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Differential Clotting Responses of Rabbits to Injections of Homogenates from Wild-Type and Tumorous-Head Drosophila Melanogaster

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DIFFERENTIAL CLOTTING RESPONSES OF RABBITS TO INJECTIONS OF HOMOGENATES FROM WILD-TYPE AND TUMOROUS-HEAD *DROSOPHILA MELANOGASTER*

BY

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THESIS

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ABSTRACT

Two groups of New Zealand white rabbits were injected with homogenates from Tumorous-head (Tuh) and Wild-type (WT) Drosophila melanogaster. A third group was used as a saline injected control. Blood collected in both acute and chronic studies was subjected to various hematological and post mortem studies.

The Tuh injected group showed a five-fold increase in thrombocytes (blood platelets) over the controls and a four-fold increase over the wild-type group. Reduced clotting times were noted from acute to chronic studies in both tumorous and wild-type studies; however, the magnitude of change between the two groups was insignificant. Investigations involving electrophoretic banding patterns, differential blood cell counts, and comparative hematocrits, provided less significant results.

The author concludes that the reduced clotting times reported in tumorous-head injected rabbits represent a decrease in bleeding time. This was caused by the more effective plugging of the damaged vessel by the increased number of platelets.

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INTRODUCTION

The term hemophilia is loosely applied to several hereditary deficiencies of coagulation, all of which cause bleeding tendencies hardly distinguishable from one another. Regardless of the precise type of hemophilia, transfusion of normal fresh plasma or of the appropriate purified protein clotting factor into the hemophilic person usually relieves his bleeding tendency for a few days (1). The present study involves the relative effects of several antigens on the hemostatic mechanism and, therefore, warrants an expanded discussion of hemostasis.

When the walls of blood vessels are severed, the subsequent blood loss is reduced by a sequence of reactions known collectively as hemostasis. There are three major facets of hemostasis: vascular spasm, the formation of platelet plugs, and plasma coagulation (1).

The nervous reflexes are presumably initiated by pain impulses originating from the traumatized vessel or from nearby tissues. The reflex signal travels first to the spinal cord, then back through the sympathetic nerves causing spasms which extend several centimeters in either direction from the point of rupture. The reaction lasts between a few seconds to a few minutes. Most spasms probably result from local myogenic contractions of vessels initiated by direct damage to the vascular wall (1). Compared to our knowledge

about the coagulation mechanism, little is known about the role of the vasculature in hemostasis (2). Zucker (3), Chen & Tsai (4), and Berman & Fulton (5) believed that the rate of blood flow through a vessel influences the local concentration of precoagulants in the blood. Increased flow rate would reduce the local precoagulant concentration whereas decreased flow rate would further enhance this concentration. Vasocontriction with consequent decreased blood flow would, therefore, be expected to be beneficial to the hemostatic process. Zucker (3,6) maintained that vasoconstriction involves not only the injured vessel but also others in the immediate vicinity, apparently because of the platelets' release of serotonin, a vasoconstrictor substance. Although vasoconstriction is inadequate to effect hemostasis, it has been suggested that the vasoconstrictor effects of serotonin may contribute to the process.

Williams (2) reported that the activation of the Hageman Factor (Coagulation Factor XII) may possibly contribute to hemostasis by initiating the reactions of blood coagulation and by the production of peptides which are able to increase capillary permeability and smooth muscle contraction. Platelets may also release substances that increase capillary permeability (7). Increased capillary permeability may improve hemostasis by increasing the viscosity of blood within the vessels (8). Active vasoconstriction plays no role in hemostasis in nonmuscular vessels such as capillaries and some venules. Blood flow in these vessels may be retarded by local tissue pressure, by adhesion of endothelial or connective tissue elements,

or by arteriolar constriction (3,4,5).

According to Williams (2), the vascular endothelium provides an inert surface for the reactions of blood coagulation and platelet aggregation. The smoothness of the endothelium, along with presence of its monomolecular layer of negatively charged proteins, prevent contact activation of the intrinsic clotting system by repelling platelets and other clotting factors. Damage to the endothelial surface causes loss of smoothness and negative electrical charge. This is believed to help activate Factor XII, thus initiating the intrinsic pathway of clotting. Sawyer (9) observed that electrical currents within and across the vessel wall may help to initiate irreversible clumping of the platelets, followed by their adhesion to the injury site.

Astrup and Buluk (10) and Kirk (11) indicated that the endothelium of blood vessels is rich in thromboplastic activity. This appears to be tissue thromboplastin (Coagulation Factor III). Tissue Factor III reacts with Factor VII and calcium to form a complex to initiate reactions to the extrinsic pathway of blood coagulation and leads to rapid formation of thrombin (figure 2). Thombin may then also effect platelet aggregation, or fibrin formation, and thus accelerate the reactions of hemostasis.

The intima of blood vessels contain significant quantities of plasminogen activator which may be released at the injury site and act as a protective mechanism against excessive local fibrin formation (2).

Guyton (1) describes the formation of the platelet plug as the second hemostasis event. This is the action by the platelets to close the vessel's trauma site. An understanding of this phenomenon warrants an explanation of the nature of platelets themselves.

Platelets, or thrombocytes, are minute round or oval discs about 2 microns in diameter and are derived from the cytoplasm of megakaryocytes, large immature cells in the bone marrow (1). After maturation, these cells fragment into cytoplasmic clumps which are released into the peripheral circulation as functional platelets (12). Each megakaryocyte is capable of producing 3,000 to 4,000 platelets (13), but the number produced and released in the bone marrow is regulated by the number already in circulation (14). In man, the normal plasma concentration of platelets ranges from 150,000 to 450,000 per cubic millimeter while their life span is approximately ten days, with a half-life of three days (12).

White (15) suggests that a platelet can be divided into three structural areas or zones, each zone being involved in a specific function. These are the peripheral zones, the sol-gel zone, and the organelle zone.

The peripheral zone is involved in adhesion and aggregation. It separates the platelet from its surrounding medium and is composed of an exterior coat, a membrane, and submembrane filaments. The exterior coat is an amorphous substance, rich in mucopolysaccharides and glycoprotein. Clotting factors identical to those present in the plasma are present. The membrane just below the exterior coat

is the source of platelet Factor 3 (PF3), a phospholipid which becomes available on the surface of the platelet during irreversible aggregation. PF3 interacts with some clotting factors in the intrinsic pathway to produce fibrin.

The sol-gel zone provides support for cell structure and mechanisms for cell contraction. The peripheral zone and the sol-zone share submembrane filaments. The submembrane filaments, along with microtubules and microfilaments in the sol-gel zone, help to support the shape of unaltered platelets and, in response to stimuli, help in the extension and stabilization of pseudopod formation. They also participate in the contractile process during irreversible aggregation and retraction. The microfilaments are the source of the contractile protein, thrombosthenin.

The organelle zone, which consists of organelles randomly distributed throughout the cytoplasm is concerned with respiratory, excretory and production functions, as well as storage and release of energy. It also regulates the platelet's chemical and physiologic response to stimuli.

Williams (2) observed that platelets undergo three changes during hemostasis: adhesion (aggregation), contraction, and secretion (releasing reaction). Adhesion is an independent facet in platelet physiology, because adhesion can occur without contraction. Secretion is dependent on the contractile system of platelets; hence, it cannot occur alone. Contraction can develop up to a point without secretion. The contractile mechanism in platelets consists of the

fusion of centrally clumped organelles and the selective release of several endogenous components that aid in hemostasis.

Platelets respond to vascular trauma by drastic reorganization of both their internal and surface structure (15). When they come in contact with a wettable surface, such as the subendothelial collagen fibers in the vascular wall, the following changes occur: they begin to swell (1); the submembrane filaments and microtubules form a constrictive band around the platelet organelles; the centrally located organelles fuse (15); they assume irregular forms with numerous irradiating processes protruding from their surfaces (1); the exterior coat acquires adhesive qualities causing the platelets to adhere to the collagen fibers at the damaged vessel site. Subsequently, release of certain chemical constituents occurs. These constituents are usually bound to the membrane surfaces and may participate in clotting by providing acceleratory influences. Several of these activities have been designated as platelet factors 1 to 4 (2). Platelet factor 1 is plasma coagulation factor V adsorbed by the platelets (16). Platelet factor 2 is a protein which accelerates the clotting of fibrinogen by thrombin, aggregates platelets from platelet-rich plasma further potentiating the platelet aggregating effect of ADP (17), and counteracts the inhibitory effects of plasma antithrombin III on a thrombin-induced fibrinogen→fibrin reaction. Platelet factor 3 or 4 are both influenced by aggregation (18,19, 20). Platelet factor 3, a lipoprotein component of platelet membrane, becomes available to coagulant enzymes and cofactors of plasma

following platelet aggregation or platelet trauma (21,22,23). The active component is the phospholipid moiety bound to both membranes and granules which accelerates plasma clotting, probably by acting as a surface catalyst (23). The lipid membrane is more readily exposed for participation in clotting (24). Activation occurs because the soluble enzymes are provided a surface on which to act, thus, having the effect of concentrating the enzymes in a localized area, leading to faster reaction rates (25). Phospholipids accelerate blood coagulation by participating in the reactions involving factor VIII and those involving factor V. Little activity can develop even when the release reaction does not occur (26,27). Platelet factor 4 is a glycoprotein (28) released from platelets following platelet aggregating by ADP, thrombin, or epinephrine (29-31). It shortens the thrombin clotting time in the presence of heparin (29-32), potentiates ADP-induced aggregation in vitro, precipitates fibrinogen, nonenzymatically clots soluble fibrin monomer complexes (24), and neutralizes certain fibrinogen breakdown products (antithrombin VI) (33). Platelets also contain intracellular fibrinogen (34-37) which appear not to be derived from plasma fibrinogen and may be synthesized in the megakarycyte (37). As indicated previously, some other constituents released by platelets are serotonin, adenosine triphosphate (ATP), and large quantities of adenosine diphosphate (ADP) (15). The ADP aids in activating nearby platelets causing them to adhere to the originally activated platelets. Therefore, at any vascular trauma site the exposed subendothial collagen elicits a continuous

cycle activating successively increasing numbers of platelets accumulating in the formation of the hemostatic platelet plug. This is a fairly loose plug; however, the blocking of blood loss is usually successful. During the process of blood coagulation, thrombin is formed. It further alters the platelets to irreversible aggregation, thus forming a tight and unyielding plug. In performing the usual coagulation tests one generally deals with the concentrations of factors present in plasma at normal or less than normal concentrations. Under certain conditions the platelet concentration may be important as well. Excessive platelet concentrations is a condition called thrombocytosis. Depending on the effectiveness of hemostatic or thromboplastic functions of this condition, a reduced bleeding time or clotting time may possibly result. Transitory thrombocytosis may result from the mobilization of extravascular platelet pools, for example, following epinephrine administration (38) or vigorous exercise (39). All other forms of throm bocytosis apparently are the result of accelerated platelet production. Preliminary thrombokinetic data would suggest that this may result from two different mechanisms (Table 1). In individuals with reactive or secondary thrombocytosis, the platelet count is directly correlated with the megakaryocyte mass and is inversely correlated with the mean megakaryocyte volume. This is the result of regulatory processes that normally reduce the stimulus to nuclear endoreduplication as the platelet count rises (40,41). A direct and linear relationship between platelet turnover rate and total megakaryocyte mass has been

Table 1

Thrombocytosis (2)

A. Primary (autonomous)
   1. Essential thrombocythemia
   2. Other myeloproliferative disorders

B. Secondary (reactive)
   1. Chronic inflammatory disorders
   2. Acute inflammatory disorders
   3. Acute hemorrhage
   4. Iron deficiency
   5. Hemolytic anemia
   6. Malignant disease
   7. Postoperative
   8. Response to drugs
   9. Response to exercise

demonstrated (40,41). The factors leading to accelerated platelet production in the various disorders associated with reactive thrombocytosis are almost totally obscure. The principal importance of this disorder is the recognition of its benign nature and its differentiation from autonomous thrombocytosis.

In autonomous thrombocytosis, platelet production apparently is unresponsive to normal regulatory processes; and the platelet count does not correlate with the megakaryocyte volume as seen in Table 2 (40). The decrease in megakaryocyte size normally resulting from an increase in the circulating platelet mass is absent. Autonomous thrombocytosis has been likened to neoplastic proliferation of other hematopoietic elements (42).

The megakaryocyte-thrombocyte system has been shown to respond to thrmbocytopenia and thrombocytosis by respectively increasing and decreasing platelet production (43,44,45). These responses have been shown to be humorally mediated (46,47). While almost nothing is established about thrombopoietin, it is known to affect both the number of magekaryocytes found within the marrow and their proliferative behavior (47). With stimulation, there is an increase in the rate of maturation of the megakarocyte size resulting from an increase in the number of mitosis within the individual cells, and possibly an increase in the size of thrombocytes appearing in circulation. With suppression, megakarocytes are smaller and contain a decreased number of nuclei. The changes affect the number of platelets produced per megakaryocyte. An inverse relationship is also normally found between

Table 2

Differentiation of Reactive and Autonomous Thrombocytosis

	Reactive Thrombocytosis (secondary)	Autonomous Thrombocytosis (primary)
THROMBOKINETIC FEATURES		
Total megakaryocyte mass	Slightly increased	Greatly increased
Megakaryocyte number	Increased	Increased
Megakaryocyte volume	Decreased	Increased
Platelet turnover or production rate	Increased	Increased
Total platelet mass	Increased	Increased
Platelet survival	Normal	Normal to slightly increased
CLINICAL AND LABORATORY FEATURES		
Thromboembolism and hemorrhage	Uncommon	Common
Duration	Often transitory	Usually > persistent
Splenomegaly	Absent*	80% of cases
Platelet count	Usually > $400,000/mm^3$	Usually > $1,000,000/mm^3$
Bleeding time	Usually normal	Often prolonged
Platelet morphology and function	Usually normal	Often abnormal
Leukocyte count	Usually normal	Increased in 90% of cases

*Unless as the result of the underlying disorder.

Thrombokinetic data from Harker (40).

the number of thrombocytes in circulation and in the size of megakaryocytes within the marrow (40). In the thrombocytosis accompanying inflammatory states and iron deficiency, however, there is dissociation between increased megakaryocyte number and the number of thrombocytes produced per megakaryocyte. At the present time, it is impossible to say whether megakaryocyte stimulation is mediated by one or more stimulating factors.

Studies of cyclic variations in the platelet count in normal individuals and in individuals with cyclic thrombocytopenia and tidal platelet disgenesis, suggest that the control of thrombopoiesis involves a negative "feedback" mechanism that contains a time delay. A result of the time lag may magnify perturbations of either a positive or negative nature, resulting in an oscillation of platelet production. More than one humoral regulation, or a combination of negative and positive feedback regulatory process, may be involved (48).

Accelerated thrombopoiesis commonly is associated with accelerated erythroopoiesis. Thus, increased platelet numbers are present in subjects with various hemolytic anemia (49), following acute blood loss (50,51,52), and in some subjects with secondary polycythemia associated with renal tumors (53). The association of thrombocytosis with various neoplasms has been recognized for many years (52). Platelet counts in excess of 400,000/mm^3 may be found in a large proportion of individuals with neoplasms, in as many as 40 percent of cases in one study (54). The persistence of post-splenectomy thrombocytosis in anemic subjects has been found. Despite the evidence

that thrombopoietin and erythropoietin are different substances (55), these findings suggest that there is some interrelationship between erythropoietic and thrombopoietic control mechanisms (56).

Several interdependent coagulation factors enter into reactions that initiate clotting (see Table 3). The deficiency of any factor may result in an abnormal hemostatic response. Any increase in factor concentration, in association with changes in the rate of blood flow, may lead to thrombus formation (2).

In 1904, Morawitz presented the classical Theory of Blood Coagulation. He postulated that:

a) prothrombin, calcium, and fibrinogen were present in circulating blood.

b) blood remained fluid in the absence of thromboplastic factor which he called "Thomboplastin" or "Thrombokinase."

c) thromboplastic factor resulted from tissue injury or platelet disintegration.

d) thromboplastic factor could convert prothrombin into thrombin with subsequent fibrin formation. (12)

Many research workers in the field of blood coagulation refer to Morawitz's thromboplastic factor or thromboplastin as prothrombin activator. Unfortunately, the detailed mechanisms of prothrombin activator formation are still not clear. However, there is much better understanding of the mechanisms of prothrombin conversion to thrombin and action of thrombin to cause fibrin thread formation. Guyton (1) indicated that this causes the polymerization of fibrinogen molecules into fibrin threads within 10 to 15 seconds. The rate

Table 3

The Proteins of Blood Coagulation (57)

Common Name	Roman Numeral	Stage of Participation in Blood Coagulation	Plasma Concentration (mg/100 ml plasma)	Electrophoretic Migration	Molecular Weight
Fibrinogen	I	Late, intrinsic and extrinsic	170-400	Between β and γ globulin	339,700 (human) 340,000 (bovine) 501,800 (horse)
Prothrombin	II	Late, intrinsic and extrinsic	7 or less	χ^2 globulin	62,850 (bovine)
Tissue thromboplastin	III	Early, extrinsic	-	-	-
Calcium	IV	Early, middle, late, intrinsic and extrinsic	9-11.5	-	-
Proaccelerin	V	Middle, intrinsic and extrinsic	Trace	Between β and γ globulin	70,000; 99,000; 180,000; 390,000; 350,000 (bovine)
Precursor of serum prothrombin conversion accelerator (ProSPCA)	VII	Middle, intrinsic	5 (or less)	Between χ and β globulin	-

Table 3 (continued)

Common Name	Roman Numeral	Stage of Participation in Blood Coagulation	Plasma Concentration (mg/100 ml plasma)	Electrophoretic Migration	Molecular Weight
Antihemophilic factor (AHF)	VIII	Middle, intrinsic	1 (or less)	β_2 globulin	1-2 million (bovine and human); 180,000 (human and pig)
Christmas factor	IX	Middle, intrinsic	0.5-1	χ^2 globulin	90,000 (bovine)
Stuart factor	X	Middle, intrinsic and extrinsic	0.5-1	χ globulin	85,000-87,000 (bovine); 54,000-56,000 (bovine)
Plasma thromboplastin antecedent (PTA)	XI	Early, intrinsic	Trace	β-γ globulin region	-
Hageman factor	XII	Early, intrinsic	Trace	β-γ globulin region	82,000 (bovine) 20,000 (human)
Fibrin stabilizing factor (FSF)	XIII	Late, intrinsic, and extrinsic	Trace	β^2 globulin	350,000 (human)

limiting factor in causing blood coagulation is usually the formation of prothrombin activator and not the subsequent reactions beyond that point. The reactions in figure 1 are a modified illustration of Morawitz's postulate demonstrating the conversion of prothrombin to thrombin under the influence of prothrombin activator and calcium ions.

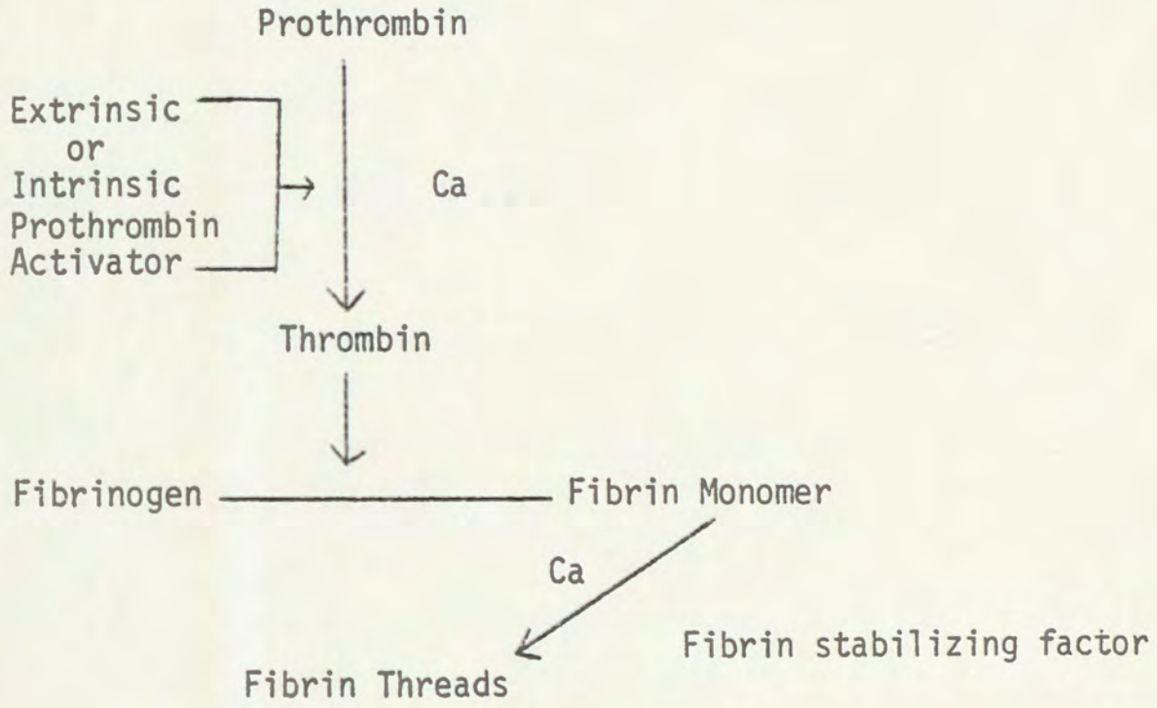


Figure 1. Morawitz's classical theory of blood coagulation

As previously indicated, the rate of formation of thrombin from prothrombin is almost directly proportional to the quantity of prothrombin activator available. This in turn is approximately proportional to the degree of trauma to the vessel wall or blood. In turn, the rapidity of the clotting process is proportional to the quantity of thrombin formed.

Guyton (1) further described the action of thrombin on fibrinogen

to form a molecule of fibrin monomer. He noted that as several fibrin monomer molecules polymerize within seconds into long fibrin threads, they form the reticulum of the clot. In the early stages of this polymerization, the fibrin monomer molecules attach to one another by loose hydrogen and hydrophobic bonds, forming weak polymer chains. However, immediately thereafter, another plasma globulin factor, the fibrin stabilizing factor, acts as an enzyme to cause covalent bonding between the fibrin monomer molecules and also between the adjacent polymer chains. Williams (2) noted this factor as catalyzing the formation of peptide bonds between glutamic acid and lysine groups in adjacent molecules of fibrin monomers in the clot. This adds tremendously to the three dimensional meshwork of the fibrin threads and yields a mechanically strong, hemostatically effective clot.

Two types of prothrombin activator resulting from two avenues of coagulation have been postulated (1):

1. Tissue extract called the extrinsic pathway
2. Plasma thromboplastin called the intrinsic pathway

The terms "intrinsic" and "extrinsic" refer to clotting inside and outside the vascular system, respectively. With respect to effective rates, the intrinsic system is relatively slower than the extrinsic system. In either case, the final common pathway is the conversion of prothrombin to thrombin. In both pathways a series of plasma proteins, especially beta globulins, play major roles. These along with the factors previously discussed are called blood clotting factors (1).

One of the more recent schemes devised to diagram the

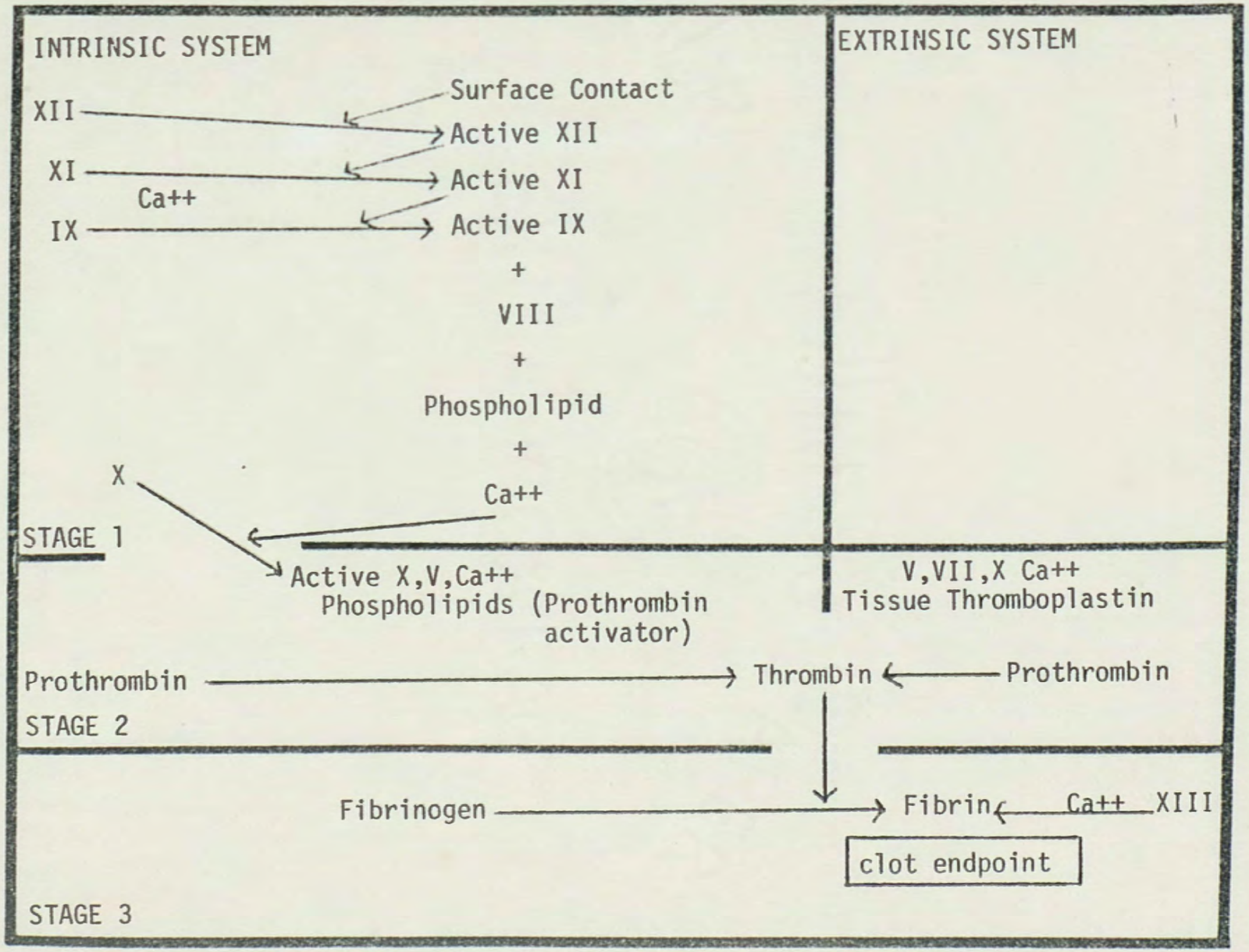
coagulation process was the cascade or waterfall sequence illustrated by Davie, Ratnoff (58), and MacFarlane (59). This is based on the theory that the blood coagulation process is primarily a series of enzyme reactions. These reactions are subdivided into three stages (figure 2).

Erslev and Gabuzda (60) describe the first phase of the intrinsic system as the surface activation of Factors XII and XI. The conversion of Factor XII to XIIa ("a" denotes activated form) seems to require prior complex formation with Factor XI, which in turn is changed to XIa. Factor XIa then triggers coagulation by activating IX to IXa, a potent procoagulant. A complex is then formed of Factors IXa and VIII with platelet phospholipid. The formation of the "Factor VIII complex" is accelerated by the presence of small quantities of thrombin. This complex then converts Factor X to Xa, which by itself has "prothrombinase" activity. The rate of reaction, however, is markedly accelerated by the presence of Factor V and platelet phospholipid.

The extrinsic system short circuits the first stage of the intrinsic system by directly activating Factor X through the formation of a complex between Factor VII and tissue thromboplastin, which is composed of phospholipid and protein (60).

Calcium is required for most of the coagulation reactions, a point of considerable laboratory importance. In vitro, blood can be prevented from clotting by reducing the calcium ion concentration below the threshold level for the clotting, either by deionizing the

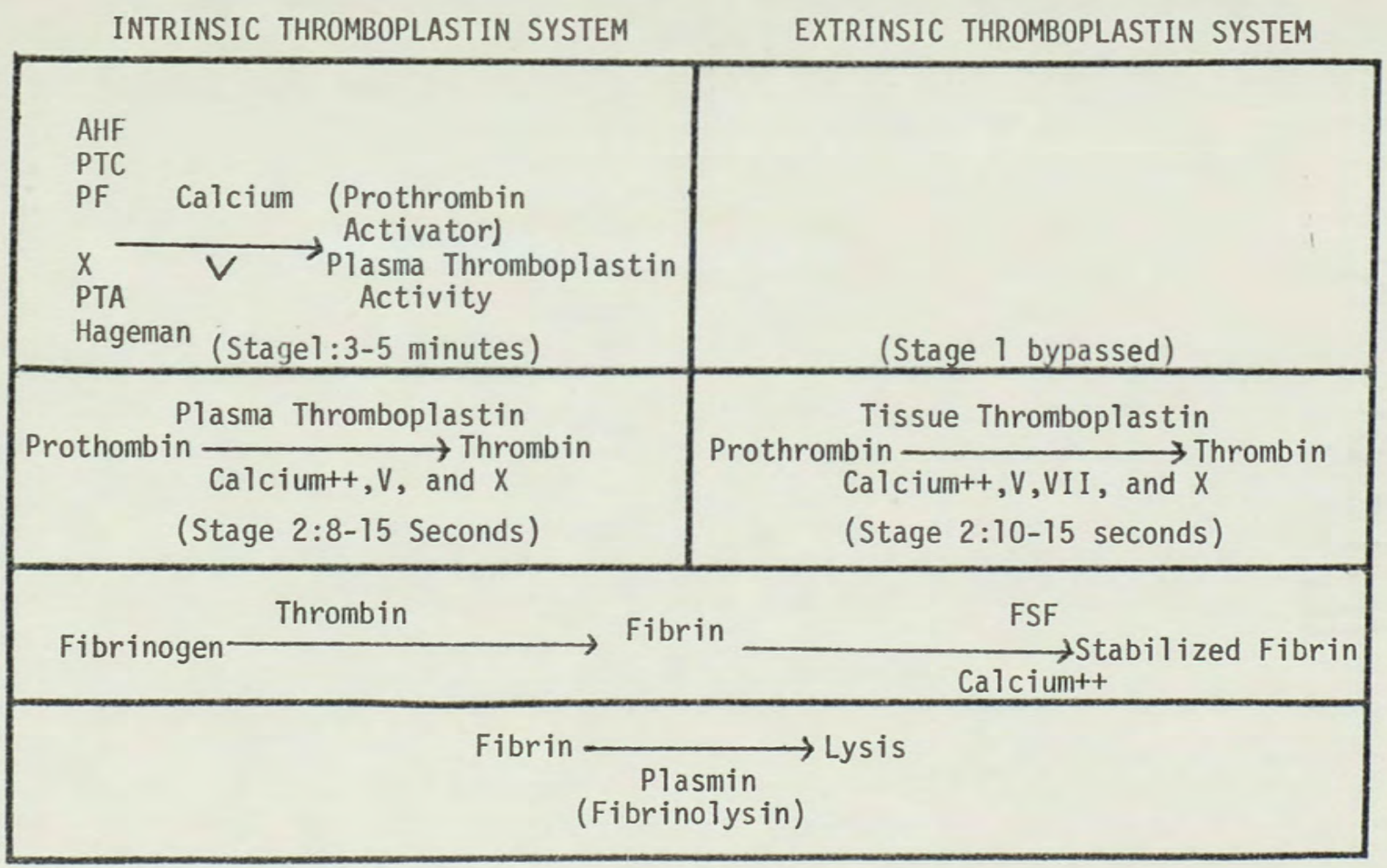
Figure 2. Cascade or waterfall sequence of coagulation (61)



calcium by reacting it with substances such as citrate ion or by precipitating the calcium with substances such as the oxalate iron, both substances commonly used as anticoagulant in laboratory coagulation testing (1). However, it is virtually impossible for hypocalcemia to be of sufficient magnitude in vivo to cause abnormal bleeding. Whether activated forms of Factors V, VII, and VIII exist or whether their activity is mediated solely through complex formation with phospholipid remains uncertain. As previously indicated, the thrombin formed as the end-product of this accelerating series of reactions, in addition to forming fibrin monomer and activating Factor XIIII, also engages in "positive feedback" by increasing platelet aggregation and promoting Factor VII complex formation. Modulating influences on the negative side come from enzyme antagonists in the plasma. The activated forms of the coagulation factors are cleared from the circulation rapidly, which is also of considerable importance in keeping the process of clot formation under physiologic control (60).

Most of the tests of the clotting mechanism depend on the appearance of a fibrin clot in the test tube. Quick (62) believed that if he added calcium and a source of prothrombin activator to oxalated plasma, prothrombin would be the rate limiting component of the clotting reaction. This test not only measures prothrombin specificially, but also factors I, V, VII, and X as well (see figure 3).

The present investigation explores the hemostatic responses of rabbits to antigens from Wild-type and Tumorous head strains of fruit



All three stages of blood clotting and the fourth stage (lysis) may be occurring continuously in minute amounts in the homeostasis of normal blood. The presence of free plasmin in more than trace amounts is abnormal.

Figure 3. Stages of blood coagulation

flies. A recent immunological investigation (63) produced results which could provide additional insights in studies concerned with the future treatment of hereditary deficiencies in coagulation. That investigation involved the intramuscular injection of New Zealand White rabbits with emulsifications prepared from wild-type and tumorous-head Drosophila melanogaster. The Tumorous-head Drosophia is a mutant strain of fruit fly in which homoetic tumors are produced on the head region. These tumors may take the shape of leg appendages or abdominal, or genital tissue. The protocol of Weihe's (63) research consisted of 5 injections at 10-day intervals. Following a six-week resting period, blood was collected by ear-vein laceration. During these collections, it was noted that the rabbits immunized with the tumerous-head flies showed decreased bleeding times and increased hemolysis in the serum relative to the rabbits immunized with wild-type flies.

Necrotic tissue areas were observed at the injection sites in both rabbit groups. The group immunized against the wild-type antigens possessed tissue that was diffuse and filled with a viscous, pus-like substance at the injection sites. The tumerous-head injected group possessed more localized and indurated tissue at the injection sites (63).

This study involves the relative effectiveness of the clotting mechanism in rabbits following intramuscular injections of homogenates from wild-type and tumourous-head Drosophila melanogaster compared with the saline injected controls.

MATERIALS AND METHODS

New Zealand white rabbits having an average weight of 4 pounds were used in both acute and chronic studies. Each study was subdivided into 3 groups:

1. Tumorous-head injected
2. Wild-Type injected
3. Saline (Control) injected

The following strains of Drosophila melanogaster were used in this study:

1. a tumorous-head mutant strain obtained from Arizona State University, and symbolized Tu-h (ASU)
2. laboratory wild-types, including Oregon R-C, Canton S, Swedish C, and Urbana S, obtained from California Institute of Technology and symbolized W-t.

Experimental cultures were maintained at 25°C in one-half pint milk bottles on freshly prepared standard Drosophila medium containing corn-meal-agar-dried yeast -sucrose-molasses with propionic acid added as a mold inhibitor (64).

The tumorous-head fly suspensions were obtained by emulsifying 100 male and 100 female flies in a tissue grinder containing 4 ml of 0.9% saline solution. Therefore, a dose of 200 Tu-h flies was used per injection. Similarly, the wild-type suspensions were obtained by emulsifying 25 male and 25 female flies from each of the four strains of W-t Drosophila, totaling 200 flies per injection per rabbit, in the 4 ml saline solution. The control group was injected with 4 ml 0.9% saline solution per rabbit. All dosages were

administered by intramuscular injections at the biceps femoris.

Whole blood was collected from all rabbits 10 days after the first injection for the acute study. The chronic study consisted of a series of 5 injections at 10-day intervals with blood collection 10 days after the final injection. The blood was obtained by ear-vein laceration and mixed with sodium oxalate in siliconized test tubes. Approximately 10 ml of each sample was immediately centrifuged at 14,900 g for 20 minutes at -5 to 5+^{o}C for plasma separation and isolation. The remainder was used for various hematological assays.

The following parameters were examined on all blood types:

1. Hematocrit
2. Red blood cell count
3. White blood cell count
4. Platelet count
5. Hemoglobin determination
6. Prothrombin times (plasma)
7. Electrophoresis (plasma)

Hematocrit

Red blood cell volume percentages were obtained from hematocrit values. They were determined by centrifuging blood in heparinized capillary tubes and measuring the packed cell volumes on the critocap chart.

Cell Counts

Red blood cell, white blood cell, and platelet counts were done by coulter counter. Cells were diluted with Isoton at 1:50,000 for RBC's, 1:500 for WBC's, and 1:3000 for platelets. All counts

were adjusted using the coulter counter coincidence correction chart.

Hemoglobin Determination

Hemoglobin concentrations were determined by hemoglobinometer (Coulter Electronics) and expressed in grams percent. The 1:50,000 cell dilution utilized in the red blood cell count was again employed in this hemoglobin determination.

Prothrombin Time

Blood collection and plasma separation were accomplished as previously described. Plasma was promptly removed to another container and stored prior to testing the same day. Reagents used were liquid activated rabbit brain thromboplastin (prothrombin activator) and 0.02M calcium chloride.

The experimental procedure followed for determination of prothrombin times was published by Dade (65). Equal quantities of activated thromboplastin and 0.02M calcium chloride were mixed and the resulting solution was pipetted in 0.2 ml aliquots into each of the desired number of 13 x 100 mm chemically clean test tubes. A small amount of test plasma was warmed at $37^{o}C$ for one minute, followed by forceful blowing of 0.1 ml of the test plamsa into the prewarmed activated thromboplastin-calcium chloride mixture. A timer, calibrated in tenths of a second, was started simultaneously. The tube was quickly shaken and rotated so as to insure mixing. The appearance of the first fibrin web denoted the end point.

Post-Mortem Inspection

All rabbits were sacrificed using sodium pentabarbital (Nembutal) and autopsies conducted following the above examinations. The objective of these studies was two-fold: 1) general inspection of gross appearance of internal anatomy, and 2) general inspection of gross appearance of the injection sites.

Special attention was given to organs concerned with the reticuloendothelial system--the liver and spleen. However, there was inspection of major organs of both thoracic and peritoneal cavities. Injection sites were examined for the reported localized, indurated necrosis versus the diffuse, viscous, purulent necrosis of the Tuh and WT specimens, respectively (63).

Electrophoresis

Plasma samples were diluted in a 1:10 ratio with 0.9% physiological saline. It was found that better banding resolutions could be established with .02 ml plasma containing 70 mg/ml of protein activity. Polyacrylamide gel electrophoresis was carried out on a 5.5% gel prepared from the following solutions mixed per 100 ml of deionized water: 1) two parts solution I [14.0 grams acrylamide (Aldrich) to 0.4 grams N,N^{1}--methylene-bisacrylamide (Isolab)], 2) one part solution II [tri-borate buffer as described later], 3) one part solution III [0.4 grams ammonium persulfate (J. T. Baker)] by volume. Added to the solution was .01 ml of N,N,N^{1},N^{1}-tetramethylethylenediamine. The solution was placed in 75 ml x 5 m ID x 8 mm O.D. electrophoresis tubes to polymerize.

A Buchler tube gel electrophoretic system was used with gel and chamber buffer composed of a tris-borate buffer. The buffer was composed of 2 20 boric acid, 4.31 g Tris (hydroxymethyl) aminomethane and 0.37 g EDTA terrasodium salt per 100 ml of distilled H_2O. The chamber buffer was a 1:4 dilution of the stock gel buffer with a final pH of 8.6. One gram of ultrapure sucrose was added to the 20 ml gel solution to eliminate mixing when water is layered over the surface. One-tenth grams of ultrapure sucrose was dissolved in 1 ml of plasma sample to facilitate layering on gel. Bromophenol blue was used as a marker to observe the migration on the sample. Gels were run at 30 milliamps for two hours at 25°C, and placed in 1% Coomassie blue (BB G-250)--trichloroacetic acid solution overnight. The required washing and destaining was done with 12.5% trichloroacetic acid, with changes performed periodically until the gel was clear.

Statistical Analysis

The animals were grouped according to the type of injection received, that is, the Control (saline), the Wild-Type (WT), or the Tumorous head (Tuh) injected rabbits. A study denotes the quantity of injections prior to assay, that is, acute Study (one injection) and chronic Study (five injections). The mean values of cell counts, prothrombin times, hematocrits, and hemoglobin determination were subjected to unpaired "T" tests (Appendix 1) to determine significant changes between Groups (Control-WT-Tuh). Likewise, within each Study, analysis of variance was applied to determine significant changes in

the following categories (66):

1) Between Groups, (Category A) that is, acute and chronic Control groups versus acute and chronic Wild-Type groups versus acute and Tumorous head groups.

2) Within Groups, (Category B) that is, acute Studies versus chronic Studies.

3) Interactions, (Category AB) that is, acute Control versus chronic Control-acute Wild-Type versus chronic Wild Type-acute Tumorous head versus chronic Tumorous head.

Following post morten inspections, tissue samples from the liver, spleen, and injection site growths were provided for pathophysiologic analysis (67).

RESULTS

The results of the prothrombin times are summarized in Table 4 and figure 4. Mean values ($\bar{x}$) are taken from both acute and chronic studies. The prothrombin times were measured in nine rabbits from each acute Study and eleven rabbits in each chronic Study. The analysis of variance revealed a significant decrease of 13.5% in the Within Groups category with an overall acute mean value of 9.12 seconds compared to 7.89 seconds in the overall chronic study (Appendix 2). There were no significant changes in the Interaction and Between Group categories; however, significant time reductions were observed from acute to chronic means in both WT and Tuh Groups as revealed by T tests (Table 5).

The results of the hematocrits are indicated in Table 6 and figure 5. Hematocrits were taken from nine rabbits in each acute study and eleven rabbits in each chronic study. No significant changes were shown by analysis of variance. Significant differences were revealed between the chronic WT and chronic Tuh and between the acute Control and acute WT as shown by T tests in Table 5. However, the 1.1% decrease from acute Control to acute Wild Type was very minimal. Mean values tended to remain close to the 39.28% Grand Mean of packed red blood cell volume. It is interesting to note a 5.2% increase in hematocrits from acute Tuh to the chronic Tuh (38.78 to 40.78%) in the Interactions category.

Table 4

Mean Values of Prothrombin Times for Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMEROUS HEAD	CONTROL	WILD TYPE	TUMEROUS HEAD
1	-	-	-	8.07	8.27	7.60
2	-	-	-	9.20	9.91	8.30
3	8.70	11.26	10.60	8.40	9.04	8.23
4	9.24	11.00	8.85	9.18	9.02	8.68
5	9.30	8.50	9.20	9.22	8.33	8.35
6	8.82	8.50	8.60	7.55	8.25	6.97
7	8.31	8.05	8.07	7.31	7.09	7.48
8	8.31	8.28	8.23	8.18	7.74	7.31
9	8.74	8.80	9.26	7.70	6.90	7.93
10	9.38	9.10	8.33	7.44	8.00	6.98
11	10.10	9.91	10.84	7.51	7.40	6.82
$\bar{X}$	8.99	9.27	9.10	8.16	8.18	7.70
S	.575	1.20	1.00	.745	.902	.641
S^2	.330	1.41	1.00	.556	.813	.411

Mean values are recorded as time in seconds. T values showed significant reductions from acute to chronic means in both WT and Tuh groups.

Analysis of variance showed significant decreases of the overall acute mean to overall chronic mean.

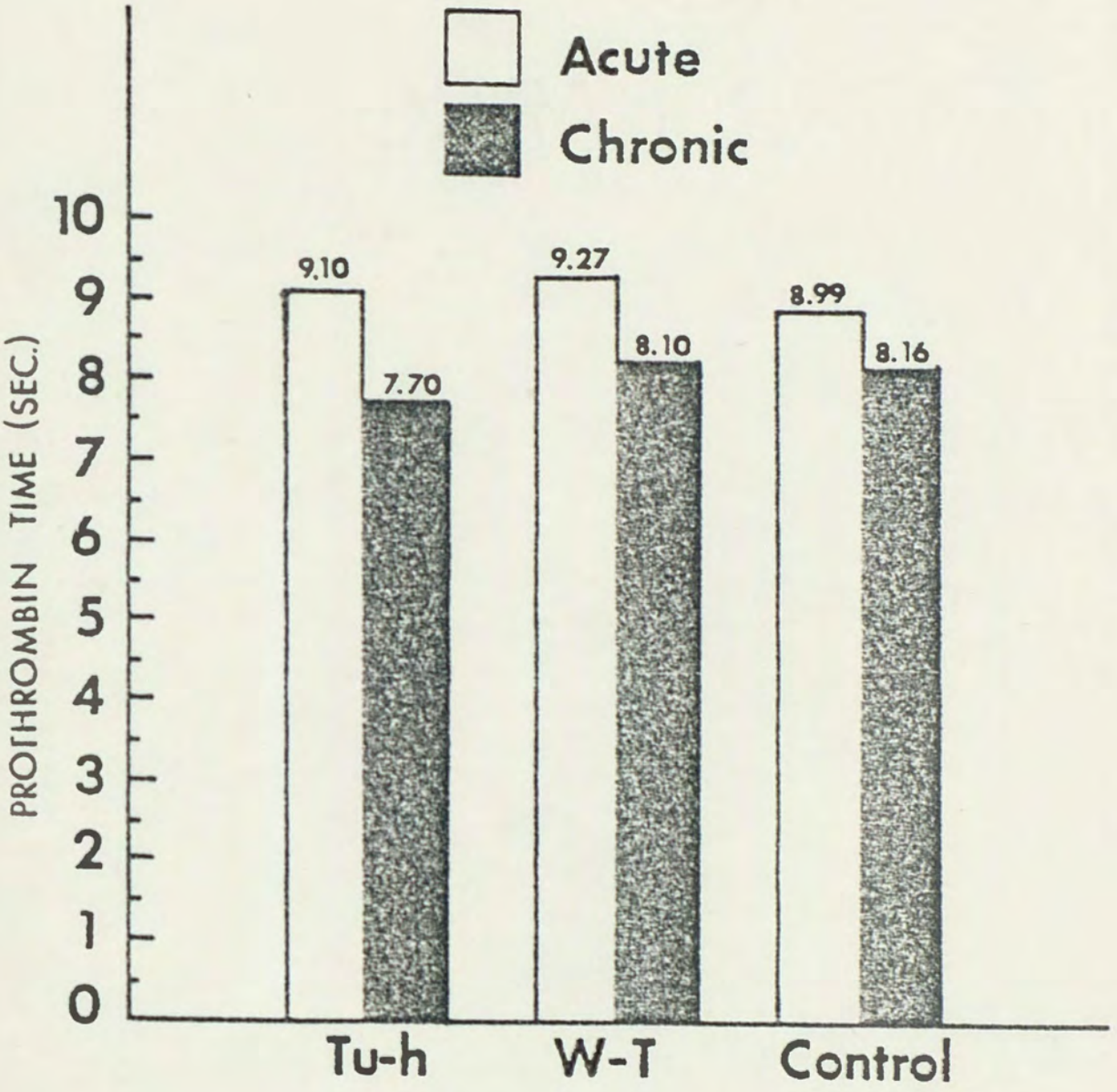


Figure 4. Changes in mean prothrombin times for Tuh, WT, and control animals in acute and chronic studies.

Table 5

List of "T" Values for the Comparisons of Control and Treatment Groups in Both Acute and Chronic Studies

	Acute			Chronic		
	Control vs. Wild Type	Control vs. Tumorous-head	Wild-Type vs. Tumorous-head	Control vs. Wild-Type	Control vs. Tumorous-head	Wild-Type vs. Tumorous-head
Prothrombin Times**	-.6371 (+)	-.2856 (+)	.3284 (-)	- .0567 (+)	1.5514 (-)	1.4384 (-)
Hematocrit	2.0234+(-)	-1.1734 (-)	- .2302 (+)	1.0988 (-)	- 1.4660 (+)	-2.4207*(+)
Hemoglobin Concentration	2.9033*(-)	1.8948*(-)	-1.3844 (+)	- .3671 (+)	- .7917 (+)	- .3766 (+)
Red Blood Cells	17.8760*(-)	9.9920*(-)	-8.7850*(+)	24.0388*(-)	- 3.4338*(+)	-5.4140*(+)
Platelets	-	-	-	- .5344 (+)	-12.8621*(+)	14.5357*(+)
White Blood Cells	-.8457 (+)	-5.7026*(+)	- .0155 (+)	.2751 (-)	- 1.8758*(+)	-7.0627*(+)

Each value represents the calculated "T" for the various groups (see Appendix 1). The * indicates significant changes at the .05 level. Each "T" value is absolute. The (+/-) signs note increases or decreases of mean values.

**T tests were also performed on acute versus chronic tumorous-head and on acute versus chronic Wild-type injected animals for the prothrombin times. Both comparative studies revealed significant reductions in the times.

Table 6

Mean Values of Hematocrits for Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMOROUS HEAD	CONTROL	WILD TYPE	TUMOROUS HEAD
1	-	-	-	42	40	40
2	-	-	-	40	38	40
3	40	36	40	39	38	41
4	41	40	40	41	41	40
5	40	39	37	40	40	42
6	39	37	40	36	35	38
7	42	39	40	41	38	43
8	40	40	40	38	38	38
9	40	40	42	39	39	40
10	40	38	35	38	41	45
11	37	38	35	40	37	40
$\bar{X}$	39.88	38.55	38.77	39.45	38.63	40.63
S	1.4	1.4	2.5	1.7	1.8	2.1
S^2	2.0	2.0	6.2	2.9	3.3	4.3

Mean values are recorded as cell percentage of whole blood. T values demonstrated a significant decrease between acute control and acute Wild-Type and a significant increase from chronic Wild Type to chronic Tumorous head. Analysis of variance showed no significant changes.

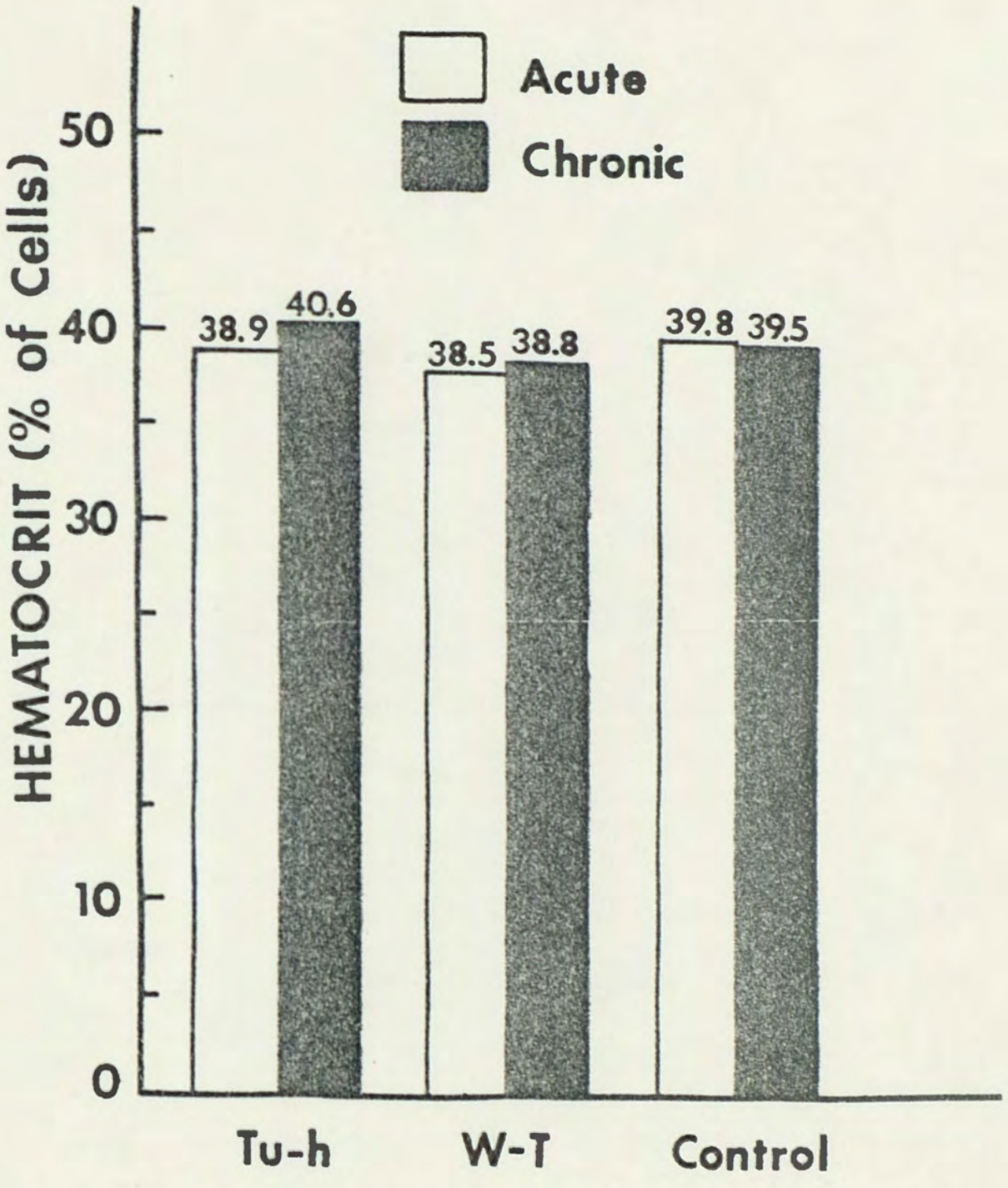


Figure 5. Changes in mean hematocrits for Tuh, WT, and control animals in acute and chronic studies.

The results of the hemoglobin determination are shown in Table 7 and figure 6. Hemoglobin determinations were taken from six rabbits in each acute study and nine rabbits in each chronic study. There were significant changes in the Interactions category (Appendix 2). These results showed a 12.0% decrease from the acute Controls to the chronic Controls (11.25 gm % to 9.90 gm %); an 11.0% increase from the acute WT to chronic WT (8.61 gm % to 9.63 gm %); and a 9.7% increase from the acute Tuh to the chronic Tuh (9.82 gm % to 10.77 gm %). The T tests showed significant decreases of 23.5% and 12.7% in both acute WT and Tuh Groups, respectively, with respect to the Control.

The results of erythrocyte counts are illustrated in Table 8 and figure 7. Erythrocyte counts were taken from six rabbits in each acute study and nine rabbits in each chronic study. The Within Groups category showed a 36.0% elevation of the chronic study mean of $5.97 \times 10^6/mm^3$ compared to the acute mean of $4.39 \times 10^6/mm^3$ (Appendix 2). All categories indicated significant changes as shown by T tests (Table 5). The acute assays showed a 30 and 16.1 % decrease in the mean erythrocyte counts of Wild-type and Tuh groups, respectively, as compared with the Control. There were 20.5% more erythrocytes in the acute Tuh than the acute WT. Chronic assay values showed a 4.5% decrease in WT and a 15.4% increase for the Tuh injected group as compared with the Control, and a 20.8% increase for Tuh compared with WT rabbits.

Table 7

Mean Values of Hemoglobin Determination for Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMOROUS HEAD	CONTROL	WILD TYPE	TUMOROUS HEAD
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	7.50	8.70	7.40
4	-	-	-	9.20	15.40	10.40
5	-	-	-	12.30	9.40	12.10
6	10.40	9.20	9.16	8.00	9.30	8.75
7	12.20	7.70	11.50	11.10	10.90	10.60
8	12.40	10.50	9.80	9.70	11.00	14.00
9	11.05	6.90	11.05	10.40	8.90	12.10
10	12.45	10.55	8.30	10.20	9.10	9.10
11	9.00	6.80	9.10	10.00	8.60	10.10
$\bar{X}$	11.25	8.61	9.82	9.82	10.14	10.51
S	1.38	1.75	1.23	1.47	2.16	2.01
S^2	1.90	3.06	1.52	2.17	4.66	4.02

Mean values are recorded as concentration in grams percent. T values indicated significant decreases in both acute Wild Type and Tumorous head with respect to the acute Control. Analysis of variance showed significant changes in the Interactions category.

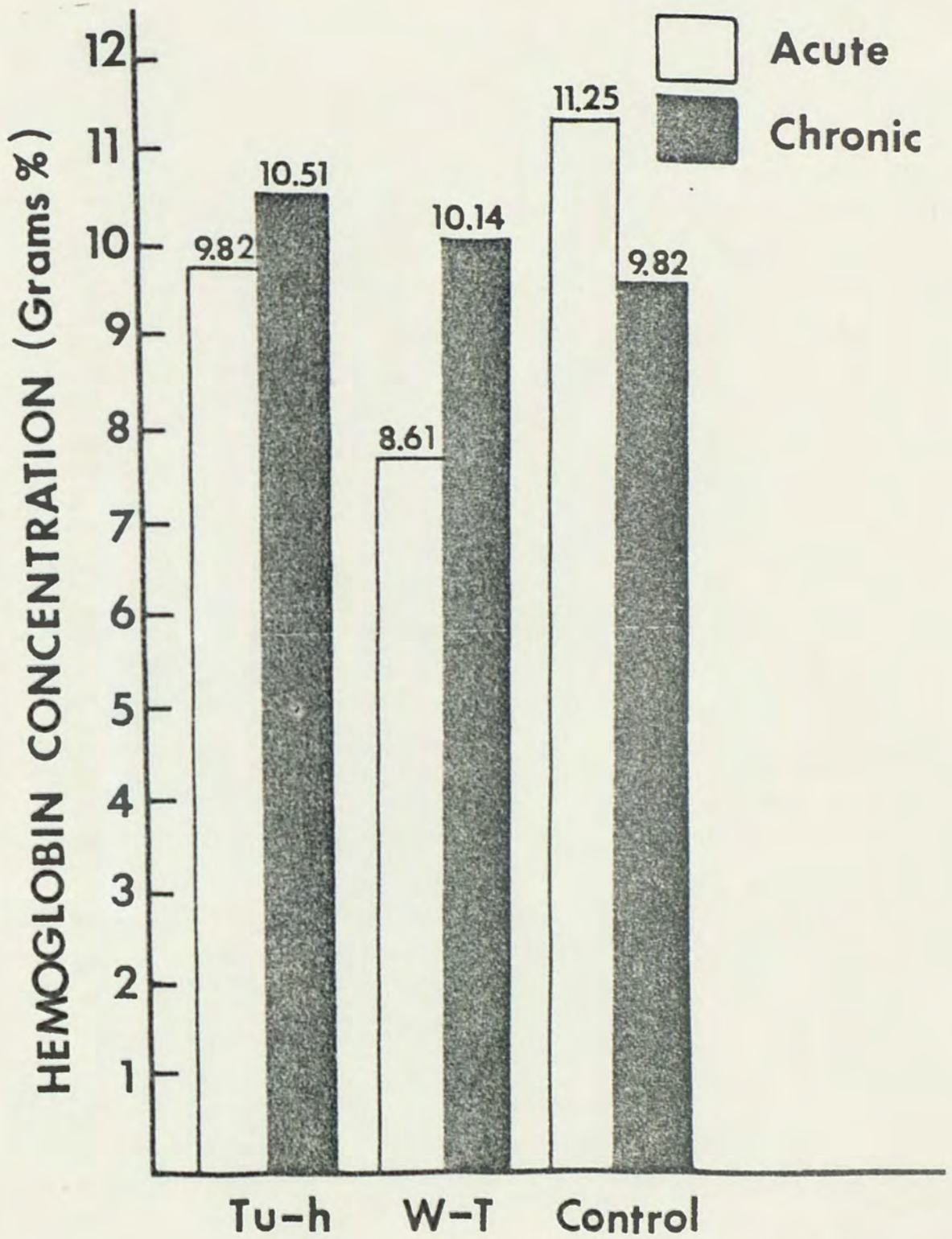


Figure 6. Changes in mean hemoglobin concentrations for Tuh, WT, and control animals in acute and chronic studies.

Table 8

Mean Values of Erythrocyte Count of Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMOROUS HEAD	CONTROL	WILD TYPE	TUMOROUS HEAD
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	3,660,000	4,480,000	3,780,000
4				4,445,000	5,360,000	5,400,000
5				5,476,000	4,660,000	5,475,000
6	5,500,000	3,340,000	4,368,000	4,203,000	4,880,000	4,786,000
7	6,160,000	3,680,000	5,520,000	10,960,000	3,940,000	10,870,000
8	5,459,000	3,520,000	4,507,000	4,310,000	8,536,000	7,980,000
9	4,903,000	4,026,000	4,600,000	5,100,000	4,646,000	5,923,000
10	5,276,000	4,145,000	3,540,000	5,356,000	4,996,000	6,503,000
11	3,863,000	2,990,000	3,620,000	4,806,000	4,636,000	5,016,000
$\bar{X}$	5,193,000	3,616,833	4,359,166	5,368,444	5,126,000	6,192,555
S	769,413.0	431,115.0	727,305.8	2,177,031	1,335,657	2,108,572
S^2	5.9×10^{10}	1.8×10^{10}	5.3×10^{10}	4.7×10^{11}	1.8×10^{11}	4.4×10^{11}

Mean values are recorded as erythrocytes per cubic millimeter of blood. T values revealed significant decreases of both acute and chronic Wild Type rabbits. In addition, T values demonstrated significant increases of both acute and chronic Tumorous head, as compared to the respective Wild Types and the chronic Tumorous head group and respect to the Control. Analysis of variance showed a significant increase of overall chronic mean with respect to overall acute mean.

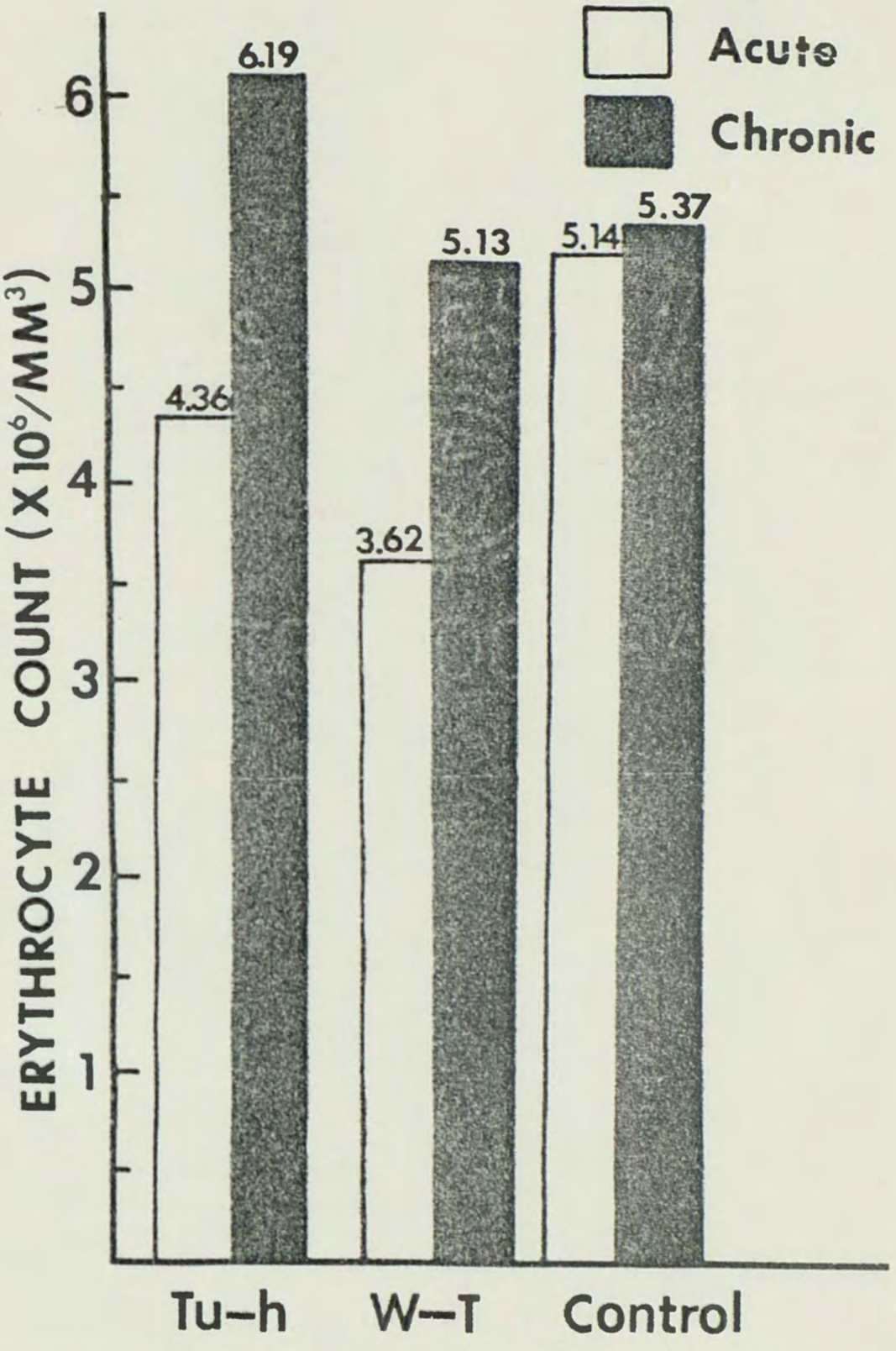


Figure 7. Changes in mean erythrocyte count for Tuh, WT, and chronic studies.

The results of the thrombocyte count are shown in Table 9 and figure 8. Only chronic studies were performed using five rabbits in the Control groups, four rabbits in the WT groups, and six rabbits in the Tuh groups. Mean values and T tests (Table 5) indicate a marked increase in thrombocytes in the Tuh Group. A hyper-elevated 501.2% increase of Tuh compared with the Control groups and a 432.2% increase of Tuh compared with WT shows a thrombocytotic tendency. Analysis of variance was not applied to this assay.

Table 10 and figure 9 show the results of leukocyte mean values. Leukocyte counts were taken from six rabbits in the acute Study and nine rabbits in the chronic Study. No significant changes were indicated by analysis of variance; however, a 19.4% leukocyte elevation can be seen from the overall acute mean to overall chronic mean (Appendix 2). These changes were indicated by T tests in Table 5. They were a 15.2% increase for the acute Tuh Group compared with acute Controls, a 30.5% increase for the chronic Tuh compared with chronic Controls, and a 30.5% increase for chronic Tuh compared with chronic WT.

The results of the post-mortem inspections are summarized in Tables 11 and 12.

The qualitative examination of the electrophoretic banding resolutions revealed no major consistent visual differences between the injected groups.

Table 9

Mean Values of Thrombocyte Count for Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMOROUS HEAD	CONTROL	WILD TYPE	TUMOROUS HEAD
1				-	-	-
2				-	-	-
3				-	-	-
4				-	-	-
5				-	-	-
6				-	-	781,720
7				98,760	122,180	346,720
8				204,040	-	521,060
9				64,840	93,720	360,060
10				88,800	114,760	575,900
11				66,060	154,120	557,060
$\bar{X}$				104,500	121,195	523,770
S				57,528.7	25,042.0	160,301
S^2				3309549600	627103566	25646714857

Mean values are recorded as thrombocytes per cubic millimeter of blood. T values showed significant increases of chronic Tumorous head with respect to both chronic control and Wild Type. Analysis of variance was not applied.

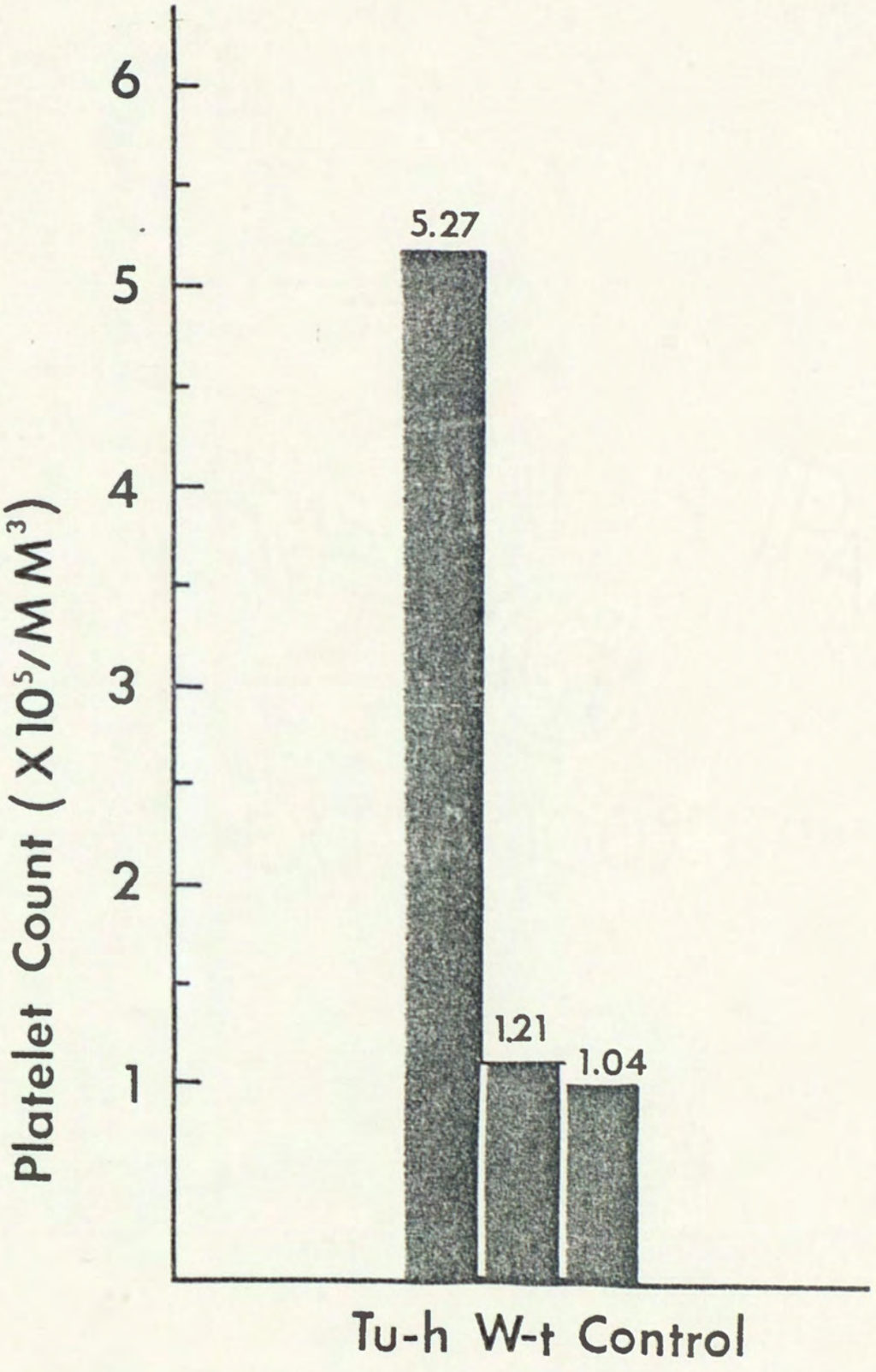


Figure 8. Changes in mean plasma thrombocyte count for Tuh, WT, and Control animals in chronic studies.

Table 10

Mean Values of Leukocytes for Both Acute and Chronic Assays

	ACUTE			CHRONIC		
Animal no.	CONTROL	WILD TYPE	TUMOROUS HEAD	CONTROL	WILD TYPE	TUMOROUS HEAD
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	10,100	9,009	15,150
4	-	-	-	7,501	9,509	9,926
5	-	-	-	7,344	7,962	9,730
6	5,070	7,664	3,263	7,163	8,881	4,786
7	6,000	9,034	10,600	13,185	8,725	9,073
8	5,717	8,804	6,945	7,655	9,023	12,962
9	6,646	2,803	4,882	4,577	6,168	9,762
10	4,788	5,252	9,036	5,785	3,397	11,033
11	6,584	6,413	5,381	6,821	4,704	9,092
$\bar{X}$	5,081	6,662	6,685	7,792	7,486	10,168
S	766.1	2,373	2,741	2,510	2,198	2,852
S^2	586,860	5,6 32,106	7,516,666	6,302,277	4,834,839	8,139,119

Mean values are recorded as leukocytes per cubic millimeter of blood. T values showed significant increases of both acute and chronic Tumorous head with respect to the Controls and chronic Tumorous head as compared to chronic Wild Type. Analysis of variance revealed no significance.

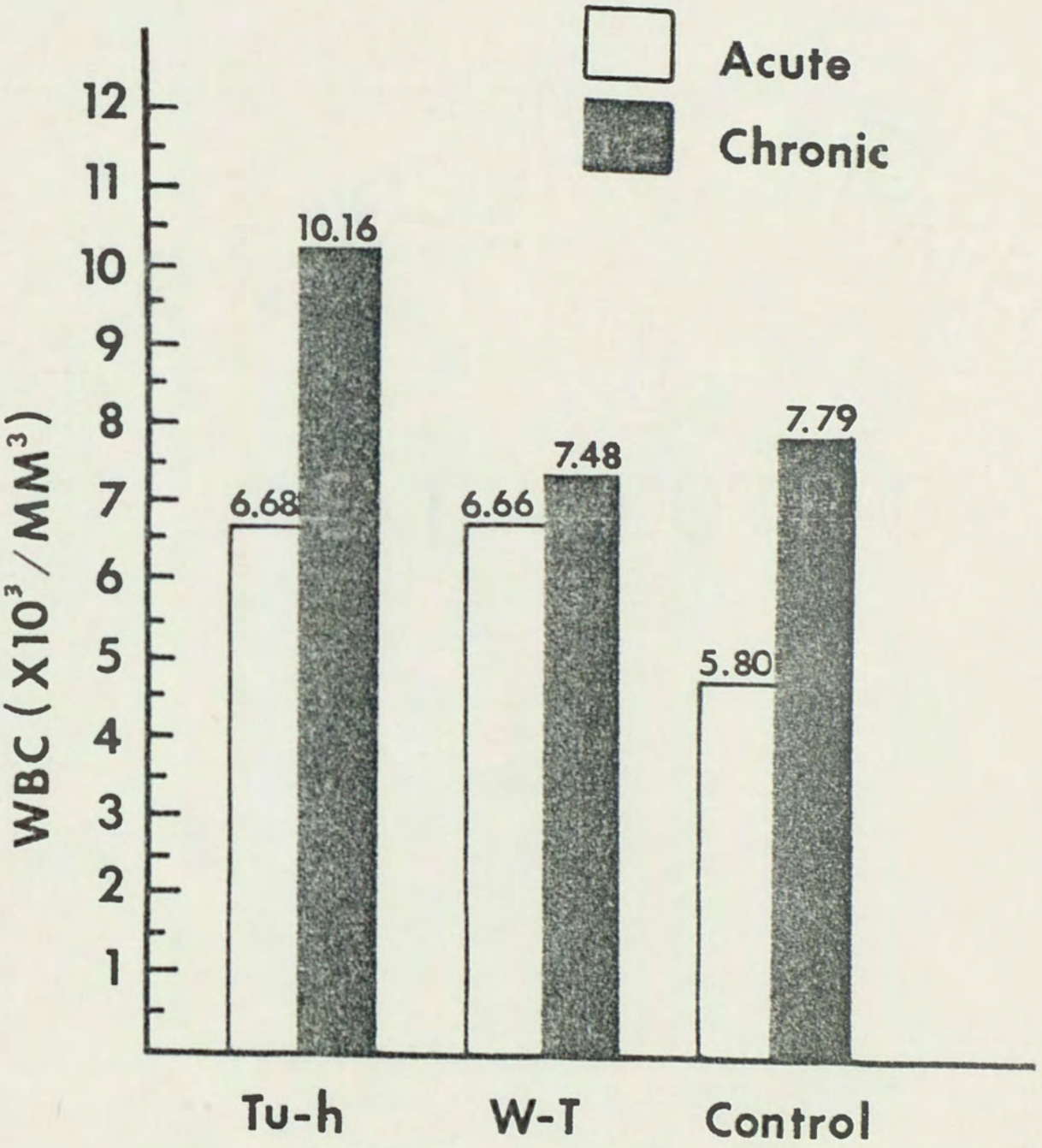


Figure 9. Changes in mean leukocyte count for Tuh, Wt, and Control animals in acute and chronic studies.

Table 11

Post-Mortem Inspection

Injection Types	No. of Rabbits	Injection Sites	Gross Inspection: Spleen	Gross Inspection: Liver	Gross Inspection: Other
Tu-h	11	9 Indurated 1 Purulent 1 Normal	2 Discolorations 9 Normal	1 Indurated modular growth 10 Normal	1 Excessive pooling in abdominal cavity
W-T	11	8 Indurated 2 Purulent 1 Normal	2 Discolorations 9 Normal	2 Discolorations 9 Normal	1 Abnormally red caecum
Saline	11	11 Normal	11 Normal	11 Normal	11 Normal

Table 12

Post-Mortem Inspection

	Tu-h		W-T		Saline	
Group	Injection Site	Gross Inspection	Injection Site	Gross Inspection	Injection Site	Gross Inspection
1	Indurated growth	Normal	Indurated growth	Normal	Normal	Normal
2	Indurated	Normal	Indurated	Normal	Normal	Normal
3	Indurated	Normal	Purulent	Massive liver discoloration (3/4 liver brown-ish-gray); abnormally red caecum	Normal	Normal
4	Indurated	Normal	Indurated	Spleen discolor-ation (spleen spotted and dark red areas	Normal	Normal
5	Indurated	Spleen discolor-ation (spleen spotted and dark red areas	Indurated	Spleen discolor-ation (spleen spotted and dark red areas	Normal	Normal
6	Indurated	Normal	Indurated	Normal	Normal	Normal

Table 12 (continued)

	Tu-h		W-T		Saline	
Group	Injection Site	Gross Inspection	Injection Site	Gross Inspection	Injection Site	Gross Inspection
7	Purulent	Spleen abnormally dark; indurated nodular growth on lateral lobe of liver	Indurated	Normal	Normal	Normal
8	Normal	Normal	Indurated	Massive liver discoloration (3/4 liver brown-ish-gray)	Normal	Normal
9	Indurated	Normal	Purulent	Normal	Normal	Normal
10	Indurated	Excessive blood pooling in abdominal cavity	Normal	Normal	Normal	Normal
11	Indurated	Normal	Indurated	Normal	Normal	Normal

DISCUSSION

The initial laboratory evaluation was designed to determine the mechanism utilized in reducing the bleeding times reported in Tuh injected rabbits. It appeared that these changes could have been influenced by vascular, platelet, or coagulation anomalies. Information concerning which processes may be involved were provided primarily by prothrombin times and platelet counts, although additional tests were performed to provide further information concerning possible coagulation changes or other hematological alterations.

The overall prothrombin times were significantly decreased from acute to chronically injected animals as shown by analysis of variance. In addition, prothrombin times showed significant reductions from both acute WT and Tuh groups to chronic WT and Tuh, respectively (Table 5). These findings suggest a slight rate change of the extrinsic and common pathways, that is, Factors V, VII, X, prothrombin, and fibrinogen (see figure 2). In general, the clotting time is shortened as coagulant activity increases, and is detected by the appearance of fibrin threads. This relationship is such that small variations in clotting times represent major differences in activity when the times are of short duration. Compared to other coagulation test systems, the prothrombin test can have a more efficient sensitivity because of its comparatively short time duration. The significant reduction of prothrombin times from acute WT and Tuh

to chronic WT and Tuh rabbits does indicate increased overall activity of the extrinsic system. When activated prothrombin was added to plasma, the plasma Factor VII reacted with the tissue factor to form a reaction product which possibly accelerated the conversion of Factor X to its activated form, Factor Xa. This, in turn, reacted with Factor V, calcium, and tissue phospholipid to form the extrinsic prothrombin activator that converted the prothrombin to thrombin. Thrombin then converted the fibrinogen to fibrin. The rate of fibrin formation is dependent on the concentration of the five extrinsic factors previously mentioned. This suggests a possible increased factor concentration in both WT and Tuh injected rabbits probably one or more of the reactions prior to Factor X formation due to their rate limiting nature. As seen in Table 6, the significant differences of prothrombin test mean values observed between both acute and chronic Tuh are greater than those for acute and chronic WT rabbits. This greater difference may be caused by the increased platelet factor concentration in the Tuh group (Table 9). This factor concentration increase is possibly due to excessive platelet thromboplastic function resulting from the observed thrombocytosis, that is, thrombocyte counts over 400,000/mm^3 (Tables 2 and 9). The possible acceleratory factors as aforementioned are platelet factors 1 through 4. These platelet constituents respectively contribute 1) Factor V through the extrinsic system, 2) a clotting accelerating protein that further potentiates platelet aggregation and counteracts the inhibitory antithrombin III, 3) the

platelet phospholipid which accelerates blood coagulation by participating in reactions involving Factor VIII and Factor V, 4) and the glycoprotein which further aids in accelerating coagulation (see pages 6 and 7). The increase of platelet phospholipid concentration in particular has the effect of concentrating the various coagulation factors in a localized area, leading to faster reaction rates. An increase in serotonin released as a result of the high platelet concentration could have added vasoconstriction effects.

Mean hematocrit values tended to remain about the same, revealing no major changes in blood viscosity. However, a slight elevation of the packed cell volume in chronic Tuh groups relative to acute Tuh groups was noted. The reason seems to be due to an increased red cell mass that could be associated with a slightly higher blood viscosity.

Changes in mean hemoglobin values indicate significant fluctuations between acute and chronic studies within the injected groups. The hemoglobin concentration in both the chronic WT and Tuh groups increased significantly over that of their respective acute study groups. There was also a significant overall increase in the mean erythrocyte count in the chronic study as compared to its acute study. This may represent a physiologic compensatory response to a tissue hypoxia indicated by the relatively low hemoglobin concentration in the acute rabbits. The elevation of erythrocytes appears to be a facilitated mutual adjustment so as to enhance oxygen transport, thus elevating the hemoglobin concentration.

Significant eythrocyte elevation in the chronic Tuh groups is consistent with the concomitant increase in hemoglobin concentration in that group. The erythrocyte content in both Tuh group studies was significantly greater than the respective WT group studies. It has been previously mentioned (page 12) that increased erythrocyte production is associated with increased thrombocyte production. Accelerated thrombopoiesis commonly is associated with accelerated erythropoiesis (49).

There was no significant difference in the hemoglobin concentration between the chronic WT and Tuh rabbits. However, there was a significant increase in red blood cell production by the Tuh injected rabbits. This rise in erythrocyte count in the chronic Tuh group was probably due to the proliferation of immature erythrocytes. The immature cells were probably those during the developmental period at which synthesis of hemoglobin is incomplete. Erslev and Gabuzda (60) note that the formation of hemoglobin begins at the earliest precursor stage of the developing erythroid cell and is completed when the anucleate reticulocyte matures to an erythrocyte. Synthesis begins just after the basophilic erythroblast stage.

Leukocyte elevations were significant in both acute and chronic Tuh groups as compared to their Controls. These tests also revealed significant increases of chronic Tuh over chronic WT groups. Increases in both WT and Tuh groups are normal responses, probably due to an increase in the counter antigens.

The data tend to support a reactive thrombocytotic mechanism

for increased platelet count especially the lack of any observed prolonged bleeding time (Table 2). Conclusive evidence of thrombocytosis of the autonomous or reactive nature was not available because qualitative thrombocyte studies have not been performed. However, slight increases in erythrocytes and leukocytes, and the association of the injected tumor element (Table 2 and page 12) all give added credence to a reactive response. The homogenates may possibly accelerate thrombopoiesis which is also associated with accelerated erythropoiesis suggesting an interrelationship between erythropoietic control mechanisms. Though little is known about the factors leading to accelerated platelet production in the various disorders associated with accelerated reactive platelet production, it is established that thrombopoietin does affect the number of megakaryocytes found in the bone marrow and their proliferative behavior. As previously mentioned, such stimulation increases the rate of megakaryocyte maturation resulting from an increase in number of mitosis within the individual cells, and possibly an increase in the size of thrombocytes appearing in circulation. Thrombocyte turnover and production rate must be accelerated, yielding a total thrombocyte mass increase. Thrombocyte life-span or survival times usually are not affected by thrombocytosis further suggesting that the mechanism is over production rather than prolonged life-span.

Post-mortem pathophysiologic analysis (67) revealed the absence of splenomegaly (Table 2). Thromboembolism or hemorrhage was not apparent. These facts coupled with relatively little

leukocyte increase, and concomitant increases in erythrocytes were evident. All these factors support reactive thrombocytotic tendency (Table 2).

The more effective occlusion of the trauma site by the increased number of platelets could be an obvious explanation for any observed reduction in bleeding time.

Autopsies were performed on 11 animals from each injection group. Post-mortem inspections indicated only four spleen discolorations (two Tuh and two WT), two Wt injected rabbit liver discolorations, and irregular indurated and purulent injection sites. There appeared to be little or no visual correlation between these discolorations, or the nature of injection sites as compared with abnormal cell counts or prothrombin times. Nothing conclusive can be derived from these findings. However, pathophysiologic analysis on three rabbit livers, one from each injection group, revealed aseptic centrilobular hepatolysis in the Tuh rabbit specimen. Microscopic analysis showed central necrosis, large zones of hemorrhagic necrosis, and disappearance of cells. Spleens were normal. Injection sites, on biopsy, displayed no apparent abnormal cells (67).

Future investigations should include thrombocyte qualitative functions such as platelet adhesive tests, platelet aggregation tests, platelet factor 3 availability tests, and bone marrow aspirations. These tests will determine if the thrombocytosis is the reactive or autonomous type. Acquired qualitative thrombocyte defects have been described in all the myeloproliferative disorders

and cannot be ruled out even though the hemostatic effects of the thromocytes appear effective. The basic defect or defects which underlie the abnormalities in thrombocyte function in the myeloproliferative syndromes remain to be determined. While these disorders have many features in common, the defects may not be identical in all cases. These functional defects usually involve aggregation, adhesion, and/or secretion dysfunctions.

SUMMARY

The hemostatic mechanism of rabbits appeared to have been disturbed in response to tumorous-head Drosophila melanogaster antigens.

The blood coagulation responses of two groups of rabbits, one group injected with wild-type Drosophila melanogaster and one group injected with a tumorous-head strain, had somewhat similar reductions in clotting times as compared to a control group. This indicates that the tumorous-head injected animals do not appear to have a peculiar accelerated coagulation mechanism as compared with wild-type injected animals.

However, rabbits injected with tumorous-head antigens had a radical 5-fold elevation in plasma platelet concentration as compared to rabbits injected with the wild-type antigens as well as control groups. This aids explanation for a previous speculated reduction in bleeding time as observed in the tumorous-head injected rabbits. The collected hematological data tend to be supportive of a reactive thrombocytosis as compared with an autonomous reaction. The precise accelerated thrombokinetic mechanism for the excessively high increase in platelets remains for future investigations.

APPENDIX 1: STATISTICAL ANALYSIS

APPENDIX 1

STATISTICAL ANALYSIS

One of the procedures used to analyze the results of the various hematological assays was the unpaired "T" test. The "T" test is used in the statistics of small samples with unknown standard deviations. The quantity t is given by the equation,

$$t = \frac{\bar{x} - \mu}{S\sqrt{n}}$$

where

$\bar{x}$ = Sample mean

S = Standard deviation

μ = Population mean

n = Number

That is, t is the deviation of the estimated mean from that of the population, measured in terms of $s/\sqrt{n}$ as the unit.

The unpaired "T" test is a derivative of the "T" test and is used when the same individuals are not subjected to each treatment. In this case, the quantity t is given by the equation,

$$t = \frac{\bar{x}_1 - \bar{x}_2}{S\bar{x}_1 - x_2}$$

where

$\bar{x}_1$ = Mean of 1st Group

$\bar{x}_2$ = Mean of 2nd Group

$S\bar{x}$ = Standard error

T Test Calculations for Prothrombin Times

	Pooled Variance	Standard Error	Unpaired "T" Distribution
Control vs. Wild-Type (acute)	$Sp^2=\frac{.3304(8)+1.4083(8)}{16}=0.8694$	$Sx_1-x_2=\sqrt{.8694(.2222)}=.4395$	$t=\frac{8.99-9.27}{.4395}=-.6371$
Control vs. Tumorous head (acute)	$Sp^2=\frac{.3304(8)+1.0045(8)}{16}=0.6675$	$Sx_1-x_2=\sqrt{.6675(.2222)}=.3851$	$t=\frac{8.99-9.10}{.3851}=-.2856$
Wild-Type vs. Tumorous head (acute)	$Sp^2=\frac{1.4083(8)+1.0045(8)}{16}=1.2064$	$Sx_1-x_2=\sqrt{1.2064(.2222)}=.5177$	$t=\frac{9.27-9.10}{.5177}=.3284$

T Test Calculations for Prothrombin Times (continued)

	Pooled Variance	Standard Error	Unpaired "T" Distribution
Control vs. Wild-Type (chronic)	$Sp^2=\frac{.5559(10)+.8134(10)}{16}=0.6847$	$Sx_1-x_2=\sqrt{.6847(.1818)}=.3528$	$t=\frac{8.16-8.18}{.3528}=-.0567$
Control vs. Tumorous head (chronic)	$Sp^2=\frac{.5559(10)+.4113(10)}{20}=0.4836$	$Sx_1-x_2=\sqrt{.4836(.1818)}=.2965$	$t=\frac{8.16-7.70}{.2965}=1.5514$
Wild-Type vs. Tumorous head (Chronic)	$Sp^2=\frac{.8134(10)+.4113(10)}{20}=0.6124$	$Sx_1-x_2=\sqrt{.6124(.1818)}=.3337$	$t=\frac{8.18-7.70}{.3337}=1.4384$

APPENDIX 2: ANALYSIS OF VARIANCE

APPENDIX 2

ANALYSIS OF VARIANCE

Often a researcher can use a single experiment advantageously to study two or more different kinds of treatment. Analysis of variance rests on a separation of the variance of all the observations into parts, each of which measures variability attributable to some specific source.

Analysis of Variance for Prothrombin Times
ℓ = .05

			F Ratio	F Values (significance < .05)
All Animals	Grand Mean = 8.50			
Between Groups (A)	Control Mean = 8.52 WT Mean = 8.62 Tuh Mean = 8.37		0.296	0.746
Within Groups (B)	Acute Mean = 9.12 Chronic Mean = 7.89		42.656	0.000*
Interactions (AB)	Control	Acute = 8.99 Chronic = 8.10	0.690	0.511
	WT	Acute = 9.27 Chronic = 7.97		
	Tuh	Acute = 9.10 Chronic = 7.63		

*denotes significant values

Analysis of Variance for Hematocrits
ℓ = .05

			F Ratio	F Values (significance < .05)
All Animals	Grand Mean = 39.28			
Between Groups (A)	Control Mean = 39.50 WT Mean = 38.55 Tuh Mean = 39.78		2.201	0.132
Within Groups (B)	Acute Mean = 39.10 Chronic Mean = 39.48		0.580	0.454
Interactions (AB)	Control	Acute = 39.88 Chronic = 39.11	2.390	0.115
	WT	Acute = 38.55 Chronic = 38.55		
	Tuh	Acute = 38.78 Chronic = 30.78		

*denotes significant values

Analysis of Variance for Hemoglobin Determination
ℓ = .05

			F Ratio	F Values (significance < .05)
All Animals	Grand Mean = 10.00			
Between Groups (A)	Control Mean = 10.57 WT Mean = 9.12 Tuh Mean = 10.30		2.514	0.114
Within Groups (B)	Acute Mean = 9.89 Chronic Mean = 10.10		0.305	0.589
Interactions (AB)	Control	Acute = 11.25 Chronic = 9.90	4.194	0.036*
	WT	Acute = 8.61 Chronic = 9.63		
	Tuh	Acute = 9.82 Chronic = 10.77		

*denotes significant values

Analysis of Variance for Erythrocyte Count
ℓ = .05

			F Ratio	F Values (significance < .05)
All Animals	Grand Mean = 5.18			
Between Groups (A)	Control Mean = 5.49 WT Mean = 4.44 Tuh Mean = 5.60		1.439	0.268
Within Groups (B)	Acute Mean = 4.39 Chronic Mean = 5.97		12.019	0.003*
Interactions (AB)	Control	Acute = 5.19 Chronic = 5.79	1.443	0.267
	WT	Acute = 3.62 Chronic = 5.27		
	Tuh	Acute = 4.36 Chronic = 6.85		

*denotes significant values

Analysis of Variance for Leukocytes
ℓ = .05

All Animals	Grand Mean = 7.00		F Ratio	F Values (significance < .05)
Between Groups (A)	Control Mean = 6.67 WT Mean = 6.54 Tuh Mean = 7.79		0.577	0.574
Within Groups (B)	Acute Mean = 6.38 Chronic Mean = 7.62		3.508	0.081
Interactions (AB)	Control	Acute = 5.80 Chronic = 7.53	0.724	0.501
	WT	Acute = 6.48 Chronic = 6.60		
	Tuh	Acute = 6.86 Chronic = 8.72		

*denotes significant values

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