

COMPARISONS AMONG GENETICALLY
DIVERSE LINES OF CHICKENS
FOR BLOOD COAGULATION FACTORS

By

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INTRODUCTION

The blood coagulation system of the body is in a state of dynamic equilibrium since anti and procoagulant activities balance each other under normal conditions. This equilibrium enables blood to remain in a fluid state and to function as an internal transportation system. Upon injury to a blood vessel, at the site of injury, the activity of procoagulants becomes considerably greater than that of the anti-coagulants and a clot develops to seal the injured vessel and prevent the loss of blood. Clotting of blood is a property of plasma and is essential for homeostasis. Increased coagulation time and prothrombin time have often been emphasized as the vital parameters of hemorrhagic diathesis.

The study of coagulation time is of considerable importance for the diagnosis and treatment of hemorrhagic disorders in chickens. Also because there is no satisfactory method for the measurement of vitamin-K in natural products it has been customary to bioassay this vitamin using plasma prothrombin time as a criterion. Prothrombin time is a measure of clotting time when an excess of thromboplastin has been added.

To our knowledge no investigation has been made of the inheritance of blood coagulation time in chickens. Since avian blood is used for the assay of vitamin-K and hemorrhagic diseases can be a large source of morbidity and mortality in flocks, there is a need for fundamental information on the inheritance of blood coagulation factors. A review

of the literature for coagulation time in chickens shows a wide range among values reported by various research workers. The probability that part of this discrepancy may be due to breed or line differences makes comparisons between various studies difficult. This thesis was therefore undertaken to make comparisons of blood coagulation factors among several diverse genetic stocks of chickens, maintained at the Virginia Agricultural Experiment Station.

REVIEW OF LITERATURE

Coagulation Time

Definition and methods. Coagulation time has been defined as the time required for blood to clot. It is a measure of the time required for sufficient fibrin to form in a mass of extracorporeal blood collected and kept under specific conditions to convert it from a free flowing to a gelatinous state (Quick, 1959). Jaques (1946) has stated that coagulation time measures the rate of fibrin formation. To measure the coagulation time, blood should be obtained with caution to avoid mixture with interstitial fluid during collection and the test should be performed in glasswares of specific dimensions and at a constant and standard temperature. In practice several methods have been used to measure coagulation time with the most common being the capillary tube method (Guyton, 1963), the untreated test tube method (Quick, 1959), and the silicone coated test tube method (Jaques et al. 1946).

Coagulation time in chickens. Coagulation time, which is a vital physiological parameter to measure the rate of fibrin formation, varies widely in different species and to some extent among the members of the same species. A range of clotting times has been reported for chickens. Both the early investigations by Delezenne (1897), Howell (1909) and the recent ones by Bigland and Triantaphyllopoulus (1960) and Bigland (1964) indicated a long clotting time for chicken blood. The mean clotting time was 69.25 minutes for 37 hens of the Leghorns,

Light Sussex and White Rock breeds (Bigland and Triantaphyllopoulus, 1960). Bigland (1964) confirmed these findings and those of the earlier workers by obtaining a clotting time of blood ranging from 26 to 69 minutes in chickens of different age groups and sexes.

Devilliers (1938) reported that the coagulation time of Ostrich blood was prolonged and that sometimes it even failed to clot whereas chicken blood clots readily. Amendt (1922-1923) cited by Dukes (1955) obtained a mean clotting time of 4.5 minutes for chickens. Didishem et al. (1959) reported that the clotting time of chicken blood was nine minutes in glass and ten minutes in silicone and suggested that the difference in clotting time was negligible between glass and silicone. Clotting times from 9 to 16 minutes were reported by Soulier et al. (1959) for chicken blood. Johnson and Conner (1933) found out that clotting time in birds varied so much, that it was doubtful if it had much significance except in extreme cases. In their observation, coagulation time in fowls varied from 1 to 14 minutes with a mean of about six minutes. Recently, Griminger (1965) has reported a normal clotting time of 2 to 10 minutes for non hypoprothrombinemic birds. In his opinion clotting time was more variable than prothrombin time.

Diet has probably received the greatest attention of the various factors that have been studied which influence the clotting time of chicken blood. Variations in clotting time has been reported as being due to either a lack or excess of various substances in the diet. Dam (1935) and Almquist and Stockstad (1935) stated that vitamin-K was the antihemorrhagic factor and clotting time was prolonged when there was

deficiency of this vitamin in the diet. Cravens et al. (1941) and March et al. (1955) obtained results consistent with this hypothesis. They observed that clotting and prothrombin time of day-old chicks was dependent upon the vitamin-K content of the diet fed their dams and that two percent alfalfa or dried cereal grass was a good source of vitamin-K. A diet that contained under heated soybean oil meal increased the clotting time of chicks (Balloun and Johnson, 1952). This was corrected by the inclusion of menadione in the diet as it reduced clotting and prothrombin time. Menadione sodium bisulphite (Frost and Spruth, 1955) and five percent alfalfa meal (Griminger et al. 1953) when fed to chickens reduce the clotting time, caused by a vitamin-K deficiency. Feeding of dicumarol has been shown by Harms and Tarver (1957) to increase the blood clotting time of chickens. Sweet et al. (1954) reported that either sulphaquinoxaline, paraaminosalicylic acid, or oxytetracycline when fed to chickens along with the above two drugs increased clotting time. Oxytetracycline when fed alone, however, had no effect. Griminger et al. (1953) stated that clotting times were increased in chickens when the diet fed contained terramycin or arsanic acid. Sulpha drugs have been observed to increase the prothrombin time in chicks by Morrison et al. (1954). Vitamin-K deficiency has been attributed as the causative agent for the prolongation of clotting time observed when drugs were administered. This was because the drugs killed the intestinal microflora concerned with synthesis of vitamin-K.

Genetics. It has been well established in humans that at least 12 factors are concerned in the process of blood coagulation. As reviewed

by Thelin (1964) the 12 clotting factors are fibrinogen (Factor-i), prothrombin (Factor-ii), thromboplastin (Factor-iii), Calcium (Factor-iv), labile factor (Factor-v), proconvertin (Factor-vii), antihemophilia factor (Factor-viii), Christmas factor (Factor-ix), Stuart prower factor (Factor-x), plasma thromboplastin antecedent (P.T.A. or Factor-xi), Hageman factor (Factor-xii) and Laki-Lorand factor (Factor-xiii). All of these factors must be present in suitable proportions to obtain a normal clotting time of blood. An absence or deficiency of anyone or more of these factors underlies bleeding and several other hemophiloid disorders. In chickens Factor-vii and Factor-xii are lacking (Didishem et al. 1959).

Considerable research has been conducted with humans on the inheritance of clotting factors. Macklin (1928) presented the hereditary pattern of hemophilia, which was due to defective transmission of antihemophilia factor. Hemophilia is transmitted as a sex-linked recessive character with the gene responsible for this condition located on the X-chromosome.

Macfarlane (1938) and Schonholzer (1939) showed that the hereditary transmission of fibrinogen (Factor-i) and afibrinogenemia in humans was qualitative. Quick et al. (1955) has suggested that prothrombin time is inherited quantitatively. Hypoprothrombinemia, which is characterized by increased prothrombin time, is familial and occurs in both sexes.

To our knowledge no such research has been conducted with chickens to account for coagulation defects which are characterized by prolonged clotting time.

Prothrombin Time

Chemistry of prothrombin. Prothrombin is a carbohydrate containing protein (Seegers, 1940) and belongs to the globulin fraction of plasma proteins. It contains 18 amino acids, a considerable amount of carbohydrate (Scheraga and Laskowski, 1957), has a molecular weight of 62,700 (Lamy and Waugh, 1954), and is formed in the liver. Prothrombin requires vitamin-K for its synthesis and is probably the only factor concerned with blood coagulation that requires a vitamin for its production. Quick and Collentine (1951) stated that although vitamin-K was essential for the production of prothrombin it was not a part of prothrombin. Rather it acted as a prosthetic group of an enzyme concerned in the syntehsis of prothrombin. Lasch and Roka (1953) have reported that liver mitochondria syntehsize prothrombin from Factor-vii in conjunction with vitamin-K.

Prothrombin and blood coagulation. Of the various factors concerned in the coagulation process, probably no other factor has received as much attention as prothrombin. The blood clotting mechanisms, as a matter of fact, are a series of chemical reactions which center around prothrombin without which blood does not clot (Seegers, 1962). Thrombin which is essential for the conversion of fibrinogen to fibrin, is derived from prothrombin. The ways in which prothrombin gives rise to thrombin is still a matter of controversy and may be the most complicated part of coagulation mechanisms. Seegers (1964) proposed that prothrombin was a multipotential protein capable of producing

a variety of derivatives depending upon the enzymes and cofactors to which it was exposed. He viewed the activation of prothrombin as a autocatalytic process because thrombin and the activities corresponding to Factors vii, ix, and x were prothrombin derivatives, which with cofactors, promoted the conversion of prothrombin to thrombin. According to this theory inactive precursors of these factors are not present in plasma except as prothrombin.

As reviewed by Thelin (1964) prothrombin requires thromboplastin, Factor-v, Factor-vii, Factor-x, and calcium to be converted to thrombin. When all of these factors are present in the appropriate concentrations, the conversion of prothrombin to thrombin occurs in about 14 seconds. Landburu (1965) has reported that when prothrombin was activated the first activity that developed was esterase activity which was followed by the development of the clotting activity. At the completion of activation, esterase activity equals clotting activity.

Definition of prothrombin time. Prothrombin time is defined as the clotting time of recalcified plasma to which an excess of thromboplastin has been added. The longer the clotting time, the lower the prothrombin concentration. The need for the determination of prothrombin time in chickens is manifold, because it is essential for the assay of vitamin-K, for the diagnosis of hemorrhagic diseases and as a liver function test.

Methods for the determination of prothrombin time. Two methods have been described in the literature for the determination of prothrombin time. One method, devised by Quick et al. (1935), is popularly known as the one stage method, while the second method, advocated by Warner

et al. (1936), is known as the two stage method. The one stage method was developed on the basis of the classical theory of coagulation. Although, initially it was thought it would measure the quantity of prothrombin present in blood, subsequent investigations showed that it also measured the factors involved in stage-II and stage-III of the coagulation mechanism. The one stage method of prothrombin time reflects deficiencies of the prothrombin-complex, which consists of prothrombin, proconvertin, the plasma thromboplastin component, and the Stuart prower factor (Thelin, 1964). The two stage method measures the total amount of prothrombin present in blood.

The method that has been widely employed for the determination of prothrombin time in chickens is the one stage method by Quick or a slight modification thereof (Griminger, 1962; Bigland and Triantaphyllopoulos, 1960; Didishem et al. 1959; and Woody et al., 1963). Evidence has accumulated that although many tissues can serve as a source of thromboplastin for Quick's one stage method, best results were obtained with brain tissue extracts. Since brain tissue thromboplastin shows species specificity (Didishem et al., 1959), chicken brain extracts were used as a source of thromboplastin in determination of prothrombin time by Griminger (1962, 1963).

Prothrombin time in chickens. Prothrombin times for normal chickens have been measured by several investigators. Chubb and Long (1957) reported a mean prothrombin time of 18.5 ± 1.5 seconds by Quick's one stage method with chicken brain as the source of thromboplastin. Evidence that age influences prothrombin time has been presented by

Cuckler and Ott (1955). They found a mean prothrombin time of 14 seconds (range - 13 to 15 seconds) in chicks of five weeks of age, 15 seconds (range - 13 to 18 seconds) in chickens 10 weeks of age, and 16 seconds (range - 14 to 18 seconds) in chickens 15 weeks of age. Didishem et al. (1959) reported a prothrombin time of 11.4 seconds and Bigland and Triantaphyllopoulus (1960) obtained a mean prothrombin time of 13.4 seconds. The latter authors (1961) also reported a prothrombin time of 11.4 seconds for birds at 10 weeks of age, from data obtained with pooled samples. Griminger (1962) has suggested that when thromboplastin was prepared and stored under optimal conditions, prothrombin time was as short as 10 to 12 seconds. Woody et al. 1963, obtained a prothrombin time of 21.6 seconds using (Difco) embryo extracts as the source of thromboplastin. Further they have suggested on the basis of their finding that oxlated blood for prothrombin time determination should not be held longer than two hours at room temperature. On the other hand blood may be refrigerated for at least 6 hours without a significant change in prothrombin time. Griminger (1962) stated that comparable results were obtained with brain tissue and Difco embryo extract thromboplastin. Griminger (1965) noted that prothrombin times similar to those of chicks were observed for adult males, whereas prothrombin times for adult females tend to be slightly longer than those for chicks.

Since prothrombin requires vitamin-K for its synthesis many attempts have been made to create vitamin-K deficiencies to facilitate the study of different diets and drugs on plasma prothrombin time.

Cravens et al. (1941) noted that the prothrombin time of day-old chicks was dependent upon the vitamin-K content of the diet fed to laying hens. They observed that two percent alfalfa leaf meal or one percent dried cereal grass furnished sufficient vitamin-K to produce chicks with normal prothrombin times. Upon the intravenous injection of menadione into vitamin-K deficient chicks, prothrombin activity returned slowly at first, and then progressively more rapidly reaching normality after 4 hours (Quick and Stefanini 1948). Davies and March (1956) have stated that addition of either beef tallow, hydrogenated animal fat, cotton seed oil or hydrogenated cotton seed oil to diets deficient in vitamin-K reduced prothrombin time. Griminger (1963) reported that thromboplastin prepared from chickens fed higher levels of vitamin-K compounds showed a tendency for shorter prothrombin time.

Genetics. Quick et al. (1955) have established that hypoprothrombinemia in humans is familial and occurs in both sexes. From their observation they concluded that a pair of dominant alleles were concerned in fixing the normal prothrombin time of 12 seconds in humans and another pair of dominant alleles were concerned in fixing a prothrombin time of 16 seconds. Prothrombin time was 14 seconds in heterozygotes. When both pairs of recessives were present prothrombin time was prolonged and severe bleeding occurred. This demonstrated a genetic control of prothrombin time in humans. To our knowledge no research of this type has been conducted with chickens.

Thrombocytes

Morphology. The thrombocytes (platelets) of chickens are elongated cells with rounded ends and of a size smaller than that of erythrocytes; thrombocytes measure about 5 x 10 microns (Blount, 1939). Lucas and Jamroz (1961), based on the measurement of 10 thrombocytes, reported an average size of 4.7 x 8.5 microns. Thrombocytes contain a large nucleus and are considered truly nucleated (Duke, 1955 and Didishem et al. 1959). Although the role of the specific granules seen on stained preparation of thrombocytes is not clearly understood, Blount (1939) suggested that they were concerned in the disintegration of the cells and essential in blood coagulation. Thrombocytes have a high glycogen content and show a tendency to clump (Didishem et al. 1959). They possess the property of adhering to foreign surfaces and also to one another (Wright, 1951). This property of adhesion to foreign surfaces is dependent upon several conditions as shown by Fidler and Jaques (1948). Thrombocytes rapidly disintegrate after the blood is withdrawn (Seegers, 1962), a property probably necessary as a part of the physiological process involved in coagulation.

Thrombocyte count. Thrombocytes may be counted in chickens from a stained smear (Lucas and Jamroz, 1961) or by the method of Quick (1959) described for platelets in mammals. Thrombocytes remain discrete in absence of thrombin and show no tendency to either clump, agglutinate or adhere to foreign surfaces. Thus blood may be drawn into a silicone coated syringe and mixed with a sodium citrate anti-coagulant solution without the formation of thrombin. The anti-

coagulant stabilizes the thrombocytes allowing them to remain intact for subsequent counting in a hemocytometer.

Didishem et al. (1959) reported a mean thrombocyte count of 150,000 per cmm of blood for White Leghorn adult females. Gordon (1926) cited by Lucas and Jamroz (1961) stated that the number of thrombocytes in avian blood varied with the number of times the blood was examined. Gordon observed that when the same birds were examined five times for thrombocytes and erythrocytes the initial thrombocyte count of 35,000 per cmm of blood increased to 86,000 with a corresponding decrease in the number of erythrocytes. He hypothesized that thrombocytes were formed at the expense of the erythrocytes. The number of thrombocytes for chickens was also reported by Lucas and Jamroz (1961) who observed that the number of thrombocytes varied from 26,000 to 60,000.

Thrombocytes and coagulation. Thrombocytes of chickens are similar in function to the platelets of mammals. Although little is known of the function of thrombocytes under normal conditions, there is evidence that they play an important role in the process of blood coagulation and formation of white thrombi (Lowell, 1909). That the rate of blood coagulation is related to the presence of blood platelets (thrombocytes) in birds was first demonstrated by Delezene (1897). Eagle (1935) has stated that platelets had nothing to do with total amount of thrombin produced but they enhanced the rate of thrombin production. However, the larger the number of platelets the greater is the production of thrombin, and the faster the coagulation process. This

strongly suggests that platelets catalyze the reaction between prothrombin and calcium without themselves contributing to the compound formed. Species specificities have not been observed in the action of the platelets.

Jaques et al. (1946) showed that platelet poor plasma prolonged clotting time and the correlation coefficient between them was $-.45$ when measured in glass tubes. The probability of this occurring by chance was 1:10. According to their view although the correlation was not significant, the effect of platelets on clotting time should not be completely discounted.

Quick (1947) has established that although platelets do not supply thromboplastin they do provide an agent which reacts with thromboplastin and other plasma constituents to form thromboplastin.

Biggs and Douglas (1953), who developed the thromboplastin generation test, agreed with the ideas postulated by Quick that platelets are essential for the generation of thromboplastin. Mann et al. (1949) reported that platelets served as the focus for the formation of the fibrin network and a part of the complex factors which enhance the rapid conversion of prothrombin to thrombin. Further they were of the opinion that the morphological and biochemical makeup of platelets accelerated the formation of fibrin network even when thrombin and thromboplastin concentration were minimal. Fenichel and Seegers (1957) stated that platelets played an important role in clot retraction. Without platelets being trapped into the network of the clot, retraction does not take place.

Quick (1949) and Conley et al. (1949) indicated that the degree and speed of clot retraction varied directly with the number of platelets. The latter workers observed that the correlation between clot retraction and platelets was better evident in silicone treated than plain glass tubes. They also observed that platelets accelerated the rate of clotting and the amount of prothrombin consumed, although they were not necessary to initiate clotting. A moderate reduction in number of platelets was sufficient to delay clotting (Quick, 1949 and Conley et al., 1949).

Quick (1949) stated that the platelets coordinate all the mechanisms of hemostasis and their activity is linked with thrombin. He mentioned that platelets were important in the production of white thrombi, the formation of thrombin, and in the maintenance of vaso-constriction; all three basic principles of hemostasis. When a blood vessel is injured it contracts promptly and it allows the platelets to adhere to the wall of the injured vessel. Sufficient thrombin is formed in the localized area which results in a platelet thrombus which remains adherent to the injured vessel.

Quick and Hussey (1950) reported that clot retraction decreased when the ratio of fibrinogen to the number of platelets was increased. Quick (1950) stated that in a system with fixed amounts of fibrinogen and a constant number of platelets, the degree of retraction became a function of the added amount of thrombin. Clotting was impaired only when the platelets level in the plasma was below critical (Buckwalter et al. 1949). In their experiment they also found out

that dog plasma required more platelets than human plasma for clotting; the reason for which could not be accounted for.

Procoagulant factors and thrombocytes. Johnson et al. (1954) found that platelets contain two factors which played an important role in the transformation of prothrombin to thrombin. One of these factors was Factor-viii or the antihemophilia factor, which has also been termed as platelet factor-1. A lack of this factor caused hemophilia. The other factor was platelet factor-2, otherwise known as the plasma thromboplastin component or Christmas factor. It is concerned in hemorrhagic diathesis referred to as deuterohemophilia or hemophilia-B.

Platelet factor-3 is probably a lipoprotein concerned with prothrombin activation (Johnson et al., 1958 cited by Seegers, 1962). Conley et al. (1948) observed a substance in platelets which neutralized the action of heparin. This substance was named platelet factor-4 or the antiheparin factor. They concluded that the concentration of heparin required to inhibit or delay coagulation was directly related to the number of platelets present. Ware et al. (1948) found a substance present in platelets which helped to establish the center of articulation in the coagulation process. It was similar in function to fibrinogen and named platelet factor-5 or platelet fibrinogen. Seegers (1962) reported two other factors which were termed as platelet factor-6 and platelet factor-7. Platelet factor-6 is the antifibrinolytic principle of platelets, whereas factor-7 shows some qualities of Factor-vii and Factor-x of blood coagulation.

Platelets and anticoagulants. Although thrombocytes may be considered as good sources of various procoagulants, evidence has accumulated which shows that they also contain anticoagulants. Chargaff et al. (1936) found that defatted horse platelets prolonged the recalcified clotting time of chicken plasma. Recent investigations have shown that anticoagulant action also exists in human plasma. Spaet (1957) demonstrated that human platelets yield two anticoagulants, one lipid in nature and the other a protein. The lipid anticoagulant of thrombocytes inhibits thromboplastin formation and is neutralized by accelerator globulin (Factor-v) concentrates. The protein anticoagulant also inhibits thromboplastin formation and is neutralized by a factor present in serum which absorbs barium sulphate. Spaet suggested that platelet anticoagulants were partially responsible for hemorrhagic syndromes in thrombocythemia and that a reciprocal relationship existed between the platelet lipid anticoagulant and the labile factor.

Genetics. It has been established in humans that thrombocytopenic purpura which is characterized by a decrease in the number of platelets and bleeding, is due to a pair of defective genes. These defective genes are inherited as autosomal recessives (Quick, 1959).

Fibrinogen

Chemistry of fibrinogen. Fibrinogen, the clotting protein of plasma and the primary substrate on which thrombin acts, is a protein in the globulin class (Scherga et al. 1957). Physiological parameters of fibrinogen have been studied in various species. The molecular weight

of bovine fibrinogen is $330,000 \pm 10,000$ as determined by the method of sedimentation and diffusion (Shulman, 1953). Although it has a high molecular weight fibrinogen may migrate into extravascular spaces as evidenced by the spontaneous clotting of lymph (Adelson, 1965).

The fibrinogen molecule is a chain of amino acid units with various side groups which are linked by identical peptide bonds (Laki, 1962). In addition to amino acids fibrinogen contains small amounts of the carbohydrates galactose, mannose, hexosamine and sialic acid (Laki and Gladner, 1964). Electron microscopic studies show that the fibrinogen molecule consists of three globules held together on a fiber. The side globules are somewhat larger than the middle one and the connecting fiber of the three globules provides stiffness to the molecule.

Craddock and Campbell (1961) have recorded that fibrinogen synthesis occurs in the liver. A similar opinion was expressed by Adelson (1965), who noted that the amount of fibrinogen produced in the liver varied from 1.5 to 5 g per day. The fibrinogen concentration of plasma regulates the production of fibrinogen by the liver and a slight decrease of concentration of plasma fibrinogen, enhances the fibrinogen synthesis many fold (Miller et al. 1961 and Adelson, 1965). Laki and Gladner (1964) suggested that a feed back mechanism was operating in this case. Fibrinogen exists in both extra and intravascular spaces with about 70 to 80 percent of it in the intravascular component (Adelson, 1965). Platelets have also been known to contain appreciable amounts of fibrinogen (Lorand, 1965).

The catabolism of fibrinogen is not completely known. Adelson (1965) has shown that the destruction of fibrinogen is extremely rapid with a half life of only a few hours.

Barnhart (1965) has reported that neutrophils phagocytosize either fibrinogen, fibrin or their break-down products. When neutrophils were challenged by additional fibrin, they accumulated and persisted at experimental inflammatory sites. The intracellular fibrin of the neutrophils have been revealed by immunofluorescence and electron microscopy. Exudative cells from inflamed joints of patients with rheumatic arthritis frequently exhibited phagosomes filled with fibrin like material.

The fibrinogen concentration in plasma is fairly constant, but shows variations under different conditions. The fibrinogen concentration becomes low during pregnancy (Phillips, 1959 and Schneider, 1959). Also there is a certain pathological condition in which fibrinogen is completely absent in the blood stream. This condition is known as afibrinogenemia (Fernando and Dharmasena, 1957; MacFarlane, 1938 and Jackson et al. (1965), which is congenital and occurs in both sexes (Frick and McQuarrie, 1954).

A complete review of plasma proteins of which fibrinogen is a component was made by Howe (1925). He showed that plasma proteins varied considerably according to species, age, sex, feeding, physiological activity, environment, disease and reactions to infection. Changes in the concentration of fibrinogen occurred more rapidly than other protein components. Sturkie and Newman (1951) reported

a sexual dimorphism in chickens for the concentration of plasma proteins with values for males being lower than those for females and suggested sex hormones as the cause of the dimorphism. Didisheim et al. (1959) found that the concentration of fibrinogen in plasma varied among species.

Monkhouse and Milojevic (1960) demonstrated that fibrinogen could be eliminated from plasma by the slow infusion of thrombin into the plasma. Under such circumstances fibrinogen was transformed through a slow and gradual process to fibrin which was deposited on the capillary walls. Fibrinogen concentration returned to normal when infusion of thrombin was stopped. Laki and Gladner (1964) postulated that low fibrinogen level in plasma in natural conditions was probably due to a naturally occurring slow infusion of thrombin.

Shainoff and Page (1960) with rabbit plasma showed that fibrinogen may exist in blood in a partially clotted state. Such partial clotted fibrinogen was named cryopofibrin. The chemical composition of fibrinogen and cryopofibrin differs slightly because cryopofibrin lacks one peptide present in fibrinogen that is released during clotting. The presence of this partial converted fibrinogen in blood suggests that fibrinogen and fibrin transition although at a low level is continuous under normal conditions, (Laki and Gladner, 1964). Cryopofibrin under certain pathological conditions may increase in human blood. The high turnover rate of fibrinogen in comparison to other plasma proteins is probably due, at least in part, to the continuous conversion of fibrinogen to fibrin (Laki and Gladner, 1964).

Fibrinogen and blood coagulation. Fibrinogen plays an unique role in the process of blood coagulation. The fibrin, which forms the clot, is derived from fibrinogen by the action of thrombin. The conversion of fibrinogen to fibrin occurs in two phases (Laki and Gladner, 1964). The first phase is called the enzymatic phase and the second phase is known as the clotting phase. In the enzymatic phase thrombin brings about a small alteration in the fibrinogen molecule. These altered molecules of fibrinogen polymerize into a network structure during the clotting phase. Polymerization occurs from the rupture of four peptide bonds and the simultaneous rupture of hydrogen bonds from fibrinogen. The reaction described above may be summarized as follows:

- i) Fibrinogen limited proteolysis Fibrin + 4 Peptides + Carbohydrates
- ii) Fibrin molecule aggregation Polymer (clot).

Electron microscopic studies by Porter and Hawn (1949) have shown that fibrinogen molecules upon polymerization by thrombin form needle shaped, crystal-like protofibrils, which become aligned in fiber strands by lateral association. The integrity of the unit of fibrils is maintained within the strand.

Thrombin, which is essential for the transformation of fibrinogen to fibrin, is a proteolytic enzyme whose action is highly specific for certain peptide bonds located between arginine and glycine in fibrinogen. The rate of the thrombin-fibrinogen interaction is a vital parameter in the blood coagulation mechanism since prolonged clotting time may lead to death. Laki and Gladner (1964) observed a

species specificity in the rate of thrombin-fibrinogen interactions and Didisheim et al. (1959) found that chicken fibrinogen was highly specific for chicken thrombin. In the latter experiment chicken thrombin clotted fibrinogen of all species, but chicken fibrinogen was only clotted by chicken thrombin. In general, thrombin from a particular species of animals or birds clots its own fibrinogen best. Laki and Gladner (1964) attribute such differences in their actions to changes that took place in the fibrinogen and thrombin during the evolution of different species.

The importance of fibrinogen in plasma lies in the fact that it is directly concerned in the process of blood coagulation. Further it is essential for the diagnosis, treatment and prognosis of various pathological conditions, including acute myocardial infarction, hepatic disorders and gangrenous lesions (Losner and Volk, 1956). Kelly (1955) reported that in patients suffering from rheumatoid arthritis there was a correlation between sedimentation rate of erythrocytes and fibrinogen. Fibrinogen concentration in plasma is also important as both fibrinogen and fibrin participate in immunological reactions (Lorand, 1965).

MacFarlane (1938) has suggested that a liability to excessive bleeding can seldom be attributed to lack of fibrinogen. Yet the efficiency of clot formation depends as much upon adequate fibrin as upon any other link in the chain of coagulation factors. Besides there is a critical level of fibrinogen concentration in plasma below which coagulation produces a clot that is too friable to be hemostatically

effective. Quick (1959) reported that the higher the fibrinogen concentration, the less marked is the clot retraction. Thus an increased fibrinogen level protects from rather than predisposes to venous thrombosis.

Dionysios (1960) stated that defibrinated plasma did not clot upon the addition of thromboplastin and either calcium or thrombin. When, however, a source of fibrinogen was added to the defibrinated plasma, clotting time upon addition of thromboplastin and calcium was inversely proportional to the concentration of fibrinogen.

Laki (1965) observed clot formation when the concentration of fibrin molecules in plasma reached a critical level and the molecules began to aggregate into a three dimensional network. The concentration of fibrin in plasma did not reach a critical level except in emergencies.

Methods for the estimation of fibrinogen. Determination of the fibrinogen concentration in plasma, involves two stages: (1) complete removal of compound from plasma and (2) actual estimation of the compound. Fibrinogen may be precipitated by a physiological solution of sodium chloride as per the method of Cullen and VanSlyke (1920). By this method oxalated plasma was diluted with the saline solution and an excess of calcium chloride was added to it for clot formation. The estimation of fibrinogen was made either by the weight of the dehydrated clot, by determination of its nitrogen content, or by the estimation of tyrosine content. The advantage of the Cullen and VanSlyke procedure is that it determines specifically the amount of

fibrinogen that actually forms the clot (Quick, 1959). A disadvantage of the method of Cullen and VanSlyke is that other proteins are precipitated along with fibrinogen because of the addition of sodium chloride. An important modification of the method was made by Hussey (1950) cited by Quick (1959), who used distilled water instead of normal saline to dilute the plasma. This modification resulted in a 20 percent increase in fibrinogen over the salting-out method.

The standard method followed for the quantitative estimation of fibrinogen in plasma is the micro-Kjeldahl method (Hawk et al. 1954). In this process isolation of fibrinogen is made as fibrin from the procedure of Cullen and VanSlyke (1920) followed by digestion, nesslerization, and a spectrophotometric reading.

Electrophoretic determinations of fibrinogen in chickens has been made by Sanders et al. (1944). A radioisotope technique for determination of fibrinogen in rabbit has been described by Atencio et al., (1965). Although electrophoresis and radioisotope techniques may be better than the older method, they have not reached a stage to be used as a common laboratory test for fibrinogen.

Sanders et al. (1944) in an electrophoretic study of chicken plasma from normal and leucosis affected chickens reported that the mobility of fibrinogen in plasma of normal chickens was 2.5×10^{-5} and the percentage composition of fibrinogen was 13.5. Didishem et al. (1959) has reported that the concentration of fibrinogen in normal chicken plasma is 79 mg percent.

Bigland and Triantaphyllopoulos (1961) measured the concentration of fibrinogen in pooled samples of chicken blood. The mean fibrinogen concentration of chicken plasma obtained with a slight modification of the method of Quick (1959) was 354.9/100 ml while the mean concentration in the same plasma obtained by a slight modification of saline method was only 243.8 mg/100 ml.

Genetics. Manifestation of congenital abnormalities of fibrinogen has been observed in three forms (Jackson et al. (1965)). A complete lack of fibrinogen in the plasma is known as afibrinogenemia, whereas moderate reduction in the level of circulating fibrinogen is known as hypofibrinogenemia. Dysfibrinogenemia, the third disorder of fibrinogen, is characterized by the presence of an abnormal amount of fibrinogen in the plasma.

Congenital afibrinogenemia occurs in both sexes (Bucek, 1951; Frick and McQuarrie, 1954). Hereditary hypofibrinogenemia was demonstrated in humans by MacFarlane (1938) and Schonholzer (1939) suggested that it was inherited as an autosomal recessive. Beck et al. (1965) cited by Jackson et al. (1965) suggested that dysfibrinogenemia was controlled by an abnormal defective gene whose presence was associated with hemostatic diathesis.

To our knowledge the inheritance of fibrinogen has not been studied in chickens.

MATERIALS AND METHODS

Experiment - I

The coagulation time of blood was determined for five lines of chickens developed at the Virginia Agricultural Experiment Station. The lines were: high weight (HW) and low weight (LW) White Plymouth Rocks (Siegel, 1962), high mating (HML) and low mating (LML) synthetics (Siegel, 1965a), and game birds (Siegel, 1965b). Although the numbers of birds per line varied with their availability, minimum sample size was 25 females with the exact number of birds per line given in Table 1. The ages of all chickens were comparable, ranging from 56 to 60 weeks. During the period of this experiment the birds were maintained in pens of 7 to 10 each and feed and water was fed ad libitum. The feed formulations are presented in Table 2. The starter ration was fed to 8 weeks of age, grower to 22 of age and breeder thereafter.

Blood coagulation time was determined by the capillary tube method. For this purpose the skin over the wing vein was excised by means of a scalpel and the wing vein was reflected. Blood was collected in two capillary tubes from each bird by puncture of the vein and a stop watch started at the exact time of collection. Capillary tubes were broken at intervals of 30 seconds to determine when clotting had occurred. Clotting was taken as the first observation of a fibrin thread sticking to both parts of the broken capillary tube and time was recorded to the nearest half minute. The average for the duplicate tubes was taken as the clotting time for that bird.

Table 1. Number and generation of birds by lines used in Experiment I.

Line	No. ($\frac{22}{2}$)	Gen.
High weight (HW)	58	F ₇
Low weight (LW)	56	F ₇
High mating (HML)	47	F ₆
Low mating (LML)	43	F ₆
Game	25	
Total	229	

Table 2. Composition of rations.

Ingredients	Starter	Grower	Breeder
	lbs	lbs	lbs
Yellow corn meal	47.12	51.65	56.55
Pulverized oats	5.00	15.00	10.00
Wheat flour middlings	10.00	6.25	5.00
Wheat bran	5.00	6.25	2.50
Alfalfa meal	5.00	3.75	2.50
Fish meal	2.50	0.00	3.00
Meat and bone scrap	2.50	2.50	5.00
Stabilized fat	0.00	0.00	1.25
Soybean oil meal	20.50	12.10	8.00
Salt, iodized	0.50	0.40	0.40
Ground limestone	1.00	1.05	5.00
Defluorinated phosphate	0.50	0.72	0.50
	g	g	g
Manganese sulphate	11.25	11.25	11.25
Vitamin-A supplement	18.00	6.75	22.50
Vitamin D ₃ supplement	4.50	4.50	11.25
Multivitamin mixture	56.25	33.75	56.25
Vitamin B ₁₂ supplement	11.25	11.25	22.50
Choline chloride	22.50	11.25	11.25
Procaine Penicillin supplement	22.50	0.00	0.00
Cocciostat	22.50	22.50	0.00
TOTAL	100.0	100.0	100.0

A difference in clotting time has been reported by Bigland (1964) between veni punctures from left and right wing. To minimize the effect of a possible bilateral difference, all measurements were made from blood obtained from the left wing. All determinations were made between 9 and 11 a.m. during a period of 14 days.

Data were analyzed by analysis of variances using a completely randomized design. When significant differences were found among lines, comparisons between the means were made by Duncan's multiple range test as modified by Kramer (1956). Heritability estimates based on full and half sib correlations, for capillary coagulation of blood were calculated by the method of King and Henderson (1954). The statistical model used was

$$X_{hijk} = \mu + l_h + S_{hi} + d_{kij} + e_{hijk}$$

where X_{hijk} was the measurement for the k th offspring of the j th dam mated is the i th sire within the h th line.

Experiment - II

Based on the results obtained in Experiment I, the HW and game lines were chosen for further study. Prothrombin time, number of thrombocytes, and fibrinogen concentration were measured to determine the contribution of these factors to coagulation time in these two lines.

Guyton (1963) noted that there were differences in coagulation time as measured by the capillary tube and test tube methods. The latter method as described by Quick (1959) was used here because it

Table 3. Number of birds used by lines and sex, Experiment II.

Line	Sex	
	♂♂	♀♀
High weight ¹	9	66
Game	11	37
Total	20	103

¹ F₈ generation

was found to be more reliable than the capillary method. Determinations were obtained as the chickens progressed from 16 to 22 weeks of age. Numbers of birds by sex and lines are given in Table 3. Five ml of blood were collected in a syringe from the left brachial vein. Syringes and needles were silicone coated with the former of either 5 or 10 ml capacity and the latter being 20 gauge. One ml of blood was transferred into each of two test tubes for duplicate determinations of coagulation time. Another ml was transferred to a silicone coated test tube containing 0.5 ml of sodium citrate and immersed in an ice bath for subsequent counting of thrombocytes. Of the remaining blood 1.8 ml was transferred to a test tube containing 0.2 ml of 0.1 M sodium oxalate solution for determination of prothrombin time. Since it was not possible in many instances to collect more than 5 ml of blood the estimations of fibrinogen were made subsequently for each bird. This procedure was employed for all birds.

When the first attempt to reach the vein was not successful, the needle was changed and another attempt was made. If second attempt failed, which was seldom, no blood was collected from that bird on that day and collection was made on a later date.

During the period of this experiment the birds were maintained in individual cages where food and water were provided ad libitum. Coagulation time by test tube method. Coagulation time of blood was determined by the method of Quick (1959). The needle was removed from the syringe immediately after collection and one ml of blood was transferred to each of two clean dry test tubes of specific

dimension (13 x 100 mm with an internal diameter of 11 mm). During the transfer of blood from the syringe to the tube every care was taken to avoid formation of foam. The tubes were placed in a water bath at $37.5 \pm 1^{\circ}\text{C}$ and tilted at 30-second intervals to determine when clotting has occurred. Clotting time was recorded for the blood in both tubes and the mean used as the clotting time for that chicken.

Prothrombin time. The one stage method of Quick (1959) was used for the determination of prothrombin time. For this purpose 1.8 ml of blood was transferred at the time of collection to a test tube containing 0.2 ml of 0.1 M of sodium oxalate anticoagulant. The blood was then well mixed with the anticoagulant and transferred to a centrifuge tube for 5 minutes of centrifugation at 3000 rpm. Woody et al. (1963) demonstrated that holding plasma more than two hours at room temperature resulted in a significant increase in prothrombin time. They found no such change occurring when plasma was stored in freezer for up to six hours. To avoid any such error plasma was stored in a refrigerator until the prothrombin test was performed, which in no instance was later than two hours.

Thromboplastin and calcium chloride solutions must be necessary in appropriate concentrations for determination of prothrombin time. Although many tissues such as lung, embryo extract and brain tissue extract have been used as a source of thromboplastin for determination of prothrombin time in chickens, the best results were usually obtained with brain tissue extracts. Since a species specificities has been demonstrated for brain tissue thromboplastin (Griminger, 1962), the

thromboplastin used here was prepared from the brains of chickens 16 to 20 weeks of age by the acetone dehydration method of Quick (1959) and stored in the freezer. Prior to use 100 mg of this brain powder was mixed with 5 ml of 0.85 percent sodium chloride solution, placed in a 55°C waterbath for ten minutes and stirred occasionally with a glass rod. Upon removal from the waterbath it was cooled and filtered. The filtrate was mixed with an equal amount of 0.025 M calcium chloride solution to form a mixture of thromboplastin-calcium chloride.

The plasma and thromboplastin-calcium chloride were prewarmed separately in a waterbath for 4 minutes at 37.5°C, then 0.1 ml of plasma was added to 0.2 ml of the thromboplastin calcium chloride and the mixture was left in the waterbath. The mixture was checked every second by tilting for appearance of clot. Appearance of fibrin threads marked the point for clotting of recalcified plasma and was considered as the prothrombin time. This procedure was duplicated for each blood sample and the mean used. The test was repeated when duplicate samples differed by more than one second.

Thrombocytes count. The method described by Quick (1959) for estimation of platelets in mammals was used for the determination of the number of thrombocytes per cmm of blood. One ml of blood was transferred immediately after collection to a silicone coated test tube containing 0.5 ml of sodium citrate solution and immersed in an ice bath. The blood was mixed with the sodium citrate fluid by covering the mouth of the test tube with paraffin coated paper and inverting the tube several times. The citrated blood was drawn

into a R.B.C. pipette to 0.5 mark and filled with sodium citrate diluting fluid to the 101 mark. The pipette was shaken for five minutes in a pipette shaker to effect thorough mixing. Both counting chambers of a hemocytometer were filled with diluted blood, which was then allowed to stand for 15 minutes to facilitate a settling out of the thrombocytes. Pieces of water-soaked sponge were placed on the hemocytometer to prevent evaporation. Thrombocytes were counted under high power in all 25 squares of both chambers and the mean value was used.

The thrombocytes per cmm of blood was determined as: mean thrombocytes count $\times 2000 \times \frac{3}{2}$.

Estimation of fibrinogen concentration. The micro-Kjeldahl method described by Hawk et al. (1954) was followed for estimation of fibrinogen concentration in chicken plasma. This method was based on the procedure of Cullen and VanSlyke (1920) in which fibrinogen was precipitated from plasma by a physiological solution of sodium chloride. A slight deviation from the method described by Hawk et al. (1954) was found necessary to obtain satisfactory results. For this purpose 1 ml of chicken plasma was mixed in a centrifuge tube with 6 ml of 0.85 percent sodium chloride solution and one ml of 2.5 percent calcium chloride solution. This was necessary because when one ml of chicken plasma was diluted with 30 ml of saline (normal) no appreciable precipitate of clot was formed upon the addition of calcium chloride.

The solution was allowed to stand for 30 to 45 minutes to enable development of a clot, after which it was centrifuged for 15 minutes

at moderate speed. The supernatant was discarded after centrifugation and the centrifuge tube was maintained in an inverted position on a paper towel for 30 minutes to effect drying of the clot. Four ml of 1 percent sodium hydroxide solution was added to the centrifuge tube which was placed in boiling for five minutes to dissolve the fibrin. Six ml of distilled water was then added and the mixture was centrifuged for five minutes at a moderate speed.

For digestion one ml of supernatant was transferred to a digestion tube and a few glass beads were added to the supernatant mixture. The digestion tube was then placed over a microburner until the appearance of white fumes and a darkening of the solution. When the digestion tube was filled with dense fumes it was covered with a watch glass and the flame was reduced so that the mixture boiled slowly and gently. When the mixture turned brownish orange in color, the burner was shut off and the tube was allowed to cool for about one minute, after which 0.5 ml of persulphate solution was added drop by drop to the brownish orange mixture. The burner was relighted and the mixture was boiled until the solution became clear. The digestion tube was removed from the burner for the immediate addition of 3 ml of distilled water. This solution was then allowed to cool for about five minutes after which double distilled water was added to the 35 ml mark. Fifteen ml of Nessler's reagent was then added and immediately mixed.

Standard and reagent blank was made exactly in the same manner described by Hawk et al. (1954). Spectrophotometric readings were

made at 475 m μ . Amount of fibrinogen in g per 100 ml of plasma of chickens was calculated as per method suggested by Siegel (1964) which is as follows:

$$\frac{\text{optical density of plasma under test}}{\text{optical density of the standard}} \times 0.1875$$

RESULTS AND DISCUSSION

Experiment - I

Means and standard deviations of coagulation time of female chickens as measured by the capillary tube method are presented by lines in Table 3. Differences among lines were highly significant (Table 4) and means were separated by Kramer's (1956) modification of the Duncan multiple range test. The mean coagulation time of the games was significantly less than that for the birds in the other lines. The mean coagulation times for the HW and LW lines were significantly longer than those for the other lines and were approximately 250 percent greater than the means for the games. Mean clotting times for the HML and LML were intermediate to those for weight and game lines.

Analysis of variance and heritability estimates of coagulation time have been shown in Table 5. The heritability of capillary coagulation time based on maternal half-sib correlations and full-sib correlations were negative. The estimate obtained from paternal half-sibs correlation was 0.13. Paternal half-sib correlations would appear most appropriate method of estimating heritability in this experiment because of the number of sires. The negative estimates based on maternal half-sib were not surprising since the number of dams per sire and number of full-sib progeny were relatively small and the standard errors of variance components are known to have wide confidence intervals.

Table 4. Means and standard deviations of coagulation time by lines.

Line	Min.
Game	0.90 ± 0.75^a
LML	1.47 ± 0.92^b
HML	1.94 ± 1.00^c
HW	2.41 ± 0.50^d
LW	2.50 ± 0.76^d

Any two means with the same superscript were not significantly different. ($P \leq .05$)

Table 5. Analysis of variance of coagulation time.

Source of Variation	D.F.	M.S.	Expected S.S.
Lines	4	16.59**	$4\sigma_E^2 + 8.8\sigma_D^2 + 24.8\sigma_S^2 + 180.2\sigma_L^2$
Among sires within lines	39	0.53	$39\sigma_E^2 + 74.85\sigma_D^2 + 197.75\sigma_S^2$
Among dams w/n sires	96	0.53	$96\sigma_E^2 + 143.11\sigma_D^2$
Among full sib progeny	89	0.79	$89\sigma_E^2$

** P ≤ .01

$$h_{2(S+D)}^2 = -.508$$

$$h_{4S}^2 = 0.13$$

$$h_{4D}^2 = -1.14$$

Table 6. Means and standard deviations of coagulation time¹ by line and sex.

Line	Sex		Wt. \bar{x}
	♂♂	♀♀	
HW	5.9 \pm 2.8	5.4 \pm 2.8	5.5 ^a
Games	4.7 \pm 1.8	2.5 \pm 1.7	3.0 ^b
Wt. \bar{x}	5.2 ^a	4.3 ^a	4.5

¹ Coagulation time by test tube method in minutes. For comparisons between either sexes or lines any two means with the same superscript were not significantly different. ($P \leq .01$)

The highly significant difference among these lines which originated from widely different gene pools indicated that blood coagulation was under genetic influences. The low heritability of the trait suggests a study of the inheritance of the specific factors which influence coagulation time. This experiment may be considered a pilot study for Experiment II.

Experiment II

Coagulation time. Means and standard deviations for coagulation time as measured by the test tube method at $37.5^{\circ} \pm 1^{\circ}\text{C}$ are presented by sex and line in Table 6. The average coagulation time of 4.5 minutes was the same as that found by Amendt (1922-23), cited by Dukes (1955), and within the range of 2 to 10 minutes reported by Griminger (1965). The values obtained in this thesis were also consistent with those observed for adult chickens by Johnson and Corner (1933), Didishem et al. (1959) and Soulier et al. (1950).

Coagulation values were, however, not in agreement with those of Delezene (1897), Howell (1909), Bigland and Triantaphyllopoulos (1960) and Bigland (1964), who have suggested a longer coagulation time for avian blood indicating a less efficient hemostatic mechanism for birds. Since conditions of our experiment and strains of birds used in this study were different, no account can be made for such varied results.

Mean coagulation times were 5.2 and 4.3 minutes for males and females respectively (Table 5), with the difference between means being not significant (Table 6).

Examination of the means in Table 5 shows a coagulation time of 5.5 minutes for birds in HW line, which is about 180 percent greater than the mean for the games. This difference in coagulation time between lines was highly significant (Table 7) and may be explained as follows. Gamecocks are used for fighting and the birds with more vigor are preferred than others. As cutting injury is inevitable during pit fights, prolonged coagulation time would reduce stamina through loss of blood. Thus in game birds there has been for many generations intensive indirect selection for non-bleeders i.e. birds with rapid coagulation times.

Conversely haemorrhagic diseases became a problem with the development of the commercial broiler industry. The longer coagulation time in the HW line may suggest that it is correlated response to selection for body weight. One may perhaps hypothesize that increased haemorrhagic syndromes in broiler flocks occurred concomitant to selection for increased body weight. Whether such a hypothesis is justified will require additional investigation.

Variation in coagulation time between the capillary tube method and the test tube method have been reported in humans by Guyton (1963). Our observation revealed an average coagulation time of 0.9 and 3.0 minutes in the games and 2.4 and 5.5 minutes in the HW line birds by capillary tube and test tube methods respectively. This confirms the observation of Guyton that clotting time was longer when measured by the test tube method than by the capillary tube method. The discrepancy

Table 7. Analyses of variance.

Source of variation	D.F.	M.S.			
		Coagulation time	Prothrombin time	Thrombocytes	Fibrinogen
Between lines	1	206**	2,052**	25,596**	513
Between sexes	1	23	18	1,219	26,196*
Line x sex	1	11	2	70	35
Error	119	6	8	513	5,993

* $P \leq .05$

** $P \leq .01$

between methods may be due to greater contamination with tissue thromboplastin with the capillary tube method resulting in quicker clotting of the blood.

Prothrombin time. Presented in Table 8 are the means and standard deviations of prothrombin time, determined by the one stage method of Quick (1935). The mean prothrombin time was 18.8 seconds for the HW line and 10.3 seconds for games, with the difference between lines being highly significant (Table 7). Although the mean prothrombin time appeared to be somewhat longer for females than for males, the difference between sexes was not significant.

Prothrombin times were reported for chickens by Cuckler and Ott (1955), Chubb and Long (1957), Didishem et al. (1959), Bigland and Triantaphyllopoulus (1961), Griminger (1962, 1965) and Woody et al. (1963). The average prothrombin time of 15.4 seconds obtained here was in close agreement with the 16 seconds obtained by Cuckler and Ott (1955) with chickens 15 weeks of age. The prothrombin time recorded for the HW birds confirms the finding of Chubb and Long (1957), who reported a prothrombin time of 18.5 ± 1.5 seconds for chicken plasma.

Griminger (1962) observed that prothrombin times as short as 10 to 12 seconds could be obtained in growing chicks when all conditions were optimum and brain tissue thromboplastin was stored under nitrogen pressure at temperatures below freezing. Since the birds used in this study were older and the conditions of thromboplastin storage were not identical, the slightly longer average prothrombin time encountered in this experiment with White Rocks was not surprising. The means obtained

Table 8. Means and standard deviations of prothrombin time¹ by line and sex.

Line	Sex		Wt. \bar{x}
	♂♂	♀♀	
HW	18.1 \pm 2.5	18.9 \pm 3.3	18.8 ^a
Game	9.1 \pm 1.1	10.6 \pm 1.8	10.3 ^b
Wt. \bar{x}	13.2 ^a	15.9 ^a	15.4

¹ Prothrombin time in seconds. For comparisons between either sexes or lines any two means with the same superscript were not significantly different. ($P \leq .01$)

for the game birds, however confirmed the finding of Griminger (1962), Didishem et al. (1959) and Bigland and Triantaphyllopoulos (1961), even though the games were of an older age. Since all of the birds in this study are kept under identical conditions of housing, management and diet, the variation may be explained as follows. Griminger (1962) stated that the prothrombin time determined by Quick's one stage method was a function of prothrombin concentration and the latter was in turn, a function of vitamin-K intake. As the diet for all birds was the same, this may be explained by the fact that synthesis of prothrombin from vitamin-K was more efficient in games than in the birds of the HW line. This may have been reflected by the increased prothrombin concentrations and shorter prothrombin times for games.

Griminger (1965) has reported in adult chickens that a slightly longer prothrombin time was obtained for females than males. In our experiment, the mean prothrombin time for males was 13.2 seconds and 15.9 seconds for females. The difference, however, between the means was not significant.

Thrombocytes. Means and standard deviations for number of thrombocytes by lines and sexes are given in Table 9. The mean number of thrombocytes per cmm of blood was 110×10^3 for games and 81×10^3 for the HW birds. The difference between lines was highly significant (Table 7) and suggest existence of genetic differences in chickens for the number of thrombocytes. The mean number of thrombocytes for all chickens was 92×10^3 with the means for males and females being 90×10^3 and 93×10^3 , respectively. The difference of 3×10^3 between males

Table 9. Means and standard deviations of thrombocytes¹ by line and sex.

Line	Sex		Wt. \bar{x}
	$\overline{\sigma\sigma}$	$\overline{\sigma\sigma}$	
HW	72.2 \pm 16.9	82.4 \pm 21.9	81.2 ^a
Game	105.3 \pm 25.6	111.3 \pm 25.0	109.9 ^b
Wt. \bar{x}	90.4 ^a	92.7 ^a	92.4

¹ Thrombocytes in thousands per cmm of blood. For comparisons between either sexes and lines any two means with the same superscript were not significantly different. ($P < .01$)

and females was not significant indicating the no sexual dimorphism for this trait.

A mean thrombocyte count of 150×10^3 was reported by Didishem et al. (1959) for White Leghorn adult females whereas Lucas and Jamooz (1961) found that the number of thrombocytes varied from 26 to 60×10^3 . The results obtained in this thesis were intermediate to those reported by Didishem et al. and Lucas and Jamooz. Since line differences were found to exist, the variation among reports may be attributed to genetic diversity of the stocks, although the general health and vigor of the various flocks may have been a source of such variation.

It has been well established that thrombocytes in birds are the counterpart of platelets in mammals (Devilliers, 1938; Lucas and Jamroz, 1961). Although the participation of thrombocytes in avian coagulation process is meager considerable evidence has accumulated on the role of platelets in blood coagulation in other species. Delezene (1897) demonstrated that the presence of thrombocytes in birds was related to the rate of blood coagulation. Since our study revealed a shorter coagulation time and a greater number of thrombocytes for games than the HW birds, we believe there exists a reciprocal relationship between the two so far as the chicken's hemostatic mechanism are concerned.

Fibrinogen. The mean fibrinogen concentration in mgs per 100 ml of chicken plasma was 144 mgs (Table 10). Mean concentration of fibrinogen

Table 10. Means and standard deviations of fibrinogen¹ by line and sex.

Line	Sex		Wt. \bar{x}
	♂♂	♀♀	
HW	108.2 ± 46.2	148.9 ± 81.6	144.0 ^a
Game	114.9 ± 49.4	152.6 ± 81.7	143.9 ^a
Wt. \bar{x}	111.9 ^a	150.2 ^b	144.0

¹ Mg/100 ml.

For comparisons between either sexes or lines any two means with the same superscript were not significantly different. (P ≤ .05)

for male and female chickens was 112 and 150 mg per 100 ml of plasma, respectively. Thus the value recorded for the females is about 135 percent greater than the males with the difference between the two means being highly significant (Table 6). This demonstrated sexual dimorphism in the fibrinogen concentration of chicken plasma. No significant differences were observed between lines for concentration of fibrinogen in the plasma.

Howe (1925) reported that plasma proteins showed considerable variation with regard to species, age, sex, breeding, physiological activity, environment, diseases and reactions to infection. These factors had a greater influence on the variation of fibrinogen than any of the other protein components of plasma. Sturkie and Newman (1951) found that sex hormones caused a difference between males and females in the concentration of plasma proteins. The data obtained in this thesis were consistent with the findings of Howe (1925) and Sturkie and Newman (1951).

Reports regarding the concentration of fibrinogen in chicken plasma has been made by Didishem et al. (1959) and Bigland and Triantaphyllopoulus (1961). A low fibrinogen concentration of 79 mg % was reported for chickens plasma by Didishem et al. and differences between methods of measuring fibrinogen were found by Bigland and Triantaphyllopoulus. The same pooled sample of plasma yielded differences between two methods of more than 100 mgs. Although the values obtained here were intermediate to those in the literature their relia-

bility may still be considered in question because of the large coefficients of variation (Table 10). Since fibrinogen concentration fluctuates under several conditions, nothing may be attributed specifically to explain this large variation.

Clot formation occurs through a series of complex reactions whose ultimate role is the conversion of fibrinogen to the fibrin that form the clot. Thus it may be assumed that the greater the amount of fibrinogen the shorter the coagulation time because of a more efficient coagulation mechanism. Interspecies comparisons of our results with those obtained by others and a short coagulation time for chick blood suggests that in chickens there is a critical level for concentration of plasma fibrinogen and that excess amounts beyond this do not impair the coagulation mechanism. Thus, a corollary may be that when fibrinogen concentration is below the critical point coagulation time is increased. This assumption is based on the finding of MacFarlane (1938) who suggested that there was a critical level of fibrinogen concentration in plasma below which coagulation produces a clot too friable to be hemostatically effective.

Correlations between coagulation factors. Simple correlation coefficients between the various coagulation factors under investigation and clotting time are presented in Table 11. The correlation between coagulation time and prothrombin time was positive in all cases with the exception of the game males. Correlations between coagulation and prothrombin time were significant for females in both the lines and the pooled data for both sexes in HW line.

Table 11. Simple correlation coefficients.

Line	Sex	Prothrombin time and coagulation time	Fibrinogen and coagulation time	Thrombocytes and coagulation time	Prothrombin time and fibrinogen	Fibrinogen and thrombocytes	Prothrombin and thrombocytes
HW	♂♂	.45	.22	-.42	-.58	-.33	-.14
	♀♀	.25*	-.09	-.15	-.08	.10	-.14
	Pooled	.26*	-.08	-.18	-.09	-.10	-.13
Game	♂♂	-.18	.34	-.84**	-.12	-.26	-.02
	♀♀	.49**	-.08	-.35**	-.26	.09	-.36*
	Pooled	.14	-.12	-.45**	-.15	.06	-.24
Pooled	♂♂	.33	.28	-.57**	-.20	-.17	-.59**
	♀♀	.55**	-.09	-.41**	-.09	.09	-.52**

* $P \leq .05$

** $P \leq .01$

The correlation between coagulation time and fibrinogen varied in sign but none was significant. This emphasizes that there is a critical level of fibrinogen in chickens and when present in excess of the minimal requirement for coagulation no consistent relationship may be seen between coagulation time and fibrinogen.

Coagulation time and the number of the thrombocytes were negatively correlated in all cases. Although the correlation was highly significant throughout for the games and not significant for birds in the HW line the pooled correlations of -0.57 for males and -0.41 for females were highly significant. These results with chickens agree with the negative correlation of -0.45 between coagulation time and platelets in humans reported by Jaques et al. (1946).

Correlations between prothrombin time and fibrinogen were negative in all comparisons, however none was significant. Correlations between fibrinogen and thrombocytes was neither consistent in signs nor significant.

A consistent negative correlation was found between prothrombin time and thrombocytes. The correlation coefficient between prothrombin time and thrombocytes was -.59 for male chickens and -.52 for female chickens, and both were highly significant.

These correlations strongly suggest a definite relationship between coagulation time and the various factors affecting this trait in chickens. The most important relationships in the lines studied were the positive correlations between prothrombin time and coagula-

Table 12. Regression coefficients.

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3$$

Line	Sex	b ₀	b ₁	b ₂	b ₃
HW	♂♂	-18.77	1.14	.05	-.02
	♀♀	3.25	0.19	-.002	-.014
	Pooled	3.29	0.21*	-.001	-.019
Game	♂♂	12.90	-0.29	.004	-.06**
	♀♀	-.53	.41*	.001	-.013
	Pooled	6.80	.02	-.002	-.034**
Pooled	♂♂	8.35	.03	.008	-.049
	♀♀	1.83	.27**	-.001	-.017

* P ≤ .05

** P ≤ .01

x₁ = Prothrombin time

x₂ = Fibrinogen

x₃ = Thrombocytes

Y = Coagulation time

Table 13. Porportion of variation accounted by prothrombin time, fibrinogen and thrombocytes towards coagulation time.

Line	Sex	Prothrombin time	Fibrinogen	Thrombocytes	Total
HW	♂♂	20.3	34.7	0.5	55.5
	♀♀	6.4	0.6	1.2	8.2
	Pooled	6.9	0.3	2.1	9.3
Game	♂♂	3.2	10.3	62.5	76.0
	♀♀	24.6	0.2	3.2	28.0
	Pooled	2.0	0.9	18.3	21.2
Pooled	♂♂	10.9	9.1	14.7	34.7
	♀♀	30.8	0.2	1.9	32.9

tion time and the negative correlations between coagulation time and thrombocytes and thrombocytes and prothrombin time.

Regression analysis. Regression analyses were used to establish the functional relationships between the dependent (coagulation time Y) and independent variables, (prothrombin x_1 , fibrinogen x_2 , and thrombocytes x_3). The values for b_0 , b_1 , b_2 , and b_3 have been presented in Table 12 for the multiple linear regression for the model:

$$Y = b_0 + b_1x_1 + b_2x_2 + b_3x_3.$$

These constants were calculated within lines and sexes and for lines and sexes pooled. The proportion of variation in coagulation time accounted for by the linear regression of prothrombin time, fibrinogen and thrombocytes to coagulation time, have been presented in Table 13. The percentage of variation attributed to these three independent variables on coagulation time was found to be 76.0 for the game males and 55.5 for the HW males. Respective values obtained for females were considerably less. Pooled data revealed that the proportion of total variation attributed to these factors was 33 percent for females and 35 percent for males. Though each of the coagulation factors may be important in the determination of coagulation time, the role played by prothrombin in coagulation, appeared to be more vital than that of the others in female chickens. This is consistent with the view expressed by Seegers (1962), who has reported that coagulation mechanism is a series of chemical reactions which centers around prothrombin, without which the blood does not clot. Seegers (1964) stated

further that prothrombin was a multipotential protein and produced several derivatives depending upon the enzymes to which it was exposed. According to him, the factors VII, IX and X are prothrombin derivatives and inactive precursors of these factors were present in plasma as prothrombin. The porportion of variation accounted for prothrombin time, fibrinogen and thrombocytes in males was found to be 11, 9, and 15 percent, respectively. This indicated that each of these factors were of comparable importance in the coagulation time of male chickens.

SUMMARY AND CONCLUSIONS

This study was undertaken to measure the coagulation time of blood in five genetically diverse lines of chickens and to investigate whether this trait was heritable. Data for this purpose were obtained for females only and revealed significant differences among the lines for coagulation time. Significant differences among lines which originated from widely different gene pools indicated that blood coagulation was under genetic influences.

Heritability estimates of capillary coagulation time based on maternal half-sib and full-sib correlations were negative, whereas estimate obtained from paternal half-sibs correlations was 0.13. The large differences between lines and the low heritability of this trait within lines suggested a study of specific coagulation factors, which influenced coagulation time.

Based on the results obtained from the above experiment two lines, the H¹ and game lines, were chosen for further investigation. Prothrombin time, concentration of thrombocytes, and plasma fibrinogen concentration were measured to determine the relative contribution of these factors in the above two lines by sex. Coagulation time by test tube method was also determined to record the difference between capillary tube method and test tube method in chickens.

Highly significant differences between lines were found for coagulation time, prothrombin time and thrombocytes concentration, whereas

none was noted for concentration of fibrinogen. Sexual dimorphism was not in evidence for coagulation time, prothrombin time and concentration of thrombocytes whereas females had significantly greater fibrinogen concentrations than males. The values recorded for coagulation time as determined by test tube method was 5.5 minutes for HW and 3.0 minutes for games. The mean prothrombin time was 18.8 seconds and 10.3 seconds for HW and games respectively. The number of thrombocytes was 110×10^3 /cmm of blood in games and 81×10^3 in HW line. The concentration of fibrinogen per 100 ml of plasma as determined by microkjeldahl method was 110 mg for males and 150 mg for females.

Comparisons of coagulation time between the test tube and capillary tube method revealed a period approximately two times longer for the former method than for the latter.

Simple correlation coefficients were determined for coagulation time with the three specific coagulation factors and between these factors. The correlation coefficient between coagulation time and prothrombin time was 0.55 for females and 0.33 for males. The correlation between coagulation time and thrombocytes concentration was 0.41 for females and -.57 for males. The correlation between prothrombin time and concentration of thrombocytes was -.52 and -.59 for females and males, respectively. Prothrombin time and fibrinogen concentration were not significantly associated. The same correlation existed for the relationship between coagulation time and concentration of fibrinogen and fibrinogen and thrombocytes concentration.

Coagulation time is known to be influenced by a number of coagulation factors, three of which are prothrombin time and concentration of thrombocytes and fibrinogen. The relative contribution of these three factors together and individually on coagulation time were determined. The percentage of total variation attributed to these factors was 33 for females and 35 for males. the proportion of variation accounted for by each factor individually was comparable for males whereas for females the prothrombin was the primary source of variation in coagulation time.

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ABSTRACT

Blood coagulation time was determined in five genetically diverse lines of chickens by the capillary tube method. Significant differences were found among lines for coagulation time suggesting genetic influences on this characteristic in chickens. Heritability of blood coagulation as estimated by paternal half-sib correlations was 0.13.

The significant difference among lines and the low heritability of this trait indicated that a study of specific coagulation factors was warranted. Prothrombin time, thrombocyte concentration and plasma fibrinogen concentration was investigated in two of the most diverse lines to determine their relative contribution towards variation in coagulation time.

Highly significant differences between lines were observed for prothrombin time and concentration of thrombocytes. Differences between lines for concentration of fibrinogen was not significant.

The coagulation time and prothrombin time were positively correlated, where as the correlation was negative between coagulation time and thrombocyte concentration and between prothrombin time and thrombocyte concentration. The correlation coefficients between coagulation time and fibrinogen concentration were not significant.

The percentage variation in coagulation time attributed to these three coagulation factors was 33 for females and 35 for males. Prothrombin time was the major source of variation in females while the contribution of each of these factors were comparable in males.