated by the very high concentration of factor V in rabbit plasma, a fact that renders comparison between the human and rabbit plasma dilutions fallible.

The method of Koller et al (1951) was therefore tried. It measures the degree of correction of Seitz-filtered bovine plasma which contains factor V in excess thus minimising the effect of variation of factor V concentration in the test plasmas. With

this method, the corrective ability of rabbit plasma was found to vary from 86.6 to 94 per cent, with an average of 89.2 per cent of the human plasma factor VII concentration (Table 95, experiments 2, 3, and 4, and Figure 55). It should be noted that the substrate plasma is also deficient in factor X which is completely retained by the asbestos filters, and that the corrective ability measured is therefore a measure of the combined activities of factors VII and X in the case of both test and control plasmas. But since rabbit plasma was found to contain about four times the concentration of factor X (as will be discussed shortly) we can exclude the fact of its having any limiting effect on the test, since even in the 1 in 40 dilutions of the test rabbit plasma the concentration of factor X would be equivalent to that present in the 100 per cent preparation of the control human plasma.

Conclusion

Rabbit plasma contains factor VII in a concentration nearly equal to that present in the human plasma.

RABBIT PLASMA FACTOR X ASSAY

The activity of factor X in rabbit plasma was measured by the ability to correct Seitz-filtered bovine plasma when clotted with Russell's viper venom and cephalin in the presence of calcium

chloride solution (method of Bachmann, Duckert, and Koller, 1958).

The rabbit plasma was found to contain 388–479 per cent of the human plasma factor X concentration, with an average of 436.5 per cent (Table 96). No species specificity towards the R.V.V.-cephalin reagent was observed. Again, the high concentration of factor V in the substrate plasma rendered insignificant any variation of the factor V concentration in the test or control plasma samples.

Though no previous quantitative assays of rabbit factor X were made, Wartelle (1957b) observed that both the stypven time and the stypven-cephalin time of rabbit plasma were shorter than those of human plasma, and inferred that rabbit factor X occurs in a higher concentration than the human factor X, though she was aware that factor V was present in rabbit plasma in very high concentrations which must have contributed to the shortening of the stypven clotting times.

Conclusions

Factor X is present in rabbit plasma in a concentration about four times more than that in the human plasma. It is not species specific towards the Russell's viper venom-cephalin reagent.

RABBIT PLASMA PROTHROMBIN ASSAY

The rabbit plasma prothrombin content was estimated by the two-stage area method of Biggs and Douglas (1953a). With human brain thromboplastin the rabbit plasma prothrombin was found to average 67.5 per cent the human plasma prothrombin content, with a range of 66.9-68.6 per cent. With rabbit brain extract the corresponding values were an average of 72 per cent and a range of 63.7-85 per cent of the human plasma prothrombin estimated similarly. The results are presented in Table 97 and experiment number one illustrated in Figure 56.

Estimation of the rabbit plasma prothrombin by the globulin fraction technique of Douglas and Biggs (1953) yielded quite different values. With human brain extract the rabbit prothrombin was 30.7 per cent of the human prothrombin concentration, while with rabbit brain extract it was 29.6 per cent (Table 98).

Discussion

The results obtained for rabbit plasma prothrombin by the two-stage area method seem to reflect the true concentration of the prothrombin relative to the human plasma prothrombin, in view of the similar findings obtained with eother human or rabbit brain extracts. It is interesting to note that the use of rabbit brain extract resulted in lower values for all the plasma samples than

when the latter were estimated with the human brain extract, except in experiment 3 (Table 97) in which the rabbit brain was used and gave higher values for both plasmas. This finding, however, does not invalidate the results since comparisons were only made between samples tested with the same species of brain extract.

Murphy and Seegers (1948) using a two-stage procedure reported rabbit plasma to contain prothrombin in a concentration very slightly less than that in normal human plasma, namely 220-250 and 290 prothrombin units per cubic centimetre of plasma respectively. Fantl (1954) utilising his pyrocatechol two-stage method reported nearly similar values for both human and rabbit prothrombin concentrations, namely 470-720 Pyrocatechol Thrombin Units per millilitre of human plasma and 450-670 P.T.U. per millilitre of rabbit plasma. Quick et al (1961) found that rabbit plasma contained the same concentration of prothrombin as present in the human plasma in a two-stage assay system, while observing that results obtained with one-stage tests varied greatly according to the type of thromboplastin, exposure to foreign surfaces, and to the serum factors.

As was previously encountered during the prothrombin assays of horse plasma by the globulin fraction technique of Douglas and Biggs (1953), this method gave much lower values for the same plasma samples than those estimated by the area method, though utilising

the same brain extracts, both experiments having been conducted on the same day. The precipitation characteristics of human and rabbit prothrombins from plasma samples at different pH values ranging from 4.2 to 6.4 was investigated. Maximal precipitation of human prothrombin occured at pH 5.2, while rabbit prothrombin was maximally precipitated at pH 5.6. This difference has a negligible effect on the results since on comparing the maximal yields the rabbit prothrombin still measured 33 per cent of the human prothrombin. Since this method entails the activation of the prothrombin in the absence of the accelerator factors over a prolonged incubation period, it may well be that rabbit prothrombin under such conditions is less prone to activation or in other words more stable. Indeed, it was noticed that during the preparation of rabbit thrombin from an euglobulin fraction of rabbit plasma, recalcification of the latter resulted in the clotting of the solution after a much longer period than is usually the experience with a similar human preparation.

Conclusion

Rabbit plasma contains prothrombin in a somewhat less concentration than that of human plasma, when estimated by the two-stage area method, namely about 67 per cent of the human plasma prothrombin concentration.

EFFICIENCY OF RABBIT EXTRINSIC THROMBOPLASTIN SYSTEM

The rabbit plasma was found to contain all the coagulation factors required for the extrinsic thromboplastin system of prothrombin activation.

Extrinsic prothrombin activation was brought about in rabbit plasma as quickly and efficiently as in human plasma, both with rabbit and with human brain thromboplastins.

RABBIT INTRINSIC THROMBOPLASTIN SYSTEM

The rabbit whole blood clotted in ordinary glass tubes in 6' 6" while in siliconed tubes it clotted in 12' 51" (Table 101). The recalcified clotting time of rabbit plasma was also found to be slightly shorter than that of human plasma, namely 114 and 148 seconds respectively (Table 102). These results indicate the efficient and rapid generation of intrinsic active thromboplastin in rabbit blood and plasma as well as the presence of the contact factors.

Salzman (1962) obtained whole blood clotting times of 2' 30" to 5' 15", and bleeding times of 1' 0" to 6' 15". The latter were obtained by making an incision 3–4 millimetres long in the dorsum of the rabbit ear with a razor blade traversing a visible

vessel.

The rabbit blood platelets occur in about the same number as the human platelets, having an average of 311,000 platelets per cubic millimetre of whole blood (Table 100).

Conclusions

The rabbit has normal whole blood clotting times, normal recalcified clotting time of plasma, and normal platelet concentration as compared to the normal human corresponding values.

RABBIT PLASMA HAGEMAN FACTOR ACTIVITY

The activity of Hageman factor in rabbit plasma was measured on two occasions by the latter's ability to correct the prolonged recalcification clotting time of plasma obtained from a patient with congenital Hageman factor deficiency. Both rabbit and human plasmas were maximally glass-activated before being tested. The method is described in full in appendix IV, 24, II.

The average Hageman factor activity of rabbit plasma was 1025 per cent that in human plasma, with a range of 910-1140 per cent (Table 103, and Figure 57). In other words, rabbit plasma contained about ten times as much Hageman factor activity as is present in human plasma. It is my impression that such high values are exaggerated due to the presence of factor V in very high concentrations

in the rabbit plasma dilution mixtures as compared to its concentration in the corresponding human plasma dilution mixtures. This is reminiscent of the interferring effect of the high factor V concentration during the assay of rabbit plasma factor VII in the one-stage test resulting in the inflation of the final result.

Attempts to measure and compare the activities of purified rabbit and human Hageman factor preparations in a similar system have failed.

Wartelle (1957b) estimated the Hageman factor activity of the rabbit plasma as 50 per cent of the human plasma ability to correct a congenitally deficient human plasma.

Conclusion

Rabbit plasma contains Hageman factor in higher concentrations than those present in human plasma. The rabbit Hageman factor is not species specific.

RABBIT PLASMA THROMBOPLASTIN ANTECEDENT ACTIVITY

Exact measurement of the plasma thromboplastin antecedent activity of rabbit plasma was not made because of the unavailability of P.T.A. congenitally deficient plasma. But in view of the longer clotting times of whole blood obtained when using siliconed tubes instead of ordinary glass tubes, and since Hageman factor alone

cannot cause such a difference, it is reasonable to assume that the rabbit plasma contains P.T.A. in a concentration that is sufficient for the normal generation of active intrinsic thromboplastin. Wartelle (1957b) found that rabbit plasma P.T.A. was 250 per cent that present in human plasma when tested in a one-stage recalcification clotting time procedure by correction of P.T.A. congenitally deficient plasma.

RABBIT PLASMA ANTIHAEMOPHILIC GLOBULIN ACTIVITY

The A.H.G. content of rabbit plasma was measured by the method of Pitney (1956). It ranged from 246.6 to 324 per cent, with an average of 288.5 per cent of the human plasma A.H.G. activity (Table 106, and Figure 59).

Discussion

The observed high A.H.G. content of rabbit plasma was also evident in the latter's greater activity than human plasma in the ordinary thromboplastin generation test when incubated with human serum and tested with either human or rabbit platelets (Figure 58), though this may have also been partly due to the high factor V concentration in the adsorbed rabbit plasma. In the A.H.G. assays by Pitney's method the use of adsorbed haemophilic plasma contributes sufficient factor V, while the high dilutions of rabbit plasma used

rendered the latter's high factor V content without significant untoward effect on the accuracy of the measurement.

Fantl and Marr (1957) reported a similar range of A.H.G. in rabbit and human plasmas. Hawkey (1960) found that a 1 in 10 dilution of rabbit adsorbed plasma caused complete correction of haemophilic plasma in the thromboplastin generation test. Wartelle (1957b) made a quantitative estimate of rabbit plasma A.H.G. content by correction of haemophilic plasma in a one-stage recalcification time test and found that rabbit plasma contained 3-4 times as much A.H.G. as human plasma.

Conclusions

Rabbit plasma contains antihaemophilic globulin in a concentration about two and a half to three times that present in human plasma. The rabbit antihaemophilic globulin is not species specific.

RABBIT SERUM CHRISTMAS FACTOR ACTIVITY

The thromboplastic activity of rabbit serum in an ordinary thromboplastin generation test when incubated with human adsorbed plasma was found to be weaker than that of human serum whether human or rabbit platelets were used (Table 104, 105, and Figure 60). When incubated with rabbit adsorbed plasma the difference was less

marked, and nearly completely eliminated when rabbit platelets were used in place of the human platelets (Table 105, mixtures 146 and 147).

Specific measurement of Christmas factor activity of rabbit serum by a modified method based on Pitney's method (1956) revealed the presence of Christmas factor in low amounts compared to human standards, the average of three estimations was 29.3 per cent, with a range of 20.4 to 40 per cent of the human serum Christmas factor activity (Table 107, and Figure 61).

Discussion

Since rabbit plasma was found to contain more than adequate amounts of factor X which is not consumed during intrinsic prothrombin conversion, it is assumed that rabbit serum also contained equivalent amounts of factor X. Its weaker thromboplastic activity in the thromboplastin generation test can therefore be ascribed to its lower Christmas factor content as corroborated by the specific assays. The masking of this weaker activity on incubation with adsorbed rabbit plasma and rabbit platelets is believed to be due to the overwhelming high content of other clotting factors in the latter reagents rather than to species specificity between rabbit serum and plasma clotting factors.

Didisheim et al (1959) and Hawkey (1960) reported that rabbit serum had a similar activity to human serum in the thromboplastin

generation test. The latter author concluded complete correction of Christmas disease serum in the thromboplastin generation test with 1 in 10 dilution of rabbit plasma or serum, though her actual findings were as follows :-

Human serum	—	—	—	10	9	10
Rabbit serum	—	120	60	27	13	11

The weaker activity of the rabbit serum in correcting Christmas disease serum is apparent, and would have been more obvious if these values were expressed in terms of percentage of active thromboplastin.

Fantl and Marr (1957) and Wartelle (1957b) also maintain that Christmas factor is present in rabbit plasma in the same concentration as in human plasma, by correction of Christmas disease plasma in a one-stage recalcification time test whose results can be easily vitiated by the very high concentration of other clotting factors in the rabbit plasma (factors V, VIII, and X) as compared to their concentration in the human control mixture, especially that they only tested low dilutions of rabbit plasma, namely 1 in 20.

Wartelle (1957b), however, observed that in the thromboplastin generation test the rabbit serum was slightly inferior to the human serum, and from her results it is obvious that even a mixture of equal volumes of human and rabbit sera did not generate active thromboplastin in a comparable concentration to that attained by

human serum alone.

Conclusion

Rabbit serum contains about one third the concentration of Christmas factor present in human serum. The rabbit Christmas factor is not species specific.

RABBIT BLOOD PLATELETS

Rabbit whole blood contains an average of 311,000 platelets per cubic millimetre (Table 100) which is similar to a normal human platelet count. Rabbit platelets are morphologically similar to the human platelets. Casey et al (1936) reported platelet counts of 388,000-798,000/c.mm. of whole rabbit blood, from 180 estimations on 15 breeds (not including the Californian breed investigated in this work). They observed that in the bigger breeds the counts were usually higher.

The thromboplastic activity of the rabbit platelets was investigated by substituting them in place of the human platelets in the thromboplastin generation test and comparing the results. The rabbit platelet preparation had less activity than the human preparation when mixed with human adsorbed plasma and human serum (Table 105, mixture 144 compared with mixture 140 in Table 104; and illustrated in Figure 62, A, curves b and a respectively).

But with rabbit adsorbed plasma and rabbit serum, the rabbit platelets exhibited more activity than the human platelets (Table 105, mixture 146 compared to mixture 142 in Table 104; illustrated in Figure 62, B, curves b and a respectively). This phenomenon may be due to species specificity between the rabbit platelets and the rabbit blood coagulation factors, or to an excess of clotting factors being adsorbed on the rabbit platelets which together with the already high rabbit plasma coagulation factors' concentration in the incubation mixture render the final concentration of these clotting factors in the mixture abnormally high, a condition that may mask the weaker activity of the rabbit platelets.

Egli and Kesseler (1955) noted similar activities for rabbit and human platelets in the thromboplastin generation test.

Humphrey and Jaques (1954) found that rabbit platelets contain much greater amounts of histamine and serotonin than those present in human platelets, namely 30-60 times as much. Kjaerheim and Hovig (1962) observed that the ultrastructure of isolated rabbit platelets was in good agreement with that of the human platelets.

The ability of rabbit platelets to bring about clot retraction was found to be similar to that of human platelets. Rabbit whole blood having similar fibrinogen and haematocrit values to human whole blood had an average of 54.6 per cent of clot retraction in one hour (Table 108).

Conclusions

Rabbit blood contains a similar number of platelets as present in human blood. The thromboplastic activity of rabbit platelets in the thromboplastin generation test was weaker than that of human platelets when used in a human system, while it was stronger when they were used in a rabbit system. Rabbit clot retraction is normal by human standards.

RABBIT PLASMA SUBSTRATE REACTIVITY IN THE T.G.T.

The use of rabbit high-spun plasma as the substrate in the thromboplastin generation test instead of the human high-spun plasma resulted in consistent shortening of the clotting times obtained, which in terms of percentage active thromboplastin would give higher values than those obtained with human high-spun plasma. This phenomenon was demonstrable with all the incubation mixtures tested (Figures 63 and 64 constructed from Tables 104, 105, 109, and 110). Such enhanced reactivity of rabbit plasma may be due to the high concentration of accelerator factors V and X present. As shown previously, both the thrombin-fibrinogen reaction and the antithrombin content of rabbit plasma were similar to human plasma.

Conclusion

Rabbit high-spun plasma is more reactive than human high-spun

plasma to active intrinsic thromboplastin, whether homologous or heterologous.

INHIBITORS AGAINST HUMAN THROMBOPLASTIN IN RABBIT BLOOD

No inhibitors were observed in rabbit adsorbed plasma or in rabbit serum against human intrinsic thromboplastin formation when investigated by the method of Biggs and Macfarlane (1957) as cited in appendix IV, 23.

The results obtained are presented in Table 111 and illustrated in Figures 65 and 66. It will be seen that mixture number 158 had less activity because of the deficiency of the Christmas factor in the rabbit serum, but the same activity was present after two hours of incubation at 37°C. (mixture 161) thus ruling out the possibility of the presence of any inhibitory effect in rabbit serum.

Conclusion

Rabbit adsorbed plasma and rabbit serum do not possess any inhibitory activity against human intrinsic active thromboplastin formation in a thromboplastin generation system.

HOMOLOGOUS RABBIT INTRINSIC THROMBOPLASTIN GENERATION

The generation of rabbit homologous active intrinsic thromboplastin by the incubation of rabbit adsorbed plasma and rabbit serum with rabbit platelet suspension, with subsampling onto rabbit high-spun plasma, occured at a faster rate and in a greater degree than that of a comparable human homologous system (Table 110, mixture 154 compared to mixture 140 in Table 104). The use of human substrate high-spun plasma still gave much higher values for the rabbit homologous incubation mixture than those obtained with a human homologous system (Figure 64, B, curves b and a respectively).

These findings are to be expected from the previously demonstrated high concentrations of most of the blood clotting factors present in rabbit plasma, combined with the absence of inhibitory activity and a normal antithrombin level.

Egli and Kesseler (1955), Wartelle (1957b), and Didisheim et al (1959) found that rabbit thromboplastin generation was similar to that of man. Wartelle (1957b) attributed the initial hyperactivity in rabbit mixtures to the presence of preformed thromboplastin activity in rabbit reagents.

EFFICIENCY OF RABBIT INTRINSIC THROMBOPLASTIN SYSTEM

All the coagulation factors concerned in the intrinsic thromboplastin system of prothrombin activation are present in rabbit plasma and serum in amounts adequate for effective normal haemostasis, with the exception of Christmas factor which is slightly deficient in the rabbit serum. In spite of this single deficiency, normal whole blood clotting times and normal plasma recalcification clotting times were obtained. Also, great amounts of active intrinsic thromboplastin were formed in a homologous rabbit thromboplastin generation mixture.

Moreover, a prothrombin consumption index for rabbit blood of about 25 per cent (Table 99) indicates the efficient intrinsic prothrombin conversion into thrombin, sufficient to effect normal haemostasis.

SUMMARY OF RABBIT BLOOD COAGULATION MECHANISM

Rabbit blood contains all the coagulation factors known to take part in the human blood coagulation mechanism. Compared to the human, the rabbit blood contained similar concentrations of fibrinogen, platelets, factor VII, and antithrombin; while the prothrombin was 67 per cent, and the Christmas factor about one third that of the human serum. Rabbit plasma contained much higher values for the other coagulation factors, namely 100 times of factor V, 4 times of factor X, 3 times of A.H.G., and about 10 times of Hageman factor.

No species specificity was observed for any of the clotting factors, except that the purified rabbit fibrinogen seemed to be more actively clotted with purified rabbit thrombin.

The rabbit blood had normal whole blood clotting times, normal plasma recalcification clotting time, normal clot retraction, and a normal prothrombin consumption index.

Both the extrinsic and the intrinsic systems of prothrombin activation in rabbit blood are believed to be efficient.

P A R T VI

LOBSTER BLOOD COAGULATION MECHANISM

LOBSTER BLOOD COAGULATION MECHANISM

The data presented in this part are based on the results of experiments conducted on blood samples obtained from lobsters of the genus Nephrops norvegicus taken as representative of the crustacean class of Arthropoda.

The blood samples were taken from fresh live healthy animals obtained at frequent intervals from Millport Marine Station, Isle of Cumbrae, Scotland. The animals were kept in fresh sea water in a large tank with constant aeration, the temperature was kept at about 10-15^{o}C. by a continuous flow of cold water in a jacket around the tank. None of the animals used were kept for more than two days in the tank. They were fed on fresh mussels.

Arthropods have an open vascular system which bathes the tissues directly. The lobster has a short tubular muscular heart which pumps the blood into a short aorta that soon breaks into several smaller arteries. From the latter the blood flows into open spaces or sinuses between the tissues from where it is collected in a sternal sinus then passes to the gills. The blood returns through a pericardial sinus and three pairs of ostia into the heart.

Because of the resemblance between this open system without veins to the lymphatic system of vertebrates, arthropod blood is called "hemolymph". The hemolymph can be easily obtained from

any of the limb joints by puncturing their membrane with a needle and withdrawing the requisite volume of blood by a syringe.

Since the coagulation of crustacean blood has unique features very different from those of human blood coagulation, it is my intention to review the literature on this subject and present the already known facts before discussing them in the light of my findings. I should perhaps note that the terminology will be that of the authors whose work is reviewed, quotation marks being used in cases where confusion with similarly named but different substances from vertebrate blood coagulation might otherwise occur.

HISTORICAL REVIEW OF CRUSTACEAN BLOOD COAGULATION

Blood coagulation was first described in Crustacea by Hewson in 1777 who noted that lobster blood clots are less firm than those of higher animals. He further observed that lobster clots contain white threads which under the microscope appear to consist essentially of cells. Carpenter (1843) was the first to maintain that fibrin is also formed in the clotting of invertebrate blood. In 1846 Wharton Jones described two kinds of cells to occur in the blood of lobsters and crabs, one granulated and the other nucleated and by far the commonest. He noted that lobster blood formed a more complete clot than crabs' blood in which the blood corpuscles

form aggregates that do not show any tendency to coagulate and form a true clot. Pouchet (1882), however, reported that no fibrin was formed in crayfish blood. Geddes (1879-1880) thought that the compact clot of lobster blood consisted exclusively of cells connected with each other by pseudopodia without the cooperation of fibrin. He termed the syncytium formed the "plasmodium".

Fredericq (1879) from microscopic studies believed that the coagulation of lobster blood starts from the blood cells. He differentiated the coagulation process into two phases, the first is that of cell agglutination, a phase that cannot be prevented even by saturated salt solutions. The second phase of coagulation which he called "plasma coagulation" occurs after the plasma has been released from the clumps formed by the first coagulation. This plasma he maintains can coagulate again, resembling more a fibrinogen coagulation, and can be prevented by the addition of certain salt solutions, and by temperatures above 50°C.

Schäfer (1883) presented evidence that in the case of the sea-urchin blood the cells were capable of exuding a coagulable material.

Halliburton (1885) made some detailed investigations on the blood coagulation of Nephrops norvegicus. He believed that the spontaneous coagulation of crustacean blood is similar in nearly all respects to that of vertebrate blood. Namely, that it does

not occur in the living vessels but only takes place after the blood is shed, that it can be prevented by the admixture with the blood of certain salts e.g. four parts of a saturated solution of magnesium sulphate to one part of blood, and that it can be hindered by cold (temperature of melting ice). He also noted that the clot consisted of fibrin which entangled the cells. The formation of the fibrin was due to the solidification of a proteid of the globulin class or fibrinogen which existed in solution in the plasma. This change he attributed to the action of a ferment yielded by the amoeboid corpuscles of the blood. He prepared this ferment from blood or serum by the alcohol precipitation method of Schmidt (1892) and found that the dry powder thus obtained from mammalian or from crustacean blood when added to the dilute lobster plasma (obtained over saturated magnesium sulphate and the precipitate filtered off) caused coagulation in five minutes; just as he found that the "thrombin" produced from the blood of crayfish, crabs, and lobsters could coagulate salted cat plasma. However, he was able to obtain a purified preparation of lobster fibrinogen by salting it out from the blood by a saturated solution of sodium chloride. He observed that lobster fibrinogen is different from human fibrinogen with respect to solubility, also that the former coagulates at 65°C. while the human fibrinogen coagulates at 56°C.

Haycraft and Carlier (1888) found that the blood of the crab

Carcinus maenas failed to clot when carefully removed with an oiled pipette and kept surrounded by oil. Contact of the fluid with any part of the glass vessel wall was sufficient to cause clotting. They observed that while the blood remained fluid the corpuscles underwent no deformation, their disintegration following contact with a non-oily substance.

Cuénot (1891) stressed the differences between the various species of Crustacea first observed by Wharton Jones. He found that Arthropods possess true blood coagulation, some have very insignificant clots and some have very compact clots; while molluscs and worms had no coagulation at all. Cuénot seemed to join Halliburton's interpretation that Fredericq's first and second phases of coagulation are one and the same process. He further observed that the blood of Crustacea kept in captivity lost the power of coagulation, assuming that they used up the fibrinogen as a reserve nutrient.

Though several authors were aware of the presence of more than one kind of cells in crustacean blood (Heitzmann, 1873; Geddes, 1879-1880; Frommann, 1884; and Löwit, 1889) it was Hardy (1892) who differentiated between their respective functions. Hardy examined fresh hanging-drop preparations and osmic acid fixed and iodine stained preparations of the blood of the crayfish Astacus. He identified three types of cells. The first which he called

the "explosive cells" are pale oval cells notorious for their great irregularity in shape and their extreme sensitivity to contact with foreign bodies, then undergoing an explosive disruption of the protoplasm. They appeared in preparations of few seconds as large distinct rounded nuclei which floated freely in the plasma. They were of about 25-30 μ by 10-11 μ in size, the nucleus being 13-14 μ by 8-10 μ. When stained with eosin after fixation with osmic acid they remained uncoloured and because of the general colouration of the plasma they became obscured as though they have vanished; while the second type of cells "the eosinophilic cells" acquired a deep opaque red colour. These latter cells are actively amoeboid in fresh preparations and contain large refractile granules or rather spherules. These cells are stable for hours, and are about 18 by 27 μ in size. The explosive cells were more abundant, occuring in the ratio of 1.2-5 to each eosinophilic cell, with an average of 3 to 1. The third type of cell, which is also the rarist, is the basophil. This is a larger cell with irregular shape and has no amoeboid movement, the granules are less refractive and colourless with great diversity in size, but stain deeply with methylene blue within few minutes without the cell generally undergoing any change. They occur more frequently in poisoned animals. Hardy noted a marked correspondence in time between the solution of the explosive cells and the solidification of the plasma, while the only other

cellular elements normally present, the eosinophils, remained unchanged and alive for a considerable time after the blood has clotted. He therefore deduced that the ferment responsible for blood clotting must originate from the explosive cells, being a product of their activity and the result of the solution of the cell granules. He also observed that iodine solution (0.25 per cent) delays the clotting time, which coincides with the delay in the solution of the granules, maintaining that iodine prevents indefinitely the explosion of the corpuscles, but not the solution of the granules; while it fixes completely the granules and cell substance of the eosinophilic cells. He stated the causes that determine the explosion of the explosive cells and the clotting of plasma to be the contact with foreign solid bodies and the presence in the plasma of the products of disintegration of other explosive cells. He ably described the process as follows "Corpuscles in contact with the glass explode practically at once, while in the hanging drop one sees a larger and larger number of intact corpuscles (as one focuses further) which in their turn explode".

Heim in his thesis (1892) considered the first coagulation to form a clot composed of cellular aggregates without true fibrin, and the second coagulation to form a clot which is chemically different, being composed of fibrin. Calcium salts were essential for the second coagulation but not for the first. He, however,

stated that lobster and mammalian purified "thrombin" could interchangeably clot lobster and mammalian blood. He prepared the "thrombin ferment" from lobster muscle, and the purified lobster fibrinogen by precipitation with saturated solutions of magnesium sulphate. He further extended Wharton Jones' observations on the differences between the various species of Crustacea, dividing them into two categories.

Bottazzi (1902) confirmed many of Halliburton's results and was of the opinion that crustacean blood clotting occured in two successive phases of the same process, observing that oxalates prevented the second but not the first coagulation. He prepared the fibrinogen solution by precipitation with ammonium sulphate solution which he found caused the fastest salting out action.

Loeb in 1903 started extensive investigations on the blood coagulation of Crustacea and can rightly be said to have contributed the most fundamental findings towards the proper understanding of crustacean coagulation. Only his most important findings shall be reviewed here. While acknowledging the presence of two different processes, he believed that coagulation occured in one phase. The first process of cell agglutination could be prevented by small amounts of oxalate or citrate, while the second process of true coagulation was prevented only by saturated salt solutions. He attributed cell agglutination to certain changes in the cytoplasmic

layers of the cells whereby they become sticky, stick together into a gelatinous mass, a pseudo-clot, which resembles a common fibrin clot and which in the course of a short time retracts. He designated the fibrin-like network the "cell fibrin". It should be noted that this clot, however, does not contain fibrinogen since his early experiments were conducted on the King crab Limulus polyphemus which has no true blood coagulation, possessing only the first process of cell agglutination. In fact, today the Limulus is classified as not belonging to the Crustacea. He was aware that this animal had only the coagulation phase identical to the lobster's first coagulation (Loeb, 1904c and 1905). In the lobster he observed that the first coagulum was usually relatively small, and in the course of twenty minutes to one hour the surrounding fluid would coagulate as a solid gelatinous mass which in the next twenty four hours retracted but little (Loeb, 1903b). In order to differentiate between the two phases he resorted to ice-cooling of the blood followed by filtration, the plasma was then stabilised by heating for 30 minutes at 56°C. to destroy the fibrin ferment (Loeb, 1904b). He made two different preparations of the coagulation ferment by extraction of either the lobster muscle or the cell fibrin with water. He thought that the enzyme occuring in the muscle was different from that present in the cells, designating them as the "tissue coagulin" and the "blood coagulin" respectively (Loeb, 1906).

Loeb (1906) believed that coagulation could occur in one of two ways. Firstly the fibrinogen is acted upon directly by the "blood coagulin" which occurs as such in the blood cells, in which reaction calcium is not needed. Secondly, the fibrinogen can be acted upon directly by the "tissue coagulin" and here calcium is required. He thus concluded that coagulation in lobsters occurs in one phase, since stabilised plasma coagulated as rapidly by the addition of muscle extract as genuine plasma did, and because no increase in activity was observed when the lobster muscle extract was previously mixed with lobster serum. He further observed that the differences between the two enzymes acting in the lobster blood coagulation are of a quantitative nature as well as of a qualitative one. The muscle extract, other than requiring the presence of calcium ions, is more heat stable and more specifically adapted than the blood cells' extract (Loeb, 1903c and 1906). Also he found that no enzyme active in vertebrate coagulation had any effect on lobster blood, and vice versa (Loeb, 1904b) which reflects his more precise observations than those previously reported by Halliburton and Heim. Similar to mammalian thromboplastin, he found that some dilution of the muscle extract was necessary for optimal activity, and that when dialyzed muscle extract was used an inverse ratio was obtained between the concentration of the extract and the clotting time, concluding that lobster muscle extract contained an inhibitory

factor (Loeb, 1905 and 1907). Later in 1910, Loeb and Fleisher investigated the species specificity aspect of lobster blood coagulation in greater detail. Using pieces of muscle from the various crabs and other Crustacea in clotting lobster plasma, they found a certain degree of species specificity to occur similar to what was known of mammalian species. Lobster muscle had the strongest effect on lobster plasma, while only three out of the five crab species studied had any effect on lobster plasma. The muscle of Limulus and of different invertebrates not belonging to the Crustacea as well as that of vertebrates had no effect. Also, when cell fibrin was used instead of the muscle extracts, the preparations from all Crustacea were active, those from other animals were inactive, while extracts of the sea-urchin eggs had a weak effect. Loeb's final contribution on the subject was a study of the blood cells of the King crab Limulus in which he stated that the Limulus possesses only one kind of cells which combine the characters of leucocytes as well as blood platelets of mammals, being nucleated, motile, and producing substances initiating or accelerating the coagulation (Loeb, 1920). He was able to isolate these blood cells by drawing the blood with an oiled canula into an open dish stood on melting ice, allowing the cells to sediment forming "an even opaque layer of tissue consisting of several rows of agglutinated cells". The supernatant blue-coloured fluid was

removed and replaced by any desired test solution. He confirmed that in Limulus haemostasis was achieved by the agglutination of the amoebocytes, being wholly a cellular process which was not dependent on the formation of fibrin that was lacking in this animal. He similarly prepared the "cell-fibrin tissue" from certain other Crustacea (not specifically cited) from which he was able to extract the "blood coagulin", noting that agglutination of the cells preceded the diffusion of the coagulin into the surrounding fluid. However, no mention was made of its potency in terms of clotting times, in fact no details of his procedure were mentioned at all.

Alsberg and Clark (1908-1909) presented evidence that the essential protein which constitutes Limulus "cell fibrin" is physico-chemically different from fibrin, more resembling an elasto-gelatin.

Nolf (1909b) believed that clotting of crustacean blood was due to the action of a substance in the blood or muscle cells (A-Fibrinogen) directly on a substance in the plasma (B-Fibrinogen). Thus he differed from Loeb in that Crustacea have only one coagulation enzyme occuring in both muscle and blood cells, and explained Loeb's contention of their difference as due to the difference in the content of inhibitory substances in each. He also observed that muscle extracts very quickly lost their activity,

had no effect on mammalian fibrinogen, and that strong thrombin solutions had no effect on crustacean plasma.

Tait and Gunn (1918) made experiments on the blood of Astacus fluviatilis, a sea-crayfish, and verified the presence in its blood of three types of cells among which, however, two types were concerned with coagulation. A first coagulation occured due to the liberation of a soluble product from the lysed "explosive cells" of Hardy, and a second coagulation which occured after after a certain interval of time due to the cytolysis of the "thigmocytes", cells that are characterised by their ability to adhere to and spread upon glass until they are reduced to a thin vacuolated sheet. They further noted that the "thrombin material" liberated by one explosive corpuscle or one thigmocyte was capable of coagulating only a limited amount of the surrounding plasma. They also stressed that there was no sharp distinction between the cells, entertaining the possibility that the explosive cells may develop into thigmocytes within the blood stream, and by the acquisition of granules may further be transformed into amoebocytes. They classified the coagulation mechanisms in Crustacea into three types:-

Type A : agglutination of cells alone, no gelation of plasma, (Cancer pagurus, Maia squinado, Inachus dorynchus, etc..).

Type B : agglutination of cells with the subsequent gelation of plasma, (Carcinus maenas, Palemon serratus, Portunus

puber, and Homarus vulgaris, in increasing order of solidness of the clot).

Type C : same as type B, but the agglutination of the cells is insignificant and restricted to the immediate surroundings of the explosive cells, after which gelation spreads to the whole plasma, (Palinurus vulgaris, and Astacus fluviatilis).

Among the species investigated they found no correlation between the type of coagulation and the distribution of other physiological or zoological classification.

Howell (1914a and 1916) found that vertebrate fibrin as studied under the microscope precipitated in the form of long needles forming a network, while invertebrate (crab) fibrin precipitated as a structureless gel, the clots were soft, transparent, broke easily into pieces which easily flowed together again, and with no tendency to retract. The addition of alkali to human fibrinogen produced essentially the same picture of a structureless gel. Pig thrombin did not clot crab blood. This was confirmed again by Zunz (1933) who demonstrated that lobster plasma was clotted by lobster muscle extract but not by vertebrate thrombin or even by fish thrombin. This specific adaptation between crustacean blood and tissue coagulains and crustacean blood or fibrinogen was also confirmed by Moore et al (1935), Silberberg (1938), Glavind (1948),

George and Nichols (1948), and Duchâteau and Florkin (1954).

Glavind in 1948 published a monograph on the coagulation of crustacean blood containing a most detailed study of the subject. His findings shall be discussed later in the text when relevant. Generally, he found that crustacean blood clotting occured in one phase only, due to the action of one enzyme occuring in the muscles and the blood cells which required the presence of calcium ions, thus resembling vertebrate thromboplastin, but this enzyme also resembled thrombin in physico-chemical properties and in its ability to clot purified fibrinogen. No enzyme active in a crustacean system was active in a mammalian system and vice versa, but the crustacean "coagulin" was active against fibrinogen from other crustacean species. He observed that clotting of crustacean blood was inhibited by heparin and hirudin, though large concentrations were required. He also noted the difficulty of keeping lobsters alive in captivity, and that great fluctuations occured in the quality of their blood in which the cell volume sometimes dropped to ten per cent of the original, while the fibrinogen level decreased so much that the blood could not be made to clot. He mentioned that half of the animals gave blood samples unsuitable for clotting experiments and therefore mainly experimented on newly caught animals.

George and Nichols (1948) from a detailed study of the marine

blue crab Callinectes sapidus and of the fresh-water crayfish Cambarus bartoni, classified their blood cells into four types, the lymphoid cells, the semi-hyaline thigmotactic amoebocytes equivalent to Hardy's explosive cells or to Tait and Gunn's explosive cells and thigmocytes, the granular eosinophlic cells, and finally cells with refractile granules that are intermediate between the second and third types. They also supported Tait and Gunn's theory of the differentiation of the cells in the blood channels, but suggested that the lymphoid cells may differentiate along two or more lines. They did not recognize any difference between the explosive cells and the thigmocytes, and believed that "thrombin" may be liberated from the eosinophils as well as from the finely granular thigmotactic amoebocytes. Again, they observed that in crabs whose blood clotted instantly after extravasation the cells disintegrated correspondingly rapidly, while a weak clotting reaction was associated with stability of the blood cells.

A comparison of the efficiency of the haemostatic mechanisms in various Crustacea in relation to their anatomical and physiological characters was made by Morrison and Morrison (1952) who studied twelve crustacean species. They found there was a degree of compensation whereby animals lacking one or more mechanisms were particularly strong in others. For example, the spider crab Libinia has no fibrinogen nor the power of autotomy but is very strongly

protected by its shell, while the Gecarcinus species have no shell nor fibrinogen but have a highly developed power of autotomy. The spiny lobster Panulirus argus with little capacity for autotomy and not too heavy a shell in relation to its large size possesses a high concentration of fibrinogen. The King crab Limulus has no shell nor fibrinogen but shows a copious cellular agglutination. Other crabs (Callinectes, Carcinides, and Grapsus) were found to utilize all of the mechanisms, each in moderate extent. Finally, they observed that in animals previously considered to possess only an initial cellular phase of coagulation (cell agglutination) the weight of the blood coagulum (deposit obtained on filtering the clotted blood, washed and dried several times) was 3-8 times greater than was expected from an estimate of the maximal cellular contribution, and therefore concluded that plasma components must be of importance in these species.

Duchâteau and Florkin in 1954 developed a method for the preparation of lobster fibrinogen by ammonium sulphate precipitation in which the third precipitation yielded an electrophoretically homogeneous fibrinogen preparation clottable in the presence of lobster muscle extract and calcium chloride solution in six minutes producing a firm gel, but in the ultracentrifuge the preparation showed two components of nearly equal proportion, the rapidly sedimented first component had an S_{20w} value similar to haemocyanin.

Because of the difficulty of isolating the coagulin of the explosive cells in vitro, they worked with the muscle extract as prepared by the method of Loeb (1905) and further enriching it by centrifugation at 48,000 r.p.m. for 30 minutes, using the supernatant as the coagulant material. This purified muscle coagulin showed three constituents on electrophoresis. Further isolation and identification of the active coagulant could not be made because of the instability of the preparation.

Gregoire in 1951 started a series of investigations by the phase contrast microscope on the blood cells of Arthropoda, mainly on insects but later also included some Crustacea, namely Limulus polyphemus and Homarus americanus. She observed that in Crustacea the coagulation mechanism was similar to her pattern I of insect blood coagulation, namely the formation of islands of coagulation around Hardy's explosive cells, while the other categories of cells did not take part but were passively entrapped in the coagulum. The process of local jellification then extended to the whole plasma. Against Loeb's and Howell's conceptions, the development of "cell fibrin" by the general cell disintegration and fusion of their cytoplasm was not observed (Gregoire, 1952 and 1955).

Jitariu and Dimitriu in 1961 presented evidence of the absence of fibrinogen in the hemolymph of the fresh-water crab Astacus fluviatilis and the marine-water crabs Carcinus maenas and

Pachigrapsus marmoratus. They explained the transformation of the hemolymph of these animals from the state of sol into that of gel by the ionic increase in lipidic and macroergic molecules provoked by the destruction of the hemolymph cells whose behaviour simulated the viscous metamorphosis of mammalian thrombocytes. The formed gel which did not retract was found to liquefy on agitation or handling with a glass rod, but gelled again on being stood undisturbed. Incomplete gelling or a prolonged gelling time of the hemolymph were observed during the months November and December during which the animals were in hibernation, and in animals kept for a long time in captivity, coinciding with a decrease in the number of the cells and a decrease in the protein and electrolyte concentration of the hemolymph.

THE EXTRINSIC THROMBOPLASTIN SYSTEM OF THE LOBSTER

THE ACTIVITY OF LOBSTER TISSUE EXTRACT

The activity of the lobster tissue extract prepared from lobster muscle tissue was tested in a one-stage procedure by clotting the lobster oxalated plasma in the presence of calcium chloride solution. Several batches of lobster muscle extract prepared by the method of Loeb (1905) as described in appendix III, 13, IIA failed completely to cause the clotting of the lobster plasma. But extracts prepared by the method of Owren (1949) as described in appendix III, 13, IIB clotted the lobster plasma in the presence of 3 per cent calcium chloride solution in about 3-5 minutes (Table 112). In the absence of the tissue extract the recalcification time of the lobster plasma was more than 10 minutes, in fact the plasma did not clot at all. That this plasma was suitable for clotting experiments was evident from the very short clotting times obtained with the lobster cell extract to be discussed later which was about 6 seconds.

The lobster muscle extract was also found to clot solutions of purified lobster fibrinogen in the presence of calcium chloride solution in less than two minutes. The potency of the muscle extract was found to deteriorate easily since its storage in the frozen condition at -20°C. for 72 hours resulted in the complete

dissappearance of its clotting activity (Table 112, experiment 3). Such great storage lability may explain the lack of activity in the extracts prepared by the method of Loeb (1905) which entails standing the preparation overnight in the refrigerator at one stage.

Species specificity of the lobster muscle extract

The lobster plasma was found to be specifically clotted by the lobster muscle extract but not affected at all by the human brain extract (Table 113). Also, the lobster muscle extract was without effect on human plasma which clotted within few minutes similar to its recalcified clotting time in the absence of any tissue extract.

Discussion

The lobster muscle extract obviously acts in a different way to human tissue extracts since it also clots solutions of purified lobster fibrinogen, suggesting the presence of preformed coagulant activity in the extract and only requiring the presence of calcium chloride solution. Thus in one aspect it resembled mammalian thrombin while in the other it resembled mammalian tissue thromboplastin. This confirms the previous findings of Loeb (1906), Nolf (1909a), Glavind (1948), and Duchâteau and Florkin (1954).

Nolf (1909a) suggested that prothrombin and fibrinogen from the lobster blood may have the same precipitation characters, inferring the contamination of lobster purified fibrinogen preparations with prothrombin. But prothrombin has never been demonstrated in any

invertebrate animal, also adsorbed lobster plasma is clotted with the tissue extract as fast as unadsorbed lobster plasma (Glavind, 1948), while the purified lobster fibrinogen which is electrophoretically homogeneous has been shown by ultracentrifugation studies to contain only two components, hemocyanin and fibrinogen (Duchâteau and Florkin, 1954). It is therefore reasonable to conclude that the lobster muscle extract acts directly on the lobster fibrinogen in the presence of calcium chloride solution. This is also corroborated by the lack of accelerator factors in the lobster plasma and serum as shall be discussed later.

The increased lability of the lobster muscle extracts on storage has been pointed out by Nolf (1909b), Moore et al (1935), Glavind (1948), and Duchâteau and Florkin (1954). Glavind (1948) found that lobster muscle extract loses all its activity within few hours if kept at room temperature, within 48 hours at 10°C., within 3-7 days at -15°C., and within 12 days at -70°C. He also noted that the dried preparation was not stable at room temperature. A fresh preparation, therefore, has to be made for each experiment.

That lobster muscle extracts and lobster plasma possess an absolute degree of species specificity as opposed to vertebrate reagents, while having a certain degree of inter-species specificity among Crustacea seems to be unequivocal as demonstrated by various workers (Loeb, 1903b, 1903c, 1904b, and 1906; Nolf, 1909b; Loeb

and Fleisher, 1910; Howell, 1916; Zunz, 1933; Moore et al, 1935; Silberberg, 1938; Glavind, 1948; George and Nichols, 1948; and Duchâteau and Florkin, 1954).

Various attempts to purify the coagulant substance of the lobster muscle extracts have failed. Glavind (1948) found that the "coagulin" cannot be precipitated from the muscle extract at any pH value, being consistently present in the supernatant; also that it cannot be adsorbed by magnesium hydroxide, aluminium hydroxide, or charcoal. Duchâteau and Florkin (1954) enriched Loeb's muscle extract by centrifugation at 48,000 r.p.m. for 30 minutes, then discarding the precipitate. They found this purified "coagulin" to be a substance of a low molecular weight, containing a negligible amount of nitrogen, and had three constituents on electrophoresis. But further isolation and identification of the coagulant substance was hindered by the instability of the preparation.

Conclusions

The lobster muscle extract clots the lobster plasma as well as the lobster purified fibrinogen solution in about the same time in the presence of calcium chloride solution, suggesting a direct action on the fibrinogen. Lobster muscle extract is very unstable compared to human tissue extracts. Lobster plasma and tissue extracts are completely species specific and are not interchangeable with vertebrate reagents.

THE ACTIVITY OF ACCELERATOR FACTORS IN LOBSTER BLOOD

In specific assays measuring the degree of correction of various artificially induced coagulation factor deficiencies the lobster plasma was found to lack completely any factor V or factor X activity, while the lobster serum lacked completely factor VII activity (Tables 114, 116, and 115 respectively). The concept that these factors may be present in lobster blood but are species specific is hard to substantiate in view of the ability of the lobster muscle extract to clot the lobster purified fibrinogen in as short a time as it clots whole lobster plasma. Indeed, their presence would have been of no avail since lobster plasma does not contain prothrombin as previously discussed, and further confirmed by the inability of Russell's viper venom-cephalin reagent to clot the lobster plasma (Table 117), though this finding is difficult to interpret due to the interference of species specificity if this existed between the R.V.V. and the lobster plasma.

Conclusions

The lobster blood lacks completely any factor V, VII, or X activities in human assay systems. Results of the lobster homologous clotting mixtures also suggest their absence from the lobster blood.

ANTITHROMBIN ASSAY IN LOBSTER PLASMA

During assays on the lobster plasma and serum for the extrinsic accelerator factors V, VII, and X, prolonged clotting times were observed for the mixtures containing lobster plasma or serum, suggesting the presence in them of some inhibitory activity which was eliminated by the further dilution of the lobster reagents in saline. Also, since the lobster plasma was not clotted at all by bovine thrombin (Table 118) presumably due to its species specificity, the presence of antithrombin may have been a contributing factor.

Antithrombin assay by the method of Astrup and Darling (1942d) conclusively demonstrated the complete lack of antithrombin from lobster plasma (Table 119). The inhibitory activity previously observed in mixtures containing lobster plasma or serum is therefore though to be due to the difference in ionic strength and colloidal composition of the lobster plasma and serum as compared to the saline or human plasma used as the control.

Conclusion

Lobster blood does not contain any antithrombin activity.

SUMMARY OF THE LOBSTER EXTRINSIC SYSTEM

The lobster blood does not possess an extrinsic thromboplastin system as exists for vertebrates. It lacks all the factors concerned in the reaction, except of course the fibrinogen and a coagulant substance present in the muscle extract. The latter, however, differs from vertebrate tissue thromboplastin in clotting lobster purified fibrinogen directly in the presence of calcium chloride solution. Thus, the lobster muscle extract resembles more vertebrate thrombin rather than vertebrate tissue thromboplastin.

No data are available on the exact role played by the lobster tissue "coagulin" in physiological haemostasis.

LOBSTER INTRINSIC THROMBOPLASTIN SYSTEM

LOBSTER WHOLE BLOOD CLOTTING TIMES

Lobster whole blood was found to clot in 15-20 minutes at 37°C. and in 3-5 minutes at 15°C. (Table 120). In siliconed tubes more prolonged clotting times were obtained in either case. In the above quoted clotting times the recorded end point was the formation of an invertible clot. In actual fact, cellular agglutination and flocculation occured almost immediately after withdrawal of the

blood and its delivery in the clotting tubes, ordinary or siliconed, at 37°C. or at 15°C. With the use of siliconed tubes, however, the cellular agglutination though rapid in onset was not of the same magnitude as occured in the ordinary glass tubes.

It was observed that animals kept in captivity until they were devitalised as judged by their sluggish movements, lack of fight on handling, and dehydrated collapsed joint spaces, had much longer whole blood clotting times than those obtained when the animals were in the fresh condition (Table 120).

The prolonged clotting times obtained at 37°C. suggest that this temperature somehow delays the process by which the final coagulant material is produced. This is not strange in view that the natural environment of Crustacea has a temperature about 10-15°C. which also seems to be most suitable for the evolution of the intrinsic coagulant material from the "explosive cells".

Conclusions

The lobster whole blood clots faster in ordinary glass tubes and at 15°C. The lobster whole blood clotting mechanism is well adapted to the temperature of its normal environment. Foreign surfaces enhance the clotting of lobster blood.

HAGEMAN FACTOR AND P.T.A. ACTIVITIES IN LOBSTER PLASMA

Assays for the Hageman factor and the plasma thromboplastin antecedent in the lobster plasma by the degree of shortening of the clotting times of human intact plasma and by the correction of human plasma congenitally deficient in Hageman factor showed that the lobster plasma did not possess either activity (Tables 121 and 122), or if it did they were species specific. It is more probable, however, that the contact factor or factors in lobster blood reside in the blood cells themselves, particularly in the explosive cells of Hardy which were observed to explode immediately on contact with foreign surfaces (Haycraft and Carlier, 1888; and Hardy, 1892). This was also confirmed by phase contrast microscopy observations of the lobster cells as shall be described later.

Conclusions

The lobster plasma lacks Hageman factor and plasma thromboplastin antecedent activities in human assay systems. These contact factors may be present in the lobster plasma but are species specific, though it is more probable that such activities reside in the lobster blood cells themselves, in particular in the explosive cells.

LOBSTER BLOOD CELLS

Lobster blood contains only nucleated cells similar to the vertebrate leucocytes. There are no red blood corpuscles, but the oxygen-carrying pigment, haemocyanin, is present in the blood as a freely soluble protein, giving the blood its blue colour when well oxygenated.

The role played by any of the lobster blood cells in the coagulation of the lobster blood was investigated by observing freshly prepared blood smears under the microscope by phase contrast which allows easy and immediate identification of any change in the consistency and physical characters of the blood, as from sol to gel, in the form of areas of increased density and granularity. Such differences are not at all discernible if examined under the ordinary light microscope. As it was the experience of previous workers that lobster blood cells agglutinate and some of them explode or disintegrate immediately on extravasation, it was therefore imperative that blood smears are examined as soon as possible. This was achieved by first setting up the microscope and lighting by the use of a dummy slide. Then the lobster under test was brought in a small water tank and as soon as the blood sample was withdrawn by a siliconed needle and syringe, a drop was placed on a previously prepared glass slide, immediately covered very gently by a cover slip, put on the microscope

stage and examined. The whole procedure from puncturing the joint space to obtain the sample till the start of actual observation should not take more than one minute, otherwise the very first changes will be missed. In such unstained preparations the exact differentiation of the cells as in stained smears was of course not possible, but generally three types of cells were recognizable. The first and by far the most important and only type of cells concerned with coagulation were the explosive cells of Hardy. They appeared as large rounded nuclei surrounded by a pale hyaline cytoplasmic area that is not sharply defined. Within few seconds clear rounded vesicles or vacuoles of various sizes were formed in a rossette shape around the nucleus (Figure 67) and again very rapidly dissapeared as if they have exploded; during which process the surrounding plasma became progressively denser (Figure 68) until the whole preparation looked granulated signifying the complete solidification of the plasma. Figure 69 illustrates the picture seen after complete plasma solidification has occured, showing two well-developed coagulation islands around two explosive cells, while the remainder types of cells were found passively embedded within the clotted plasma. The explosive cells did not show any amoeboid movements and did not agglutinate together. They appeared to be fixed to the glass slide, while the shape of veils formed by the coagulation process seemed to be governed by the direction of current

flow in the preparation. The size and density of the coagulation islands formed varied greatly from cell to cell.

The second type of cells observed was very large cells of irregular shape containing large highly refractile granules, almost obscuring a relatively small nucleus. These were the only type of cells still evidently seen as luminous bodies when the condenser was racked from the phase contrast position to the dark-field position. They corresponded to Hardy's "eosinophilic cells" (1892), Tait and Gunn's "amoebocytes" (1918), and to the "granular cells" described by George and Nichols (1948). They showed some amoeboid movements, but seemed to have no role in the process of plasma solidification.

The third type of cells observed was of intermediate size between the explosive cells and the eosinophilic cells, with abundant well-defined cytoplasm that tended to spread on the glass slide forming thin large sheets around a globular nucleus. This last type seems to correspond to the "thigmocytes" described by Tait and Gunn (1918) as highly phagocytic cells whose cytolysis contributed to the process of plasma coagulation. However, in phase contrast preparations, they were never seen to disintegrate, nor to produce any clotting of their surrounding plasma, but were scattered adherent to the glass.

All the above findings are very similar to those previously reported by Gregoire (1951, 1952, and 1955) to occur in insects'

blood, as well as in the blood of the giant lobster Homarus americanus.

The total cell counts in lobster blood varied from 13,200 to 56,000 cells/c.mm. of whole blood, with an average of 25,910 (Table 123). The higher counts were observed to occur in the larger lobster specimens, while on one occasion the re-counting of the cells in the blood of a dying lobster showed great diminution in their number compared to that obtained when the animal was in the fresh and healthy condition (Table 123, experiment 6).

The differential count of the explosive cells was made as described in appendix IV, 1, C. They were found to constitute about 20 per cent of the total cell count (Table 123), thus their absolute number averaged about 5,200 cells/c.mm. of whole blood. In the blood of the crayfish Astacus fluviatilis, Hardy (1892) found a total cell count of 250-400 cells/c.mm., with an explosive cell-eosinophil cell ratio of about 3 to 1; while Tait and Gunn (1918) reported an explosive cell ratio of 40-50 per cent of the total cell count in the same species of crayfish. Yeager and Tauber (1935) made total counts on 13 crustacean species and reported an average of 18,700 ± 6,500 cells/c.mm. of whole blood.

Conclusions

The lobster blood contains a type of cells, the "explosive cells" of Hardy, which are very fragile and whose cytoplasm disintegrates

on contact with foreign surfaces releasing a substance which either by itself coagulates or there induces coagulation in the surrounding plasma. In the lobster Nephrops norvegicus these cells constitute about twenty per cent of the total blood cells' count.

LOBSTER FIBRINOGEN

1. Identification

Fibrinogen is known to normally occur in all vertebrate plasmas. It was also demonstrated in plasma of cold-blooded vertebrates, namely the reptiles, amphibians, and fish (Zunz, 1933; and Fantl, 1961). Among the invertebrates, Crustacea form a class which is a large division of the animal kingdom comprising crabs, lobsters, crayfish, prawns, shrimps, and a vast multitude of less familiar forms that are not distinguished by any popular names. Fibrinogen does not occur uniformly in the plasma of all crustacean species, a state which led to much controversy and confusion. Geddes (1879-1880) first reported that lobster blood clots consisted only of cells without any fibrin. Pouchet (1882) maintained that there was no fibrin in the blood of Nephrops norvegicus, while Halliburton (1885) stated that the blood clots of Nephrops norvegicus consisted of fibrin entangling the blood cells. Alsberg and Clark (1908-1909) demonstrated conclusively that the blood of the crab Limulus

polyphemus did not contain fibrinogen. Later several species were reported to have no fibrinogen in their plasmas, for example Cancer pagurus, Maia squinado, and Inachus dorynchus (Tait and Gunn, 1918); Libinia, and Gecarcinus (Morrison and Morrison, 1952); Astacus fluviatilis, Carcinus maenas, and Pachigrapsus marmoratus (Jitariu and Dimitriu, 1961).

It was therefore necessary to establish conclusively the presence or absence of fibrinogen in the blood of the lobster Nephrops norvegicus. Lobster plasma (appendix III, 3) and lobster serum (appendix III, 5) were electrophoresed in barbitone buffer at pH 8.6 for 2 hours, alongside control samples of human plasma and serum. The strips were then stained with Ponceau S stain and their densitometric scannings made as described in appendix II, 7. The proteins in the lobster plasma were found to separate into three components. The fastest and most abundant was haemocyanin (h), the oxygen-carrying pigment of lobster blood; a second slower and less abundant component but with a definite peak was component f which is believed to represent the true lobster fibrinogen; and finally an even slower and smaller component (x) which is as yet unidentified (Figure 70). In fact component x was only encountered in one plasma sample, all other plasma samples failed to show this component (compare with Figures 71 and 73). The lobster serum while showing the haemocyanin component, failed to show the fibrinogen one in all

the preparations tested (Figures 70 and 71), and was not clottable with the lobster blood cells' extract (Table 125, mixture 6).

It should be noted that the lobster fibrinogen (component f) had a mobility very similar to that of human albumin, a fact which denotes its different nature from human fibrinogen which hardly moves at all from the origin (Figure 72).

That this component f actually represents a clottable protein present in the lobster plasma was shown by its absence from the lobster serum obtained from spontaneously clotted lobster blood. Also, it was absent from lobster plasma clotted with lobster blood cells' extract (Figure 73). This fact is further substantiated by the coincicence of the f peak in lobster plasma electrophoresis strips with that obtained by the electrophoresis of a purified lobster fibrinogen preparation (Figure 74, II) which proved clottable by the lobster blood cells' extract in six seconds.

2. Assay of fibrinogen in lobster plasma

Oxalated lobster plasma diluted 1 in 10 in saline was clotted with lobster blood cells' extract in the presence of calcium chloride solution. The formed fibrin was collected on ballotini glass beads, and after several washings in saline the protein content of the deposit was estimated by the modified Greenberg method described in appendix IV, II. On two occasions the fibrinogen was also estimated by the micro-Kjeldahl method described in appendix IV, I. The

average fibrinogen content of lobster plasma by the Kjeldahl method was 251.6 mg/100 mls absolute plasma, and by the Greenberg method was 213.2 mg/100 mls absolute plasma (Table 124). Experiment 6 made on the plasma of a lobster kept in captivity until it showed signs of devitalisation had a fibrinogen concentration of 30 mg as compared to the original level of 186.6 mg/100 mls of plasma. This lowered fibrinogen content together with the diminution of the total cell count in devitalised lobsters as previously demonstrated account for the prolonged whole blood clotting times observed for samples obtained from devitalised specimens, and confirms the previous experiences of Cuénot (1891) and Glavind (1948).

Though marked variations in the fibrinogen levels occured from one specimen to another, this method seems to be the most accurate yet utilized, since the lobster cell extract used contained only minute amounts of protein as shown by its electrophoresis pattern (Figure 73) in which the main component was haemocyanin, a contaminant which is soluble and easily removed in the washing stage. Glavind (1948) modified Gram's gravimetric method (1921) by clotting 1 ml of the lobster plasma with 0.5 ml of muscle extract in the presence of 0.5 ml of 3 per cent calcium chloride solution. The clot was dried between filter paper, washed in distilled water for 30 minutes then dried in alcohol and ether to remove the pigment, and finally weighed on a microbalance. He reported average fibrinogen

values of 294 mg/100 mls of Homarus americanus plasma citrated 1 in 5 (equivalent to 367 mg/100 mls of absolute plasma), and still higher values of 400 mg/100 mls of citrated plasma of newly captured animals (equivalent to 500 mg/100 mls of absolute plasma). However, the use of undiluted plasma in addition to the presence of the protein-rich muscle extract in a high proportion in the mixture (1 to 4) might lead to the entrapping of excess insoluble protein matter in the meshes of the clots, giving rise to inaccurate results.

3. Preparation of purified lobster fibrinogen

Several method have been used for the preparation of lobster fibrinogen from the plasma by precipitation with solid sodium chloride, saturated magnesium sulphate solution, or acidification with acetic acid (Halliburton, 1885; Heim, 1892; Bottazzi, 1902; Loeb, 1905; and Glavind, 1948). The best devised method, however, was that of Duchâteau and Florkin (1954) which mainly relies on the precipitation of the fibrinogen with saturated magnesium sulphate solution in three stages. Their first precipitate obtained from plasma of the giant lobster Homarus americanus by 25 per cent saturation with magnesium sulphate contained three electrophoretic components, haemocyanin, fibrinogen, and an unidentified protein. The fibrinogen was greatly increased in proportion to the haemocyanin on comparison with the original plasma. The second precipitate obtained with 31 per cent saturation of magnesium sulphate contained fibrinogen

and the unidentified protein; while the third precipitate obtained with 45 per cent saturation with the magnesium sulphate consisted of an electrophoretically homogeneous fibrinogen preparation that was clottable with lobster tissue extract and calcium chloride solution in six minutes producing a firm gel. However, in the ultracentrifuge, this preparation showed two components of equal proportion, of which the slower sedimenting one with a Svedberg sedimentation constant in water at 20°C. (S_{20w}) of 16.9 was identified as the fibrinogen. The faster sedimenting component was haemocyanin (S_{20w} = 21). This was the method used in this work to prepare purified fibrinogen from the plasma of the lobster Nephrops norvegicus, with the slight modification of dissolving the various precipitates obtained in saline solution instead of distilled water, and dialysing them immediately against saline in the cold overnight to get rid of the excess magnesium sulphate ions. The method is described in detail in appendix III, 6, B. In my hands, the solution of precipitate 1 contained nearly equal amounts of fibrinogen and haemocyanin on electrophoresis (Figure 74, I) and was clotted with the lobster blood cells' extract and 3 per cent calcium chloride solution in 7.5 seconds (Table 125, mixture 9). Solution of precipitate 2 was the most pure preparation, consisting mainly of fibrinogen and only a trace of haemocyanin (Figure 74, II). It was clotted with lobster blood cells' extract and 3 per cent calcium chloride solution in

6.5 seconds (Table 125, mixture 10). Solution of precipitate 3 contained little fibrinogen and an appreciable amount of haemocyanin (Figure 74, III). It was clotted in 134 seconds forming a small gel floating amidst the surrounding fluid (Table 125, mixture 11).

The fibrinogen bands in the first two precipitate solutions had the same fast mobility of the fibrinogen in the original plasma, having an electrophoretic mobility of 5.5×10^{-5} cm./second/volt/cm. (Figure 74, I and II; and Figure 71).

It seems that the lobster fibrinogen is not a globulin since Duchâteau and Florkin (1954) have separated it from the precipitates by solution in distilled water, while globulins are not soluble in this medium but require the presence of an electrolyte such as sodium chloride in the solution. They also have demonstrated that the lobster fibrinogen was more soluble than the bovine fibrinogen, also that it had a very rapid electrophoretic mobility of 5.7×10^{-5} compared to a mobility rate of 2.7×10^{-5} for the bovine fibrinogen.

Conclusions

The lobster plasma contains a soluble coagulable protein that is missing in lobster serum or lobster plasma clotted with lobster blood cells' extract, in a concentration averaging 213-251 mg/100 mls of absolute plasma. It had a fast electrophoretic mobility similar to that of human albumin; and was best separated from plasma by precipitation with 31 per cent saturation with magnesium sulphate.

LOBSTER BLOOD CELLS' EXTRACT

Most of the earlier investigations on the clotting of crustacean blood have been carried out using muscle extracts as the coagulant material. These extracts varied greatly in potency and were usually of weak thromboplastic activity, giving clotting times ranging from several minutes to several hours, without a sharply defined end point. This seemed strange in view of the fact that the explosive cells of the blood almost instantaneously explode on contact with foreign surfaces releasing a potent coagulant material that immediately clots the surrounding plasma.

Loeb (1903b, 1905, and 1920) made use of the thromboplastic activity of the lobster blood cells in his clotting experiments on the plasma of the giant lobster Homarus americanus. He allowed the carefully withdrawn blood to stand in a clean sterile dish over melting ice, avoiding agitation of the blood. As soon as the cells settled forming an opaque layer of tissue, the supernatant fluid was drawn off and replaced by the desired test solution. Later, he extracted this "cell fibrin" preparation in a volume of saline for 25 minutes and used the extract to clot the plasma samples under test. With this preparation he obtained clotting times varying from 5 to about 16 minutes. He noted the great temperature and storage lability of the cell-fibrin extracts. Glavind (1948) also

attempted preparing similar extracts by collecting the freshly spun down cellular sediment whenever blood samples were available, storing it meanwhile in dry ice at -70°C. until a sufficient amount was collected, usually from 10-20 animals. The sediments were then ground in one part quartz and one part distilled water to which has been added an amount of calcium chloride calculated so as to compensate for the oxalate added to the plasma. The mixture was then stored in the ice-box overnight, and finally centrifuged. He found that the extracts prepared in this manner were as a rule active but weaker than the muscle extracts similarly prepared, giving clotting times of 2-30 minutes. They had the same lability characteristics as described for the muscle extracts.

A potent lobster blood cells' extract was prepared by a very simple and rapid method described in detail in appendix III, 16. Briefly, the freshly withdrawn oxalated blood was centrifuged immediately for 5 minutes at 2,000 r.p.m. in siliconed conical centrifuge tubes. The supernatant plasma decanted (usable for all clotting experiments) and the tube washed gently with saline without dislodging the deposit which is then thoroughly emulsified in a small volume of saline and immediately centrifuged in the cold. The supernatant which should be as clear as saline is used without delay in the clotting experiments. In the presence of calcium chloride solution, this cell extract clotted lobster oxalated plasma

in 5-7 seconds (Table 125, mixture 1) which is the shortest clotting time yet recorded for crustacean blood with any extract. The cell extract itself did not contain any fibrinogen as judged by its electrophoretic pattern (Figure 73, a) as well as by its inability to form a clot by itself in the presence of calcium chloride solution (Table 125, mixture 3). The lobster blood cells' extract also had the ability to clot lobster adsorbed plasma and lobster purified fibrinogen preparations in as short a time as obtained for the lobster plasma (Table 125, mixtures 7, 9, and 10 respectively). This suggests that the extract contained a preformed active coagulant material that acts directly on the fibrinogen, and only requires the presence of calcium chloride solution. The optimal concentration of calcium chloride solution was 3 per cent; higher concentrations (11 per cent or molar solution) hardly improved the clotting times, while an M/40 (0.27 per cent) solution gave much longer clotting times (Table 125).

The lobster blood cells' extract had no activity at all on human plasma or on human purified fibrinogen (Table 125, mixtures 4 and 5, and 12 respectively), thus resembling the lobster muscle extract in its species specificity.

While the lobster blood cells' extract behaved as a thrombin-like material by directly clotting purified fibrinogen preparations, it showed no esteratic activity when using TAMe as a substrate (Table

126). This demonstrates that whatever its mode of action is, by which it causes the clotting of lobster fibrinogen, it almost certainly is not through the splitting of arginyl-glycine bonds as occurs when human fibrinogen is clotted with human or bovine thrombin.

Cell extracts stored frozen at -20°C. for the short period of three hours, when rethawed and used, were found to have lost all clotting activity as compared to the original fresh extract (Table 127). The same findings were observed when the extract was kept at ordinary room temperature on the bench for the same period. Such great storage lability of the cell extract necessitated the frequent preparation of fresh aliquots, their use immediately on preparation, and keeping them at the temperature of melting ice during the period of the various experiments.

Conclusions

An extremely active lobster blood cells' extract was made from the freshly sedimented blood cells by a simple and rapid method.

The extract clotted oxalated lobster plasma, adsorbed lobster plasma, and purified lobster fibrinogen solutions within few seconds, in the presence of calcium chloride solution. It seemed to act directly on the fibrinogen, but had no esteratic activity on TAMe substrate. It was very labile on storage and is highly species specific.

FURTHER INVESTIGATIONS ON THE ACTIVITY OF THE LOBSTER BLOOD CELLS' EXTRACT

Since the lobster blood cells' extract by directly clotting adsorbed lobster plasma or purified lobster fibrinogen solution behaved as a thrombin-like material on one hand, while on the other hand it required the presence of calcium chloride solution for its action to be manifest, resembling vertebrate thromboplastins, further investigations of its characters were necessary.

Effect of heparin on the activity of the lobster cells' extract

Mixtures of lobster oxalated plasma were incubated with various concentrations of heparin (varying from 0-500 units in each mixture) for three minutes before clotting them with lobster blood cells' extract and calcium chloride solution. Heparin was found to prolong the clotting times progressively as its concentration rose in each mixture, up to 360 seconds obtained with the mixture containing 500 units of heparin, compared to a control clotting time of 27 seconds (Table 128, line 1). While at all the heparin concentrations the action of bovine thrombin (50 units/ml with a final concentration of 12.5 units/0.4 ml of the clotting mixture) on human plasma was completely antagonised resulting in an incoagulable mixture (Table 128, line 2), mixtures of lobster plasma were still coagulable though in prolonged periods. This observation can be explained either by

the use of a highly concentrated and active cell extract, or that heparin is not so much specifically antagonistic towards lobster blood cells' extract as it is to bovine thrombin. The latter explanation seems more attractive, since the cell extract could not have been that strong since in fact its dilution by one-fold in saline immediately resulted in the prolongation of the clotting time of the plasma from 5 seconds to 8 seconds, suggesting there was no excess active coagulant material present in the extract.

The addition of toluidine blue (a heparin antagoniser) to the lobster clotting mixtures containing heparin resulted in a diminution of the previously observed delay in clotting times, more pronounced with the more concentrated toluidine blue solution containing 50 mg per cent (Table 128, lines 3 and 5). It should also be noted that such concentrations of toluidine blue were still insufficient to prevent the action of heparin in the human clotting mixtures (Table 128, lines 4 and 6).

The addition of hexadimethrine bromide, a heparin neutralizer, in the form of Polybrene (Abbott) to the various plasma-heparin mixtures resulted in the greatest diminution in clotting times. At a concentration of 1 mg in each mixture, it was able to completely neutralize heparin concentrations of up to 250 units in the lobster mixtures, and of up to 125 units in the human mixtures (Table 128, lines 7 and 8). However, it was observed that hexadimethrine

bromide by itself slightly retarded the clotting time of lobster plasma from a control reading of 27 seconds to 56 seconds; while 50 seconds was the shortest clotting time obtainable for any lobster clotting mixture containing hexadimethrine bromide, whether heparin was present or absent.

Whatever the nature of the coagulant material present in the cell extract is, it again bears some resemblance to vertebrate thrombin in being antagonised by heparin. Haycraft (1884) who discovered hirudin, the coagulation-preventing substance obtained from the medicinal leech, observed that it was unable to prevent the coagulation of crustacean blood; a finding later confirmed by Loeb (1903c). Numanoi (1938) using very high concentrations of heparin and hirudin found that they inhibited the blood coagulation of the crustacean species Ligia exotica. He reported that the degree of anticoagulant action of heparin and hirudin for the blood of the Ligia was of the order of 1 to 400, while that for human blood was 1 to 100,000, again stressing the need for greater concentrations of heparin to retard the clotting of crustacean blood. Glavind (1948) confirmed that hirudin in amounts which strongly inhibit human coagulation was without effect on lobster blood clotting, though large amounts had some inhibitory action. He also found that the heparin concentration required to inhibit the clotting of the lobster plasma with the muscle extract was much

greater (about 1000 times) than that required for vertebrate blood, and was of the same magnitude as the concentration required to protect vertebrate platelets from agglutination and disintegration. He further observed that the prevention of agglutination of the lobster blood cells was very difficult and much higher concentrations of heparin than those required to inhibit spontaneous lobster blood coagulation (3.3 gm per cent in lobster blood) were still unable to prevent the cellular agglutination and disintegration. Because he was able to inhibit the clotting of the lobster plasma - muscle extract mixture by using a heparin concentration of 0.9-1.25 gm per cent (equivalent to 0.9-1.25 mg in the mixture or 90-125 units) as compared to 3.3 gm per cent (equivalent to 3.3 mg in the mixture or 330 units) required to inhibit spontaneous coagulation, he inferred that the native blood cells contain "coagulin" in a much larger concentration than present in the muscles, not being necessarily a different enzyme as Loeb previously suggested (1906).

The hepatopancreas of various Crustacea was found to contain a substance which very strongly inhibited the coagulation of both lobster and vertebrate bloods, its mode of action, however, is as yet unknown (Abelous and Billard, 1898; Nolf, 1909b; Gautier, 1913; and Numanoi, 1938).

Lobster blood cells' extract-fibrinogen reaction

When lobster plasma was clotted with various dilutions of

lobster blood cells' extract, the clotting times were found to increase proportionately in the first two dilutions, after which the lobster blood cells' extract diluted 1 in 8 in saline seemed to lose its activity more than was expected until with the 1 in 32 dilution the plasma was incoagulable (Table 129).

Clotting of the lobster purified fibrinogen (solution of precipitate 2) with various dilutions of the lobster blood cells' extract gave progressively increasing clotting times which when plotted on double logarithmic paper against the concentration of the cell extract were found to produce a straight line, as obtained with a human thrombin-fibrinogen dilution curve, suggesting that the reaction of the cell extract with the lobster fibrinogen is also a first order reaction (Table 129).

Clotting progressive dilutions of the lobster fibrinogen with the concentrated cell extract also gave clotting times inversely proportional to the concentration of the fibrinogen.

The prolonged clotting times of the lobster plasma obtained with the 1 in 8 and the 1 in 16 dilutions of the cell extract as compared to the shorter clotting times of the lobster fibrinogen clotted with the same dilutions of the cell extract suggest the presence of some sort of inhibitor in the lobster plasma, but may have been due to the presence of oxalate ions in the plasma whose effect became apparent only with the higher dilutions of the cell extract,

although excess calcium chloride solution was always added to each mixture.

Conclusions

The active coagulant material present in the lobster blood cells' extract resembled vertebrate thrombins in two other features.

Firstly, its clotting activity was antagonised by heparin whose action was again reversed by the addition of either toluidine blue or of hexadimethrine bromide. However, much higher concentrations of heparin were needed to completely antagonise the cell extract activity than was required to inhibit a potent vertebrate thrombin preparation.

Secondly, an inversely proportional relationship was observed between the concentration of the cell extract and the clotting time of purified lobster fibrinogen solution, similar to that observed in vertebrate thrombin-fibrinogen dilution curves.

PRECURSOR THROMBOPLASTIC ACTIVITIES IN LOBSTER BLOOD

The presence of antihaemophilic globulin activity in adsorbed lobster plasma was tested for in a thromboplastin generation test using human plasma as the substrate. The lobster adsorbed plasma was found to lack completely any antihaemophilic globulin activity, also it had no inhibitory effect when added to a human thromboplastin

generation mixture (Table 130).

Similarly, the lobster serum did not show any Christmas factor activity in the thromboplastin generation test, did not correct the defect of Christmas disease serum, and did not possess any inhibitory activity against human thromboplastin generation mixtures (Table 131).

Discussion

The lack of factor VIII and factor IX activities in lobster blood, together with the previously demonstrated lack of factors II, V, VII, X, XI, and XII activities suggest that the lobster blood does not possess either the extrinsic or the intrinsic thromboplastin mechanisms. It seems more reasonable to conclude that these factors are actually missing from lobster blood rather than present but species specific, especially that it has been observed that the coagulation of the lobster blood is mainly dependent on the disintegration of the explosive cells, while an extract of the lobster blood cells was found to contain a coagulant material possessing many similarities to vertebrate thrombin. All these findings lead to the conclusion that lobster blood coagulation occurs in one phase only, namely the conversion of a soluble protein present in the blood (lobster fibrinogen) into an insoluble product resembling fibrin, by the action of a coagulant material released from a certain type of blood cells, the explosive cells, characterised

by their ability to disintegrate very rapidly on contact with foreign surfaces. It is only assumed that such a mechanism is also operative in vivo as it is in vitro, possibly substantiating and/or substantiated by cellular agglutination as described by Loeb (1903b, 1904a, and 1920); Glavind (1948), and Gregoire (1952) to achieve proper haemostasis.

Conclusion

The lobster blood does not contain any of the precursor thromboplastic factors known to take part in vertebrate blood coagulation.

DEMONSTRATION OF THE DEVELOPMENT OF CLOTTING ACTIVITY IN THE LOBSTER NATIVE BLOOD

Nascent clotting activity was demonstrated to develop in native whole lobster blood by subsampling aliquots of the latter onto aliquots of purified lobster fibrinogen. The clotting activity was maximal within one minute after the blood withdrawal, after which time the clotting activity became progressively weaker (Table 132). The appearance of this clotting activity is believed to coincide with the disruption of the lobster explosive cells releasing the coagulant material. The gradual disappearance of the clotting activity on incubation is thought to be due to the

consumption of the coagulant material as well as to its great lability, rather than to the presence of inhibitory factors similar to vertebrate antithrombin.

Conclusion

An active coagulant material which clots purified lobster fibrinogen is generated in native lobster blood within the first minute of its withdrawal from the animal, thereafter the clotting activity disappeared progressively on further incubation.

LOBSTER BLOOD CLOT RETRACTION

Clots formed from lobster whole blood were found to have very negligible retraction varying from 0-4 per cent within one hour, and only very slightly improved on further standing for 24 hours (Table 133). The lobster blood clots are not as firm as vertebrate blood clots, and are in the form of clear gels which break easily into gelatinous masses on handling.

Conclusion

In vitro clots of lobster blood do not possess the power of retraction.

SUMMARY OF THE LOBSTER BLOOD COAGULATION MECHANISM

The lobster blood does not contain any of the coagulation factors known to take part in vertebrate blood coagulation, except for a soluble protein, the lobster fibrinogen, present in a concentration of about 250 mg per 100 mls of absolute plasma, and has an electrophoretic mobility similar to that of human albumin.

Lobster fibrinogen is directly clotted by an active coagulant material present in the lobster muscle and in the explosive cells of the blood. The coagulant material is highly unstable on storage, resembles vertebrate thrombins in many aspects, but requires the presence of calcium chloride solution for its activity to be manifest. The coagulant material had no hydrolytic activity on arginine esters.

No enzyme active in the lobster blood coagulation was active in the human blood coagulation and vice versa, indicating the absolute species specificity of the crustacean coagulation mechanism.

Lobster whole blood containing about 5,200 explosive cells per cubic millimetre was found to spontaneously clot in ordinary glass tubes within 3-5 minutes at 15°C. and in moderately longer times at 37°C. suggesting that the lobster coagulation mechanism is well adapted to function at this lower temperature of their normal

environment. Such in vitro blood clots showed very negligible retraction.

The rapid occurence of lobster blood coagulation in one phase, together with the lobster's moderate power of autotomy as well as its protection by a strong shell show that the haemostatic mechanism of the lobster is well adapted for its survival.

GENERAL DISCUSSION

GENERAL DISCUSSION

Findings reported in this work have been already discussed under the headings of the separate subjects. This is now a discussion of general aspects not already covered, with brief reference to personal findings where relevant.

The process of evolution of the mammalian coagulation mechanism shall first be presented, followed by discussion of the differences and specificity of the various coagulation factors in the different species, and their implications.

In discussing the process of evolution of the coagulation mechanism, two facts should be kept in mind. Firstly, a biological species is defined as an evolved or evolving, genetically distinctive, reproductively isolated, natural population. Professor Emerson stated that "Artificial populations compounded by man through domestication are not given species rank, even when they are genetically distinctive and reproductively isolated populations. Under natural conditions such man-produced populations would seldom maintain their genetic distinctiveness even if they were able to survive" (Emerson, 1945). It follows therefore that the domestic cattle or fowl for example, though classified as full species, the separate breeds are not. Though the separate breeds investigated here do not represent natural species, however, it is reasonable

though not certain, to suppose that similar findings would have been observed were these breeds natural species. Secondly, the coagulation process is not necessarily a defensive mechanism in all the species, it may well be part of a metabolic process (Nolf, 1908) or indeed wholly concerned with cellular activities as occurs in primordial organisms (Heilbrunn, 1937; and Robb-Smith, 1955).

The phylum Protozoa represents the simplest but by no means simple animals. In such unicellular organisms, for example the amoeba, a surface precipitation reaction has been described to occur in the form of changes in the cytoplasmic consistency from sol to gel and vice versa. This reaction, a character of the cell protoplasm, is necessary for the cellular activities of pseudopodial formation, phagocytosis, as well as division, and is thought to be analogous to a clotting reaction (Heilbrunn, 1961). The reaction occurs normally only in the presence of free calcium ions. It can be prevented by the removal of the free calcium ions through the addition of oxalates or citrates. It can also be prevented by the addition of heparin.

In invertebrates of more complex structure (multicellular organisms with a body cavity) as the Echinoderms exemplified by the sea-urchin and the starfish, haemostasis takes place only in the form of cellular agglutination, again dependent on the presence of calcium ions (Davidson, 1953).

As we go up the scale of evolution, achievement of haemostasis by cellular agglutination seems to be the only mechanism observed in several phylae, namely Molluscs represented by the oyster "Ostrea edulis" (Takatsuki, 1934), the boring mussel "Pholas" and the gastropod "Buccinum" (Geddes, 1879-1880); Worms represented by the common earthworm (Geddes, 1879-1880); and the Arthropoda represented by insects and crustacea. In insects, however, four types of reaction were described by Gregoire (1951). The first and most important is the formation of coagulation islands or granular clouds caused by the disintegration of the fragile hyaline cells of the haemolymph. The second type of reaction is the production of pseudopodial meshworks by the hyaline cells with the occasional formation of diffuse plasma veils. The third type is the first two combined. The fourth type showed no visible plasma coagulation nor any reaction in the hyaline cells. Oxalates and cocaine hydrochloride were found to delay or prevent the alterations in the hyaline cells and consequently preventing the coagulation of the haemolymph. Similarly, in crustacea there is wide variation of the process of haemostasis. Several species rely on cellular agglutination alone, for example the king crab Limulus polyphemus (Loeb, 1903b, 1904c, and 1920), Cancer pagurus and Maia squinado (Tait and Gunn, 1918), as well as the species Libinia and Gecarcinus (Morrison and Morrison, 1952). In the latter species the power of

autotomy is particularly well developed. When a limb is injured it is reflexly shed off at its origin from the body, a diaphragm extends across the breaking plane and a valve closed by the blood pressure from within shuts off the blood sinus when the limb is finally lost. In other species, cellular agglutination is followed by the gelation of the plasma, for example Homarus vulgaris (Tait and Gunn, 1918), Homarus americanus (Glavind, 1948), and Nephrops norvegicus as demonstrated in Part VI. These species possess a fibrinogen-like substance present in the plasma, coagulable by a thrombin-like substance released either from the blood cells or from muscle tissues on injury, in the presence of calcium ions. The active coagulant material can be inhibited by heparin in large concentrations, which action is again neutralized by hexadimethrine bromide. Up to this point in evolution, the blood cells played the predominant role, being contact sensitive, and releasing the active coagulant material.

Next in the scale of evolution are the cold-blooded vertebrates, namely fish, amphibians, then reptiles. These were not investigated in this study. However, it is reported that the blood of fish contains fibrinogen that can be clotted by thrombin which is evolved from prothrombin (Russel et al, 1962). Warner et al (1939a) found that dogfish blood contains eight per cent of the dog's plasma prothrombin level when estimated by a two-stage method utilising

either human brain extract or dog lung extract. Hawkey (1960) reported that dogfish blood lacks factors VII, IX, and X in human assay systems; while Fantl (1961) found that trout plasma is clottable with Russell's viper venom in 13 seconds and with trypsin in 8 seconds suggesting the presence of prothrombin and perhaps factors V and X in ample amounts. Fibrinogen was present in a concentration of 250 mg per 100 mls of plasma, and was clottable with bovine thrombin with a reactivity of 80 per cent that of human fibrinogen. Amphibians possess even greater similarity to the mammalian coagulation mechanism. They have an efficient extrinsic thromboplastin system, though most of the toad reagents were species specific (Hawkey, 1960; and Fantl, 1961). They also have an intrinsic thromboplastin system with rapid formation of active intrinsic thromboplastin when their plasma was incubated with human cephalin and calcium chloride; their serum, however, very slightly corrected Christmas disease serum, while their adsorbed plasma did not correct haemophilic adsorbed plasma in the thromboplastin generation test (Hawkey, 1960). Their blood contains nucleated thrombocytes instead of the platelets, the removal of which by filtration renders the plasma incoagulable (Tait and Green, 1926). Anstall and Huntsman (1960) and Hawkey (1960) have demonstrated that the coagulation mechanism of the toads' blood is well adapted to function properly at the low temperatures to which this species

is frequently subjected. The phylum of Reptiles comprises snakes, lizards, and tortoises. The blood of the tiger snake Notechis scutatus was investigated by Fantl (1961) who found that the only clotting factor present in it in adequate concentration was the fibrinogen. Prothrombin was present in very little amounts since the plasma was clottable with Russell's viper venom in 24 seconds and with trypsin in 22-49 seconds. Snake plasma did not correct the defect of congenitally Hageman factor deficient plasma. On the other hand, lizard and tortoise bloods had efficient homologous extrinsic thromboplastin systems, could be activated by contact with kaolin, but had reduced intrinsic thromboplastin formation (Fantl, 1961).

A further step in evolution is represented by the non-mammalian vertebrates, namely the Avian phylum. As demonstrated in Part II fowl blood contains nucleated thrombocytes instead of platelets, but the intrinsic thromboplastin system lacks many factors (IX, X, XI, and XII), while factor VIII activity was only demonstrable when the adsorbed plasma was incubated with fowl thrombocyte suspensions.

In mammalian vertebrates represented by cattle, horses, and rabbits (discussed in Parts III, IV, and V respectively) we find that the extrinsic thromboplastin systems are very efficient while the species specificity of some of the factors concerned in this reaction starts to disappear, for example factor V of the three

species, and factors VII and X of the rabbit. Moreover, they were found to possess efficient intrinsic thromboplastin systems containing all the factors known to take part in the human blood coagulation mechanism, including the Hageman factor and the plasma thromboplastin antecedent. All these factors also lost their species specificity and were completely interchangeable in a human intrinsic thromboplastin system.

That the human blood coagulation mechanism as it now exists has undergone evolution along the broad lines described above depends in the first place on our acceptance of Darwin's theory of evolution. However, the above findings strongly confirm Heilbrunn's suggestion that plasma clotting in higher animals is a result of a progressive evolutionary extension into the extra-cellular plasma of the cytoplasmic sol-gel mechanism which is present in the cells of all living organisms (Heilbrunn, 1937 and 1956). Activities formerly characteristic of the cells are now detectable as free factors in the plasma, while all the time during evolution new factors appeared forming a second thromboplastin system. Because higher vertebrates possess two thromboplastin systems instead of one, it is believed that their haemostatic mechanism is more efficient, though its growing complexity may well be also a safeguard against undue clotting or thrombosis.

Great differences were observed in the levels of the different

coagulation factors concerned in the vertebrate coagulation mechanism, as seen in Table 134 which because of its importance is again reproduced below and shows the various concentrations of the different factors in the species investigated, compared to normal human blood, in terms of percentage values except in the case of the platelets and the fibrinogen.

	SPECIES				
	Lobster	Fowl	Cattle	Horse	Rabbit
Blood platelets /c.mm. whole blood	5,200*	19,650**	312,350	117,000	311,000
Fibrinogen in mgms. per 100 mls plasma	230	264	697	436	228
Prothrombin	Nil	13-23 %	100-300 %	160 %	70 %
Factor V	Nil	Present (sp.sp.)	268 %	643 %	10558 %
Factor VII	Nil	Present (sp.sp.)	Present (sp.sp.)	80 % (sp.sp.)	89 %
Factor VIII	Nil	Present (sp.sp.)	816 %	209 %	288 %
Factor IX	Nil	Nil	20 %	203 %	29 %
Factor X	Nil	Present (sp.sp.)	101 % (sp.sp.)	85 % (sp.sp.)	436 %
Factor XI	Nil	Nil	100 %	—	—
Factor XII	Nil	Nil	100 %	118 %	1025 %

* Explosive cells.

** Thrombocytes.

(sp.sp.) = species specific.

While fowl plasma lacked several of the intrinsic factors (IX, XI, and XII) and had little prothrombin, plasmas of the other

vertebrates contained all the coagulation factors in ample amounts and in many instances in apparent great excess as compared with the human, with the exception of factor IX which was partially deficient in cattle and rabbits' sera. If we correlate such activities as determined by laboratory tests with a haemorrhagic condition in the case of deficiencies, or with thrombosis in the case of increased activities, as is the practice in human blood coagulation, we would expect these species to be greatly compromised with haemorrhages or thromboses, yet they appear to survive satisfactorily without suffering from these two diseases. Bigland and Triantaphyllopoulos (1960) suggested that birds perhaps bypass the plasma coagulation system and rely on a mechanism of vaso-constriction and rapid liberation of tissue thromboplastin following trauma. Fantl (1961) has also suggested that the relationship between the activity of a clotting factor as found in laboratory tests and the clinical symptoms observed in mammals is apparently not applicable to birds and snakes, and suggested that the level of fibrinogen is perhaps the deciding factor in haemostasis in these two species.

The role, reason, and implication of the presence of many factors in apparent excess as compared with the human in some vertebrate species is beyond the scope of this investigation. However, two important facts are revealed. Firstly, it is perhaps misleading to compare the various species on the same basis. For instance,

if all the species investigated were compared using the rabbit plasma as the reference standard, they all would seem to have very deficient coagulation mechanisms, including man, which is not actually the case. Secondly, that horse serum is a rich source for Christmas factor, while rabbit plasma is a very rich source for factors V and XII, similar to the previously observed finding that bovine plasma contains a high concentration of factor VIII as well as of fibrinogen.

As regards the specificity of the various coagulation factors, it was demonstrated that those factors active in crustacean coagulation were absolutely species specific towards vertebrate reagents, while possessing some degree of specificity among the various crustacean species inside the class. Also, it was demonstrated that all the coagulation factors present in fowl blood were absolutely species specific. In the mammalian vertebrates, namely cattle and horses, the species specificity was only encountered in the case of factors VII and X, and in the case of cattle was only of a minor degree that can be eliminated by prolonged incubation of the factors concerned with the heterologous tissue thromboplastin extract. Such species specificities would naturally interfere with the proper interpretation of tests in which these animal reagents are used in conjunction with human reagents. Several authors have devised methods for the assay of human blood coagulation factors in which animal reagents are used, either as thromboplastic agents, for example bovine brain extracts

(Owren, 1959; and Nour-Eldin, 1962), or as the deficient substrate to be corrected by the human test plasma, for example Seitz-filtered or adsorbed bovine plasma (Koller et al, 1951), chicken plasma (Wartelle, 1957b), and bovine serum (Bergna, 1960). Unless the limitations of the use of these animal reagents are well recognized, and until fuller and proper understanding of the effects these reagents have on human assay systems, particularly in one-stage techniques, the frequent use of these reagents requires further critical review.

SUMMARY

SUMMARY

Blood coagulation has been investigated in lobsters, fowl, cattle, horses, and rabbits. Comparison has been made with the human blood coagulation mechanism, on the basis of current concepts of that system.

Blood coagulation in the lobsters was found to occur in one phase, namely the conversion of lobster fibrinogen from the state of sol into that of gel by the action of an active thrombin-like material, in the presence of calcium ions. This material is released directly from one particular kind of blood cell, the explosive cell, characterised by its great sensitivity to contact with foreign surfaces which causes it to disintegrate almost instantaneously. The thrombin-like material had no hydrolytic activity on arginine esters, but its property of coagulating lobster plasma or purified lobster fibrinogen was inhibited by large concentrations of heparin, which action was reversed by hexadimethrine bromide. The lobster blood did not contain any of the other coagulation factors known to occur in man. The lobster coagulation reagents were absolutely species specific and were not interchangeable with human coagulation reagents.

Fowl blood lacked completely factors IX, XI, and XII activities,

while factors V, VII, VIII, and X were present but species specific. Fowl thrombocytes possessed a weak thromboplastic activity even in homologous thromboplastin generation mixtures.

In the mammalian vertebrates, species specificity was only demonstrable in the case of factors VII and X in bovine and equine blood; all the other factors were not species specific and mostly present in high concentrations, especially bovine factors I, II, and VIII, equine factors II, V, and IX, and in the rabbit factors V and XII. The exception was factor IX which was partially deficient in both cattle and rabbit sera.

All vertebrate plasmas and the extracted fibrinogen solutions were coagulated most rapidly with the homologous thrombin solution, again revealing the species specificity of the thrombin-fibrinogen reaction.

Adsorbed plasmas from the fowl and horses had an inhibitory effect when added to human thromboplastin generation mixtures. This inhibitory property can be removed by heating the adsorbed plasma prior to its addition, to precipitate the fibrinogen which is believed to be, or associated with, the responsible agent, since both fowl and horse sera had no such inhibitory activity at all.

The presented findings reveal that during the process of evolution, several phenomena occur. Firstly, functions which were the sole property of the cells become attributed to factors freely

soluble in the extra-cellular plasma. Secondly, the coagulation mechanism becomes more complex due to the appearance of more coagulation factors; clotting then occurs after several stages of interactions, with the consequent differentiation of two thromboplastin systems, the more primitive extrinsic or tissue thromboplastin system, and the more intricate intrinsic or plasma thromboplastin system being acquired in higher animals. Thirdly, the mammals which are regarded as the most highly evolved species, have an increased concentration of the coagulation factors, thereby probably ensuring the efficiency of the coagulation mechanism.

Finally, factors which in lower animals were completely species specific gradually lose their specificity, eventually becoming fully interchangeable with human blood coagulation factors.

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