Backbone Title

STUDIES ON VASCULAR FRAGILITY - N. F. GANG

# STUDIES ON THE MECHANISM OF VASCULAR FRAGILITY

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science.

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August, 1965.

## ACKNOWLEDGEMENTS

The present study was carried out in the Department of Biochemistry, McGill University under the direct supervision of Professor O.F. Denstedt. I wish to express my sincere gratitude for his unlimited help, advice, stimulating criticism and suggestions during the investigation and in the preparation of the manuscript.

I am deeply indebted to Dr. M. Saffran for valuable criticism during the first part of the study.

I am grateful to Dr. D. Rubinstein, Dr. E.A. Hosein and Dr. J.H. Spencer of the Department and to my student colleagues for helpful criticisms during seminars, and throughout the course of the investigation.

I wish to thank Professor Yves Clermont of the Department of Anatomy, McGill University, for his expert advice concerning the histochemical part of the study, and Dr. C.H. Stary of the Department of Pathology for assistance in the interpretation of the histological slides.

I am indebted to Dr. D. Kahn, Director of Laboratories at St. Mary's Memorial Hospital, Montreal, for providing laboratory space and facilities during our study of hemophilia and to Dr. Cecil Harris, Director of the Department of Hematology, for making available blood samples from normal and hemophilic patients, and for his helpful interest in the study.

I am especially grateful to my wife for her help, patience and encouragement throughout the study and the preparation of the thesis.

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# PART ONE

# PREFACE

The volume of literature on the morphology and physiology of the vasculature is enormous. The concepts of the structure and activity of the blood vessels have changed greatly in the past fifteen years with the availability of revolutionary tools such as the electron-microscope, and the tremendous advancement in the knowledge of physiology and metabolism.

Nowhere has the change in concept been more dramatic than in the understanding of the structure and physiological behaviour of the small vessels - the capillaries and precapillaries.

While the literature is extensive on the physiological aspects of the blood vessels, there is a notable lack of knowledge on the biochemical side. This situation led Dr. O.F. Denstedt of the Department of Biochemistry at McGill University to explore the literature from the chemical point of view, and to initiate a programme of research into the biochemical changes involved in the development of vascular fragility and spontaneous internal bleeding.

The first graduate student to participate in the programme - H.B. Sells - made a remarkably comprehensive survey of the widely scattered and uncorrelated literature pertaining to the biochemical aspects of capillary fragility and spontaneous internal hemorrhage. To our knowledge it represents the first effort to assemble and correlate the heterogeneous body of experimental

data on the vasculature from the chemical point of view. The bibliography comprises part of his Ph.D. thesis (I).

The present author has extended Sells's study to encompass the biochemical mechanism of connective tissue breakdown in relation to vascular instability.

From the vantage point of biochemistry the numerous hemorrhagic diseases appear to fall into two categories: one, in which disturbance of connective tissue metabolism is a prominent involvement and the second group, associated predominantly with differences in the clotting properties of the blood. The following is a list of some of the conditions that are accompanied by vascular fragility and bleeding.

1. Vascular disorders associated with primary involvement of the small vessel wall and connective tissue ground substance:

   Hereditary hemorrhagic teleangiectasias
   Allergic purpuras
   Thromboasthenia
   Scurvy
   Collagen diseases
   Hypothyroid myxoedema
   Paraproteinaemias
   Anoxia
   Erosion of vessels
   Terminal stages of malignant diseases
   Poisoning (chemical, vegetable, animal toxins)
   Autoerythrocyte sensitization
   Acute infections (bacterial toxins)
   Uraemia
   Shock
   Burns
   Magnesium deficiency
   Cold injury (without freezing)
   Virus infections

Lathyrism
Hemorrhagic fever
Purpura senilis
Ehlers-Danlos Syndrome
Radiation damage

2. Vascular disorders associated with primary deficiencies of the blood clotting system:

Thrombocytopenia (hereditary, poisoning, malignancy)
Thromboplastin deficiency
    hemophilia, Christmas disease
Prothrombin deficiency
    liver diseases, dicumarol poisoning, vitamin K deficiency
Proaccelerin deficiency (congenital)
    liver damage, malignancy
Proconvertin deficiency
Fibrinogen deficiency (congenital)
    liver damage
Hemorrhagic states associated with conversion of prothrombin to thrombin
Hemorrhagic states associated with circulating anticoagulants (antithrombin, antithromboplastin)
Increase in ~~anti~~fibrinolysin (shock, anaphylaxis, etc.)
Fibrinase deficiency (hereditary)

To classify the hemorrhagic diseases into the two categories may not be completely accurate, but it suffices to give perspective to the problem.

It is evident that there must be a biochemical connection between the two categories of diseases since they apparently involve a common mode of vascular breakdown. What the relationship is between the state of the wall of the small blood vessels and the state of blood coagulability is not yet known, and the elucidation of this question is one of the ultimate aims of our research.

# GENERAL INTRODUCTION

The most obvious function of the hemostatic mechanism is to prevent blood loss from the vasculature in the event of external or internal injury. Until recently the primary role within the framework of hemostasis was considered to be the activation of the blood clotting mechanism.

In certain individuals with a defective coagulation system resulting from deficiency of one or other of the blood clotting factors, as in hemophilia, physical injury may result in excessive and even fatal, external or internal blood loss if treatment (infusion of plasma) is not promptly administered. These individuals, even without exposure to physical trauma, are prone periodically to internal bleeding, usually into the joints. In these individuals the level of the deficient factor in the blood tends to fluctuate and only when it falls to a low value do the small vessels, usually in the working parts of the extremities - in the knee, ankle, elbow or wrist - , become 'fragile' and permit the escape of blood into the tissue spaces. Little is known about the conditions which initiate the periodic bleeding episodes. It is of particular biochemical interest that the infusion of large quantities of plasma or plasma derivatives, which contain the needed blood clotting factor, is necessary to arrest the bleeding.

In order to ascertain the biochemical changes involved in the development of vascular fragility and in the process of hemostasis, it is essential to have a correct appreciation of the structure of the small vessels and the

dynamics of their function.

One can have nothing but praise and admiration for the pioneers who built up a remarkable though incomplete body of knowledge of the capillary vessels,having the microscope as their only aid. Until as recently as a decade ago progress was retarded by the incorrectness and inadequacy of the concepts that prevailed about the fine structure of the small vessels, intercellular 'cement', 'stickiness' of the membrane surface, 'permeability', 'pores', the common sites where spontaneous bleeding occurs and other details. With the rapid extension of the application of the electronmicroscope, the micromanipulator and radioautography, revolutionary changes have taken place in many concepts. In some instances these aids have substantiated the older hypotheses.

The concept of hemostasis is becoming more and more complex. It now takes into consideration the coagulation system of the blood, the state of the endothelium and the supporting connective tissue, metabolism, hormonal and immunological influences, the magnitude and nature of the injury and many other factors.

Among the serious obstacles to the biochemical study of hemostasis in the past has been the lack of a suitable method for obtaining a reproducible hemorrhagic state in an experimental animal. Numerous workers have used the standardized application of negative pressure to the skin of animals or to the surface of the cheek pouch of the hamster and counting the number of petechiae produced. The administration of toxic substances and the ex-

posure of animals to radiation also have been used. None of these methods has been found to have any merit for biochemical investigations.

We have been fortunate in evolving a suitable method for the experimental production of hemorrhage in the rat. This has enabled us to study the biochemical aspects of vascular breakdown.

# SURVEY OF THE LITERATURE

## 1. THE CONCEPT OF THE 'CAPILLARY'.

The arteriovenous circulation was first described in the 16th century by William Harvey in his famous treatise De Motu Cordis. Less than half a century later Marcello Malphighi described the circulation of the blood through 'hair like' tubes or 'capillaries' (capillus, hair).

The capillary vessel normally is about 7 microns in diameter. Its wall consists mainly of two structural units (2,3):

(a) a mosaic-like lining of endothelial cells, and

(b) an outer layer, the pericapillary sheath composed of supporting reticulin fibers and condensed connective tissue ground substance.

Each of these components is known to be heterogeneous in makeup (4). From the evidence of research accumulated in the past ten years it is evident that there is a wide variation in structure as well as in regulation (5) and response (6) of the capillaries in different organs and also between species. Each organ having a special function, the small vessels show evidence of adaptation to serve the function (7).

### (1) Classification of Kisch

According to the studies of Kisch (8) with the aid of the electronmicroscope, the capillaries are of three types: (a) the "protocapillaries", the smallest vessels, consisting of a succession of single endothelial cells,

each curved so that the opposite edges make contact to form a tube, (b) the "mosaic capillaries" composed of two or more endothelial cells which span the capillary circumference in a mosaic fashion, and (c) the pre- and the postcapillaries consisting of endothelial mosaic, surrounded by smooth muscle elements.

The inner membrane (membrana interna) of the endothelial cells has a rippled surface. Kisch observed microvilli or tentacles projecting from this surface. These features are especially abundant in the capillaries of the heart (8). The wavy surface and the protuberances present a large area for the absorption and transfer of materials to and from the neighboring tissues. This inner membrane is thought to be coated with a monomolecular layer of protein derived from the plasma (9).

Between the endothelial cells there are slits rather than pores, as revealed by the electronmicroscope. They occur between the overlaps of adjacent cells (8, 10). In vivo, these openings may have a transitory existence and may open or close in various places along the mosaic according to physiological needs. The formerly held view that the endothelial cells are kept in contact by an intercellular 'cement' or by 'cytoplasmic bridges' which form a syncytium was largely hypothetical notion (8, 11).

(2) The 'Unit-of-Circulation' concept of Zweifach

Zweifach (12) views the capillary more from the standpoint of function. He regards the capillary 'bed' as the basic functional unit of

the circulation. The capillary bed is considered to include the capillaries proper, the metarterioles, precapillary sphincters and the collecting venules. He makes reference also to "arteriovenous (AV) capillaries", "preferential channels" and "sinusoidal capillaries", each type with a different and specific function (13).

The arteriovenous capillaries serve as 'shunts' or direct connections between the arterial and the venous circulations. Thus, when a precapillary sphincter closes, the blood may be shunted directly into the venous system without going through the capillary network. The opening or closing of the capillaries depends on the metabolic needs or state of the particular tissue segment. The sphincters are thought to be controlled both by the nervous system and, humorally, by chemical substances which act directly upon the contractile elements of the pericapillary sphincter.

(3) The 'Dynamic-Equilibrium' concept

Numerous investigators (14, 15, 16, 17) hold that the capillary should be considered as an integral part of the surrounding connective tissue. This view is based on the observations (1) that injury inflicted on the perivascular connective tissue causes a change in the physicochemical state of the ground substance and results in the escape of proteins and other macromolecular materials from the capillaries, and (2) that the ground substance of the capillary wall is chemically identical with, but appears to be more dense than that of the ground substance of the perivascular

connective tissue.

Accepting the dynamic view, it is relevant to consider the cellular elements in the connective tissue in relation to capillary structure and function.

(a) Pericytes or 'Rouget cells'. These cells have been described and speculated upon by histologists and physiologists in the past decades (18, 19, 20). They were thought to play a role in the regulation of the flow of blood in the capillaries by contraction of their filamentous appendages which run through and around the pericapillary sheath. This mechanism was thought to be responsible for the contractility of the capillary vessels. Later, Clark and Clark (21), with the aid of the micromanipulator, established that the Rouget cell is incapable of responding to direct stimuli. The presence of the pericyte has been confirmed by electronmicroscopists, (22) but their functional significance is still obscure.

(b) Mast cells. These are often seen in aggregations near by the pericapillary sheath. They had received considerable attention also in relation to capillary structure and function. These basophilic cells were first described by Ehrlich (23). Fulton and Lutz (24) have suggested that the pericapillary and connective tissue mast cells may play a role in the maintenance of the capillary wall. Mast cells are known to be associated with mucopolysaccharides (25, 26). Whether they actually produce mucopolysaccharides or function merely as storage depots, as was originally suggested by Ehrlich and has been reaffirmed more recently by Riley (27), remains to

be established.

The mast cell has been shown to contain also numerous other substances, histamine (28), 5-hydroxy-tryptamine (29), heparin (30), beta-glucuronidase (31), hyaluronic acid (32), trypsin- and chymotrypsin-like enzymes (33) and a number of other substances (34), all of which can act directly on, and facilitate the passage of materials through the capillary wall.

(c) Fibroblasts. These cells are known to play a prominent role in the maintenance of the connective tissue. They have been shown also to be directly associated with the embryonic development of the capillary (35). The endothelium is first surrounded by a thin layer of proteinaceous material secreted by the primitive endothelial cell. This layer merges imperceptibly with the pericapillary connective tissue and thus is not a distinct structural element. At this stage of development the fibroblasts in the neighborhood line up and stretch themselves over the outer surface of the newly formed vessel. They then secrete various materials which form a gel with embedded protein (collagen) fibers running through it. The tough jelly-like material is mainly hyaluronic acid.

Riley (27) has suggested that the fibroblasts and the mast cells are functional components of a common system responsible for maintenance of the connective tissue.

(d) The collagen fibers. These fibers which run through the perivascular ground substance contribute to the strength of the capillary wall. Interference

with collagen metabolism as occurs in lathyrism (36) and in scurvy results in an extreme susceptibility of the vasculature to toxic agents (37). In scurvy, the deficiency in the formation of connective tissue gives rise to widespread hemorrhages (38).

(4) The classification of Bennett, Luft and Hampton

These investigators (39) have proposed a simple classification of the capillary vessels based on a comparative study of the capillaries of various organs and in various species. The capillaries are classified according to the appearance of the endothelial cells, the presence of a continuous basement membrane and the nature of the pericapillary cellular elements.

The most significant function of the capillary is to supply nutrients to the tissues and maintain their physicochemical milieu. Two views are current as to the mechanism of transport of substances across the capillary wall: (a) Active transport by engulfment and excretion of the material by the endothelial cell, (b) by passive ultrafiltration through the pericapillary basement membrane. Both these hypotheses take into consideration the role of the various structural elements of the capillary wall.

## 2. THE VASCULAR ENDOTHELIUM

The endothelial lining of all the blood vessels, regardless of size, is made up of a unicellular layer of cells. The shape of the endothelial cell depends on the balance between the intraluminal and perivascular pressures. The intraluminal pressure is regulated by the tone of the metarterioles (40), while the external pressure is contributed by a combination of conditions: the physicochemical state of the perivascular sheath, the degree of hydration of the surrounding ground substance gel and the functional activity of collecting venules and lymphatic capillaries (14, 41).

The endothelial cell, like other cells, possesses all of the subcellular structures: nucleus with nucleoli, endoplasmic reticulum, Golgi apparatus, mitochondria (42). Numerous microvesicles also may be visible in the cytoplasm. These apparently develop from projections of the inner surface of the plasma membrane. The vesicles move into the interior of the cell where they may become fused with the endoplasmic reticulum (43) or with the perinuclear cysternae (44). This behaviour is suggestive of the mode of fluid transport known as 'pinocytosis' (cell drinking). Moore and Ruska (45) have suggested that this term should be used when referring to fluid uptake for the cell's own use, and that the term 'cytopemesis' is more suitable when referring to the transport of a membrane enclosed fluid globule through the cell.

Alksne (46) suggested that the appearance of vesicles, folds, clefts or caveolae signifies an acceleration of the normal transport mechanism. This interpretation has been refuted by Majno and Palade (47) who demonstrated that exudation occurs only between venular endothelial cells. Their view has been substantiated by other investigators (48, 49, 50).

Lysosomal-like bodies containing acid-hydrolases, first described by De Duve (51), were observed also by Marchesi (52) in inflamed endothelial cells. He was unable to demonstrate the presence of these subcellular particles in normal cells. He surmised that lysosomes may be present in a latent state in normal cells and become activated in response to injury. The role of the lysosomes in autolytic processes has been reviewed by De Duve (51). Janoff (53) suggested that the same organelles in the leucocyte may play a role in phagocytosis. Whether there is a relationship between these particles and the previously described vesicles in the endothelial cells, and whether they have any function in the normal cell or under pathological conditions must await further elucidation.

Large fenestrations between the endothelial cells lining the walls of the sinusoids under physiological conditions have been described by various workers (54). Luft and Hechter (55) observed that the endothelial cells of small vessels under conditions of anoxia tend to assume a per-

forated appearance. The observed reversibility of this type of behaviour led them to suggest that the phenomenonmay be involved in the transport of metabolites across the cell.

The normal endothelial cell displays the properties of tone (56) and contractility (57). Despite the extensive study given to these characteristics (58, 60, 61), their significance in the transport of materials across the vessel wall remains obscure. The capillaries in the adrenalectomized animal are atonic and abnormally permeable (62,63). The same conditions prevail in extreme vasoconstriction (64, 65).

The endothelial cells of the small vessels and of the lining of the sinusoids, by virtue of their potential phagocytic properties,belong to the reticuloendothelial system (RES) (66). Thus, when necessary, as in tissue injury and inflammatory states, they play a major role in the protection and defence systems of the body (67).

Jancso (68) observed that colloidal carbon particles, administered intravenously in fluid suspension, tend to accumulate at the site of histamine application. This he attributed to an acquired stickiness of the endothelial cellsand he considered histamine to be the activator. There appeared also to be a relationship between the degree of stickiness of the membrane of the endothelial cells and the observed increase in capillary permeability, after the application of histamine. These phenomena were extensively investigated by Gozsi and Kato (69, 70, 71, 72). By

the use of various antihistamine agents these workers showed that increased capillary permeability and stickiness of the endothelial cells are two distinct and unrelated phenomena. The concept and significance of stickiness have recently been challenged. Majno and Palade (47), by the use of electronmicroscope, have revealed that colloidal particles are accumulated between the endothelial cells, rather than on the surface of the cells. Their finding was substantiated by Hurley and Spector (73).

Florey and Grant (74) have obtained evidence that injury to the capillary will cause irreversible damage to the endothelial cells. Majno and Palade (47) substantiated their view by demonstrating complete disintegration of endothelial cells following injury.

A biochemical approach regarding the mechanism of endothelial permeability has recently been reported by Cascarano et al. (75). Combining tissue slice metabolic studies with histochemical procedures these workers were able to demonstrate that the permeability of the endothelium is intimately associated with oxidative metabolism and ATP generation. Thus the passage of substances and materials across the endothelial wall is a dynamic process under metabolic control.

## 3. INTERCELLULAR BINDING

The question of how contiguous cells, particularly those of the endothelial mosaic of the blood vessels, are held in contact has been a subject of study by cytologists and histologists for more than 30 years. The view that the cells are held together by means of a cement substance has been accepted for decades and still is widely held. However, in the light of more recent findings it appears to be giving way to modern concepts of intermolecular attractions and dynamic control.

### (1) Concept of intercellular cement

According to this view, the cells secrete a jelly-like cement substance (76), closely related to hyaluronic acid but more likely a mucoprotein. This substance combined with or containing calcium ions is presumed to be the intercellular binding material. The cement has been thought to be actively involved also in the capillary filtration mechanism (77). The boundaries of intercellular cement can be made visible by the application of silver nitrate to a flat preparation of endothelium and subsequent exposure to light (78, 79). The black, silver-stained boundary or layer outlining the cells has been found to be about 1 micron in thickness.

Pappenheimer (80) estimated the area available for capillary filtration to be about 1 per cent of the endothelial surface area. This value

was in apparent agreement with the estimate of the area of the intercellular contact (cement).

The importance of calcium ions in the maintenance of intercellular cohesion was demonstrated by Chambers and Zweifach (81) by perfusion of the capillaries of the frog's mesentery. They found that the removal of calcium ions or alteration of the pH of the perfusion medium resulted in accelerated permeability, apparently from 'softening' of the cement.

Perfusion experiments utilizing the escape of dyes of relatively large colloidal dimensions, such as Evans Blue (82), Trypan Blue (83), demonstrated the escape of an excessive amount of these macromolecules from the capillaries following changes in the cement substance.

Curran (84) and Curran and Collins (85) observed that endothelial cells concentrate radio-sulfate and they suggested that this mechanism may be connected with the formation of the cement material. However, Jennings and Florey (86) and Jennings (87) prepared radioautographs of tissues of animals injected with $S^{35}O_4$ and were unable to substantiate the interpretation.

(2) Concept of binding by intermolecular attraction

The application, in recent years, of electronmicroscopy to the study of endothelial structure has prompted reexamination of the findings of earlier workers (88, 89) and has raised scepticism about the

'cement' and the 'pore' concepts. Among the reasons for doubt are the following observations:

(a) Measurements with the electronmicroscope indicate that the space between adjacent endothelial cell surfaces does not exceed 0.02 microns under physiological conditions. This contrasts greatly with the estimate of 1 micron with the silver stain method and indicates the absence of any substantial layer of cement material. The apparent silver stained layer is now considered to be the adsorption of silver particles on the surface of the cell membrane and even in the cytoplasm. Extension of the silver stain on the membrane beyond the margin of the area of intercellular contact furthermore is believed to contribute to the apparent thickness of the 'cement'.

(b) Stehbens (88) suggests that the prolonged exposure used by earlier workers in the preparation of radioautographs could account for the localization of radioactivity at the intercellular junction and in the endothelial cells.

(c) Electronmicroscopy reveals the presence of irregularly shaped channels (Fig. 1) between the overlapping surfaces of the endothelial cells. Thus the notion of the 'pore' also has undergone modification.

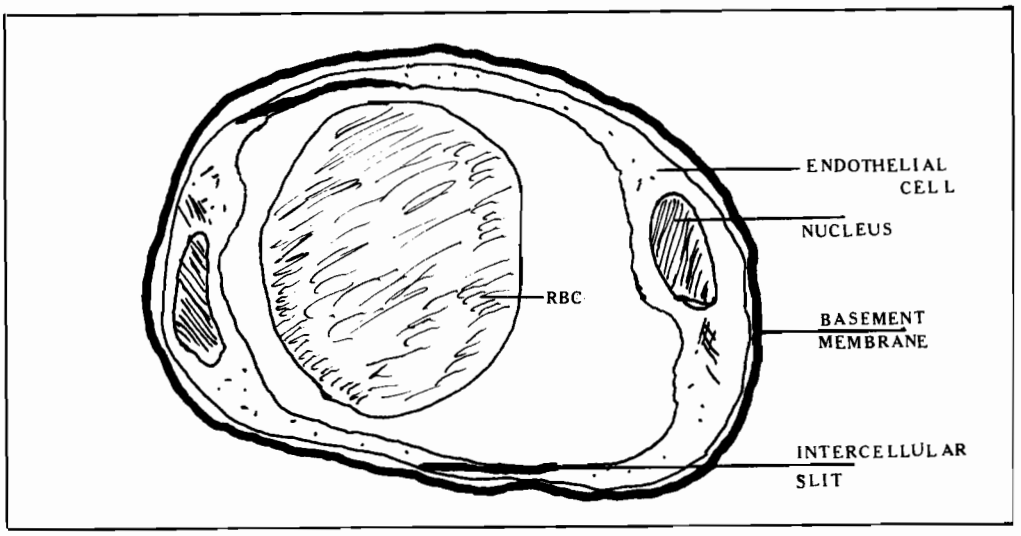
Workers now consider that the endothelial cell surfaces are held together by molecular attractions (90), which would imply the predominant presence of polar groups. The earlier view, however, considers the endo-

thelium of the blood vessels as a 'nonpolar' and nonwettable surface. Recently Roka (91) and Zubairov et al. (92) challenged the above concept. According to the experimental evidence obtained by these workers the endothelial membrane, while having a lipoprotein surface, has both hydrophilic polar and hydrophobic groups in random distribution. This view, if correct, necessitates revision also of the prevalent notion that the circulating blood is prevented from clotting in the blood vessels by virtue of the nonwettable property of the endothelial lining.

The phenomenon of cell adhesiveness has been reviewed recently by Bangham (93). The author postulates that if the surfaces of neighboring cells have numerous protuberances with a small radius of curvature it would permit close approach at ordinary thermal energies. Calcium also could be involved in binding under these conditions. It is of interest that in perfusion experiments, as described earlier in this section, the use of perfusion fluid lacking in calcium always resulted in increased permeability. Taking Bangham's view into consideration, it is thus reasonable to consider that the presence of calcium bridges plays an important role not only in endothelial cell binding but also in the control of capillary permeability. Thus there is no need to postulate the existence of an intercellular cement.

Figure 1

Representation of Intercellular Space in the Capillary




## 4. ENDOCAPILLARY LAYER

Danielli (94) obtained evidence of the existence of still another component of the capillary wall in the form of a thin layer of material, covering the inner surface of the capillary tube. The layer is thought to be adsorbed from circulating plasma proteins. Evidence in support of the presence of this layer is based on (a) perfusion experiments (95) and (b) on the observation that epsilon-amino caproic acid, a fibrinolysin inhibitor, has a potent inhibitory action on histamine-induced permeability changes in the guinea pig skin (96).

As yet there is no direct microscopic or electronmicroscopic evidence of its existence.

## 5. THE PERIVASCULAR SHEATH

The capillary endothelium is covered with a supporting layer about 500-600 A in thickness and composed mainly of mucopolysaccharides with embedded argentophyl reticulin fibers (97).

The mucopolysaccharide material is chemically similar to the ground substance of connective tissue (Table I), but differs from it in density and in solubility characteristics (98).

TABLE I

Mucopolysaccharides of Connective Tissue

| |
|---|
| Neutral mucopolysaccharides containing no acidic groups (e.g. chitin, composed of acetylglucosamine only). |
| Acid Mucopolysaccharides:<br>(a) Simple - acid component uronic acid (e.g. hyaluronic acid composed of acetylglucosamine and glucuronic acid).<br>(b) Complex - acid component of complex polysaccharide include uronic acid, sulphuric acid or phosphoric acid (e.g. heparin, chondroitin sulfuric acid which contains acetylgalactosamine, glucuronic acid and sulfuric acid). |
| Mucoprotein - made up of small amount of polysaccharide conjugated with protein. |

Alksne (46) observed that the passage of colloidal particles of mercuric sulfide across the dermal capillaries of the mouse was obstructed

by the pericapillary sheath. The same phenomenon has been observed by other workers under different experimental conditions (22, 99, 100, 101). It is now accepted that this layer represents the main barrier against the transport of materials across the capillary wall.

The multifunctional role of the ground substance sheath has been discussed in a recent review by Haynes and Rodbard (102). These authors ascribe to it the various functions of a filter, a hygroscopic component, adsorption medium, ion exchange medium, lubricant, cement and a prosthesis in the maintenance of connective tissue and vascular metabolism. Despite the relative ease with which it can be deformed by slight pressure exerted in the vicinity, it has a definite structure and is amply strong to provide support to the endothelium. It takes up water avidly (pericapillary edema after slight injury to the vessel wall), with an accompanying change in state from gel to sol, but compression tends to expel the water and favour the return to the gel state. The high viscosity of the material of this layer provides resistance to passage of fluid across the vessel wall. Metabolites passing through this barrier may be liable to adsorption, binding or exchange as on an ion exchange column.

The significance of the pericapillary sheath in vascular resistance has been studied recently by Schiff and Burn (103). These workers also subscribe to the view that the pericapillary sheath is closely related to the connective tissue ground substance (104). On intravenous administration of

conjugated equine estrogens they observed a close parallel between capillary strength and the amount of acid mucopolysaccharide in the connective tissue and the perivascular sheath. These workers postulated also that the change in the mucopolysaccharide content in the perivascular area is connected to an 'activation' of the neighbouring mast cells (105). The hemostatic effect of estrogens has been recently reviewed by Rona (106). A close relationship between the pericapillary sheath and the connective tissue ground substance is further indicated by the circumstance that agents which affect the connective tissue matrix act similarly on the vessel wall. Application of hyaluronidase to the pericapillary structures produces a depolymerization of the connective tissue ground substance with the prompt development of microscopic hemorrhages (107). The intravenous administration of hyaluronidase on the other hand will increase capillary permeability, but without extravasation of red blood cells (108). It has been demonstrated further that bacterial toxins, which are known to contain hyaluronidase, likewise produce hemorrhages (Shiga and Clostridium welchi exotoxins) (109, 110).

Zweifach (111) considers, that Duran-Reynals's view (112) that hyaluronidase controls capillary permeability, should be interpreted as a change in 'fragility' (vascular stability) rather than in 'permeability'.

Evidence, in support of Zweifach's hypothesis comes from the recent

work of Wayne et al. (113), who demonstrated that the hemostatic effect of estrogens is related to their capacity to counteract the action of hyaluronidase by increasing the serum concentration of the hyaluronidase inhibitor.

Numerous workers (114, 115, 116, 117, 118, 119, 120) agree that a change in the composition of the perivascular connective tissue can greatly modify its property as a supporting tissue. This is manifested by altered permeability and tensile strength, and the inability to maintain hemostasis, thus giving rise to purpura or massive hemorrhages.

## 6. PATHOLOGY OF THE MICROCIRCULATION

### (1) General considerations

It is evident that a pathological state of the microcirculation in any region, as a consequence of any type of injury, represents the end result of a chain of biochemical events. The small vessels and the surrounding connective tissue are very sensitive to external or internal stimuli. Even light pressure, as with the mild rubbing of the skin, is sufficient to cause local dilatation and increased permeability of the capillaries with demonstrable escape of plasma proteins (albumin and other small macromolecular material) and with an accompanying loss of water (production of edema). Early in the sequence of events is the vasodilatation, that appears to be initiated by the release of chemical mediators (histamine, serotonin) from injured mast cells. The resulting increase in capillary permeability involves an increase in intraluminal pressure with opening of the endothelium or widening of existing openings. This process points to the weakening of forces and breaking of bonds, perhaps calcium bridges, that maintain the contact between endothelial cells. Little is known about the detailed biochemical nature of the sequence of steps involved, except that they are accompanied by changes in the concentration of sodium and potassium ions in the cells of the vessel walls.

The extreme difficulty in making a differentiation between the physiological and the pathological states of the small vessel wall arises from the lack of information concerning the nature of the changes and the observation that, in both instances the same chemical mediators seem to be operative.

However there appears to be a clear distinction as to condition and mechanism between the two pathological conditions: (1) vascular fragility, which is accompanied by hemorrhages and (2) increased vascular permeability, characterized by a leakage of material and fluid from the vasculature, but without hemorrhage.

(2) The concept of vascular fragility

The understanding of the nature of vascular fragility was obscured by the introduction of the negative-pressure or Rumpel-Leed methods (121, 122) as a means of measuring the state of the small vessel wall. The number of petechiae produced per unit area of the skin subjected to negative pressure was supposed to give an index of vascular fragility. This procedure may be effective for the diagnosis of vascular instability and therefore has some clinical usefulness. However, it is of no value in scientific studies on vascular stability (123). For one thing, the exposure of the skin or mucosa (e.g. the cheek pouch of the hamster) to negative pressure, subjects the microcirculation (capillaries, venules, arterioles)

in the tissues to a heavy trauma. Even in normal tissue, the subsequent upset between the intra- and extraluminal pressures is sufficient to promote the diapedesis of red blood cells through the walls of the venules. Fulton (124) has questioned that the escape of red blood cells under these conditions is at all indicative of a prevailing state of vascular fragility in the individual. It is of interest that ecchymosis under such stressful circumstances appears to occur at the venules and not at the capillaries as was formerly believed (125). Furthermore Wilbrandt (126) considered it highly improbable that vascular fragility represents merely a state of highly increased vascular permeability. There is no physiological relationship between the two phenomena (127).

The important distinction between vascular fragility and increased vascular permeability comes from the observation that the former is initiated from the extravascular side and proceeds by gradual breakdown of the ground substance of the perivascular sheath, while permeability changes may be initiated from either side of the vessel wall, but will involve only the endothelium proper with no alteration of the perivascular sheath (128).

Various authorities (129,130) believe that vascular fragility involves only the small venules, and that it is brought about by the action of hydrolases - hyaluronidase, proteases, phosphatases, etc. - which are associated with the lysosomes. Application of these enzymes to the peri-

vascular region has been shown, as mentioned previously, to produce hemorrhage.

(3) Increased vascular permeability

The increased vascular permeability which accompanies the inflammatory state is reversible as long as the inflammation remains under physiological control.

Increased vascular permeability associated with inflammation, according to Cotran and Majno (131), appears to be of three types, which the authors say, are experimentally differentiable. They differ mainly in degree and as to the site of occurrence.

(a) The histamine type

This condition of abnormal permeability can be produced by application of histamine, bradykinin or serotonin to the small vessels, or, since histamine and serotonin are localized in the mast cells, by application of a dilute solution of an agent such as 48/80 or lecithinase A, which causes disruption of the mast cells (132).

Bradykinin, a nonapeptide, undergoes proteolytic activation in the plasma by the action of tryptic enzymes (133, 134). It has been suggested that the activation of the tryptase, in turn, may be initiated by the action of histamine. The anti-inflammatory action of salicylates is thought to be attributable in part to the inhibitory action of these agents on the

activation of the kinin precursor (135,136).

There is evidence that the histamine type of altered permeability is confined largely to the endothelium of the venules (137). It is a transitory and readily reversible condition. Intravenously injected colloid material such as carbon (India ink), thorium dioxide, etc., is rapidly taken up by the Kupffer cells of the liver, the sinusoidal endothelium of the spleen and by other phagocytic cells. Some of the material can be shown to escape from enlarged interendothelial spaces, in the venules, between the endothelial cells and the periendothelial sheath. This process may be greatly accelerated by topical application of any one of the mediators (serotonin) of the 'histamine' type of inflammation. Within a short period of time the bulged portion of the venular sheath overlying the accumulated colloidal material becomes detached from the endothelium and undergoes breakdown (at that site only) with liberation of the material into the extravascular space (138), where it is taken up by macrophages and transported into the lymphatics.

(b) Vascular permeability resulting from tissue damage

Direct damage to the tissue, in the form of mechanical trauma or chemical irritants, may vary in degree and extent of involvement. The damage from these agents is more severe and extensive than that represented by the histamine type. Depending on the location of the trauma, it can involve any part of the microcirculation- the capillaries, venules and

arterioles. The periendothelial sheath of the small venules or the pre-capillaries tends to remain intact. The injury is confined to the endothelium.

(c) 'Delayed' prolonged type of increased permeability

This variety of vascular permeability develops after the application of moderate heat to any epithelial surface. At the site of heat injury there will be an immediate histamine-type of response, followed in the neighbouring area by a delayed and prolonged vascular damage. While the immediate reaction is mediated by histamine, serotonin, etc., and is confined to the venules, according to Wells and Miles (139), the delayed response is not mediated by chemical means and is confined to the capillaries. The escape of macromolecular materials ultimately is more extensive than in the other conditions of altered permeability. This phenomenon has been discussed in a review by Miles and Wilhelm (140).

The foregoing classification, while largely hypothetical, nevertheless is based on observation and experimentation, and thus may be helpful in the elucidation of the phenomenon referred to as 'increased permeability'.

From the biochemical point of view the classification is hardly adequate since it does not include certain conditions such as the vascular permeability caused by injection of L-ascorbic acid (141). This agent, injected subcutaneously, or applied to tissues in vivo, or tested in vitro on a solution of hyaluronic acid, produces oxidative depolymerization of

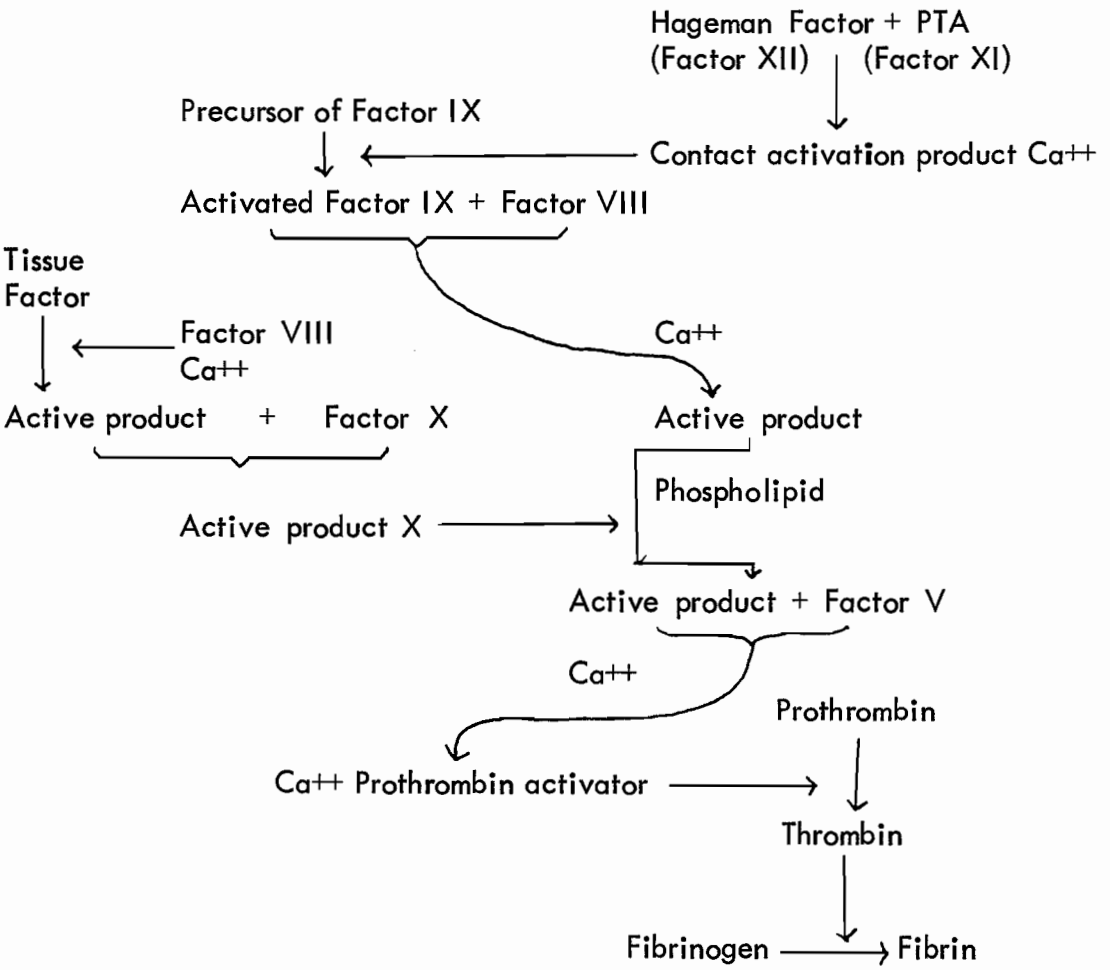
the macromolecule (142). Since ascorbic acid is closely associated with connective tissue metabolism, the thought occurs that the vitamin may play a direct role in the mechanism of tissue permeability.

There are, in addition, immunological implications which are not taken into consideration. Cochrane et al. (143) recently have reported an observation which may warrant a complete re-examination of existing concepts. These authors report that the presence of leucocytes is essential to the development of increased vascular permeability in immunological inflammation.

## 7. BLOOD COAGULATION

The mechanism of the blood clotting system is of concern in our study only insofar as it affects the stability of the small blood vessels. As mentioned previously, a severe deficiency of any of the factors associated with the activation of prothrombin or the operation of thrombin, tends to give rise to spontaneous hemorrhages. Therefore, we are interested in the subtle relationship between the state of the blood clotting mechanism on the one hand, and the state of the wall of the small vessels on the other. Since we use an anticoagulant, dicumarol, for the experimental production of vascular fragility, and have occasion in the discussion to refer to certain of the components of the blood clotting system, it may be helpful to the reader if the author includes a representation of the coagulation system to

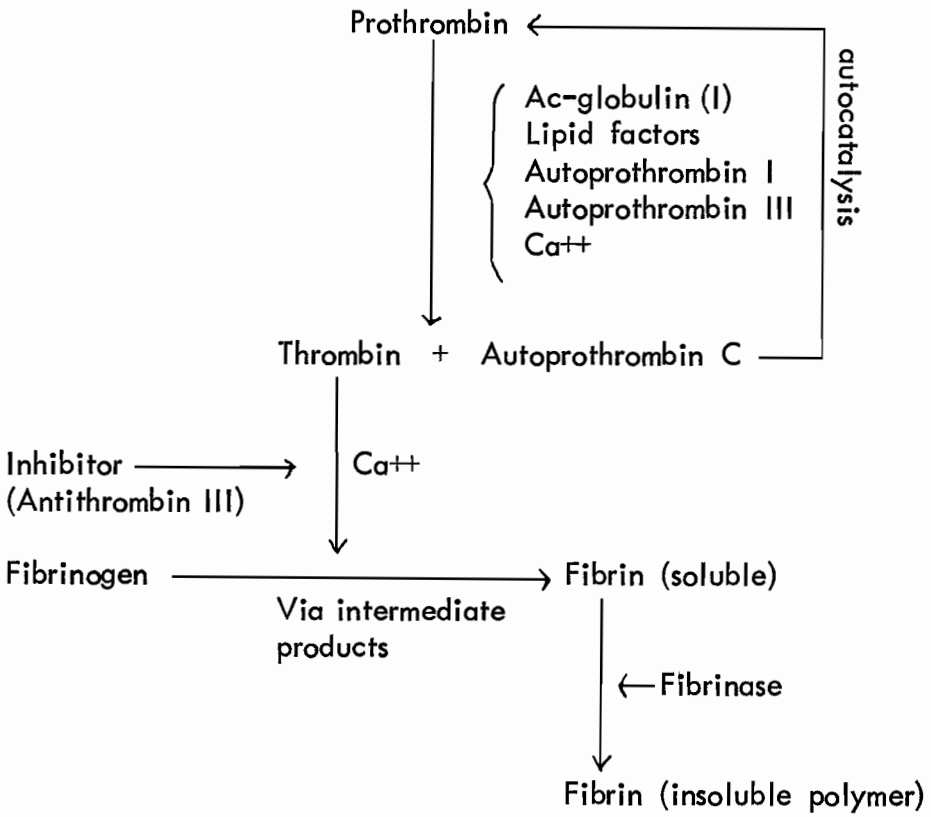
show where the various factors are involved in the system. It is impossible to give an adequate schematic representation of the blood clotting sequence, because of the involvement of feedback mechanisms, and the vagueness of existing knowledge. Most of the current hypotheses are deduced from the results of coagulation tests (144, 145, 146). One representation, that of Biggs and Macfarlane (147), is shown in the following scheme and listed nomenclature:



Nomenclature for the blood clotting factors (148):

| | |
|---|---|
| Factor I | Fibrinogen (has been purified). |
| Factor II | Prothrombin (has been purified). |
| Factor III | Thromboplastin (tissue extract) not found in plasma. Tissue extract which has 'thromboplastin' activity has been found to contain Cathepsin C, which, by itself, could account for the activity (149). |
| Factor IV | Calcium |
| Factor V | Proaccelerin, labile factor, accelerator globulin, Ac-globulin (has been purified). |
| Factor VI | Regarded as an intermediate with prothrombin accelerating power. |
| Factor VII | Autoprothrombin I, serum prothrombin conversion accelerator (SPCA), proconvertin (has been purified). |
| Factor VIII | Platelet cofactor I, antihemophilic globulin (AHG), antihemophilic factor (AHF), antihemophilic factor A (has been isolated). |
| Factor IX | Autoprothrombin II, Christmas factor, platelet cofactor II, plasma thromboplastin component (PTC), antihemophilic factor B (has been purified). |
| Factor X | Stuart-Prower factor. Not yet purified. According to Seegers, is associated with abnormal prothrombin molecule. |
| Factor XI | Plasma thromboplastin antecedent (PTA). |
| Factor XII | Hageman factor. |

Seegers and associates (150) have contributed much to the understanding of the nature of the blood clotting mechanism, by purification of prothrombin, thrombin and a number of intermediates formed during the coagulation of the blood. The scheme shown below is based on the results of studies on the interaction of 'pure' or highly purified materials, and on the chemical identification of the reaction products, rather than on blood clotting tests.



In their interpretation of the nature of the blood clotting disorders, Seegers and Marciniak (151) have suggested the following classification:

(1) Abnormality of prothrombin molecule (present in 'Stuart plasma')

(2) Disorders related to alteration in the derivatives of prothrombin:

    (a) Autoprothrombin I

    (b) Autoprothrombin III

(3) The molecular abnormality or deficiency of accessory substances essential for the generation of Autoprothrombin C thus interfering with its function as an autocatalyst:

    (a) Platelet Cofactor I (antihemophilic factor)

    (b) Other factor(s)

(4) Deficiency of accessory factors required for the functioning of autoprothrombin C after it is split from prothrombin:

    (a) Ac-globulin

    (b) Platelets (Platelet cofactor III)

Dicumarol, a substance, 3'3'-Methylener-bis (4-hydroxy-cumarin), isolated by Link and associates (152) from spoiled (mildewed) sweet clover, was found to interfere with the formation of prothrombin and components of the prothrombin complex (factors V, VII and X) in the liver. It interferes in some way with the function of vitamin K (153), which is essential for the production of these blood clotting factors. The inhibitory action can be counteracted by the administration of vitamin K. Dicumarol often is referred to erroneously as an "anticoagulant" but it has no effect on

coagulation of the blood or plasma *in vitro*.

It is of historical interest that Sweet Clover Disease, a hemorrhagic and often fatal disease manifested in cattle, horses and sheep fed mildewed sweet clover, was originally described in North America in 1924 by F.W. Schofield (154) of the Ontario Veterinary College in Guelph. The same type of hemorrhagic disease was found to occur in poultry fed a synthetic diet lacking in vitamin K. Many animals on a good diet are remarkably resistant to the effect of dicumarol added to the diet or given by injection.

# PART TWO

# EXPERIMENTAL

## 1. A NEW METHOD FOR THE PRODUCTION OF EXPERIMENTAL HEMORRHAGE IN THE RAT.

### INTRODUCTION

For the biochemical study of vascular 'fragility' it is necessary to have a method of producing nontraumatic bleeding in laboratory animals. Several procedures have been used by various workers. The administration of benzene is known to cause thrombocytopenic purpura in the rat, by reducing the number of platelets in the circulation (155). Benzene also accelerates the destruction of circulating erythrocytes and poisons the hemopoietic tissue with consequent aplastic anemia and agranulocytosis (156).

Another chemical, aminoacetonitrile, is an 'osteolathyrogenic' agent (157) which prevents the crosslinking of newly formed tropocollagen (158). This interference with connective tissue metabolism produces exostosis, an irregular proliferation of cartilage and dissecting aortic aneurysm in the rat (159). When aminoacetonitrile and dicumarol are administered together, intracranial and subperiosteal hemorrhages occur (160).

The hemorrhagic tendency in scurvy is well known. Severe vitamin C

deficiency is characterized by gingival bleeding, subcutaneous ecchymosis and wide-spread minute hemorrhages in the skeletal muscles, and, in the periarticular soft tissues (161). These lesions can be reproduced in the guinea pig by administration of a diet deficient in vitamin C (162), or, in the rat by administering a vitamin C analogue (inhibitor) such as glucoascorbic acid. The morphological changes attributed to disturbed connective tissue metabolism (163) are accompanied also by impairment of odontogenesis and bone formation (164).

## BACKGROUND OF EXPERIMENTATION

Previous workers in our laboratory have used dicumarol by serial injection to induce hemorrhage in the rat and observed that older animals display a marked resistance to the drug.

Having encountered this resistance in experiments with the rat, the author reviewed the literature on the conditions which favor the onset of vascular fragility. In the search he noticed incidentally a report that hypothyroid myxoedema is associated with capillary bleeding (165). This suggested that the thyroidectomized animal may be less resistant to dicumarol. In preparing the first lot of animals the submaxillary salivary glands were removed in error and the animals, after recovery, were placed on the dicumarol containing diet. All died from internal bleeding within five days. A follow-up

experiment was then carried out with thyroidectomized animals and to our surprise, they proved to be more resistant than the normals. It was, thus, through an error that we discovered that the salivarectomized animal is a very satisfactory subject for the production of experimental hemorrhage. The method has proved to give reproducible results and it has definite advantages over the simultaneous use of two drugs for producing bleeding.

## MATERIALS AND METHODS

### (1) Preparation of 'hemorrhagic' diet

Rat Chow was obtained in powdered form from the Ralston Purina Company, Montreal. The powder was converted into a moderately stiff paste by the addition of water in proportion 2 parts of Purina to one of water (v/v). Dicumarol (sodium salt) in finely crystalline form was added to give a concentration of 0.5 per cent in the final mixture when desiccated. After mixing the paste thoroughly with a mechanical mixer, to ensure uniform distribution of dicumarol, the paste was spread in a half inch thick layer on aluminum plates divided into 2x2 cm squares and dried to a biscuit texture in a vacuum oven at 38°C for 24 hours.

Preliminary growth-rate tests with young rats had established that the method of preparation of the diet, without addition of dicumarol, caused no alteration in its nutritive quality compared with that of Purina Chow.

(2) Procedure for production of experimental hemorrhage in the rat

Male albino rats, of uniform body weight (60 grams), were purchased from a local breeder and kept for a week in the animal room on the regular Purina ration for conditioning to the surroundings and the temperature of 74°F.

They were then anesthetized with ether and a 1.5 cm midline incision made in the neck. In the 'experimental' group the submaxillary salivary glands were detached from their fibrous capsule, the salivary ducts and blood vessels ligated, and the glands removed. The incision then was sutured with silk surgical thread (Figure 2).

The 'control' animals were subjected to a sham operation involving the making of the incision but not removing the glands.

The animals were kept, 5 in a roomy cage, provided with food and water during a 'recovery' period of two to three weeks. They then weighed about 180 grams.

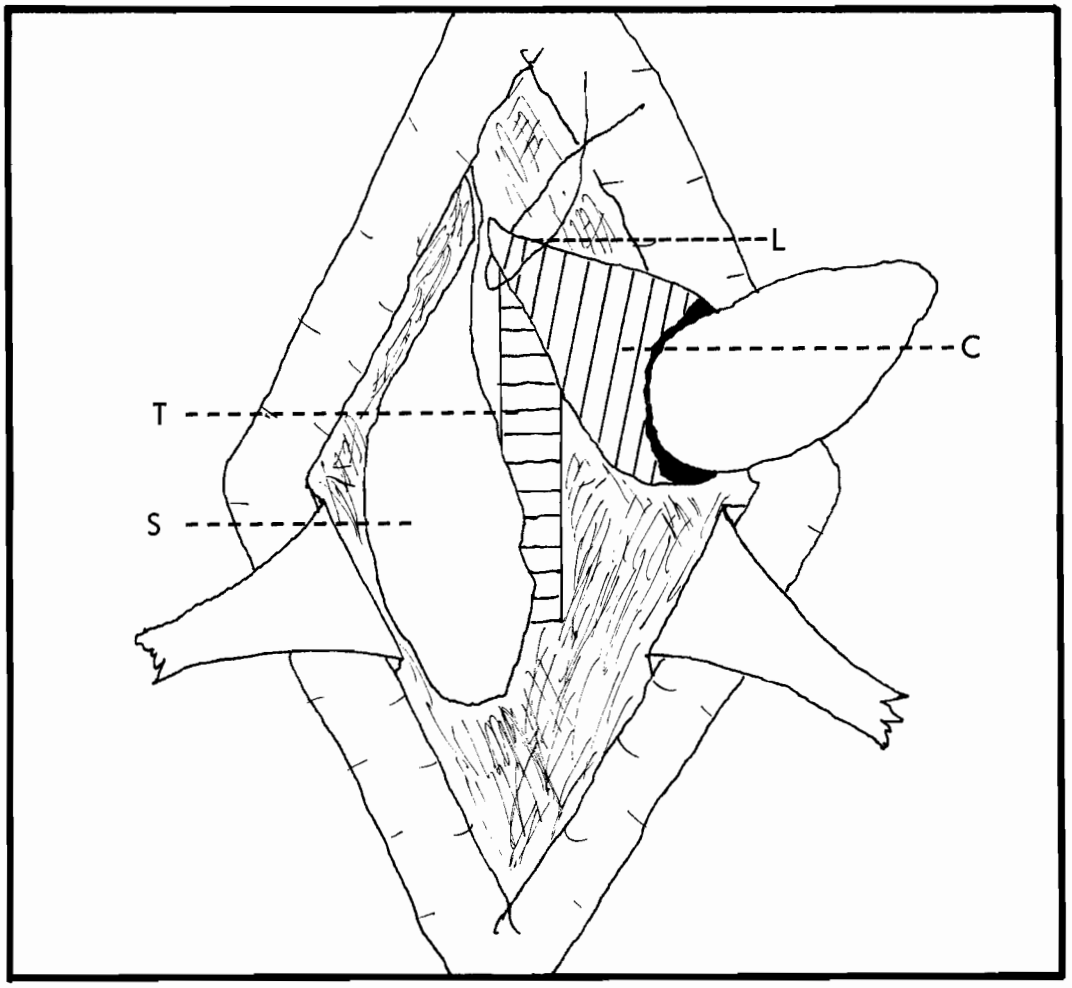
At this time the two groups were fed the 'hemorrhagic' diet. A record was kept daily of the food consumption, body weight and the general condition of the animals. Post mortem examination was made of the ones that succumbed.

Five experiments were performed to test the reproducibility of the method. In all 200 male albino rats, of initial body weight of $60 \pm 5$ grams, were used. One hundred of the animals were salivarectomized and the other

Figure 2

Representation of the Submaxillary Salivary Glands

before Surgical Removal




L - Position of ligature.

C - Fibrous connective tissue capsule.

S - Submaxillary salivary gland.

T - Trachea.

hundred subjected to the sham operation.

## RESULTS

### (1) Symptoms

Both groups of animals suffered a transitory weight loss after surgery chiefly from the reduced food intake. Thereafter, as indicated in Figure 3, both groups showed the same growth rate during the recovery period.

The only difference observed between the groups was a rather pronounced 'rusting' of the fur in the salivarectomized animals. All were given the hemorrhagic diet when the body weight was about 180 grams (180 ± 10 grams).

Table II shows the observations made from day to day after starting to feed the experimental ration.

The mortality rate in the two groups was as indicated in Table III.

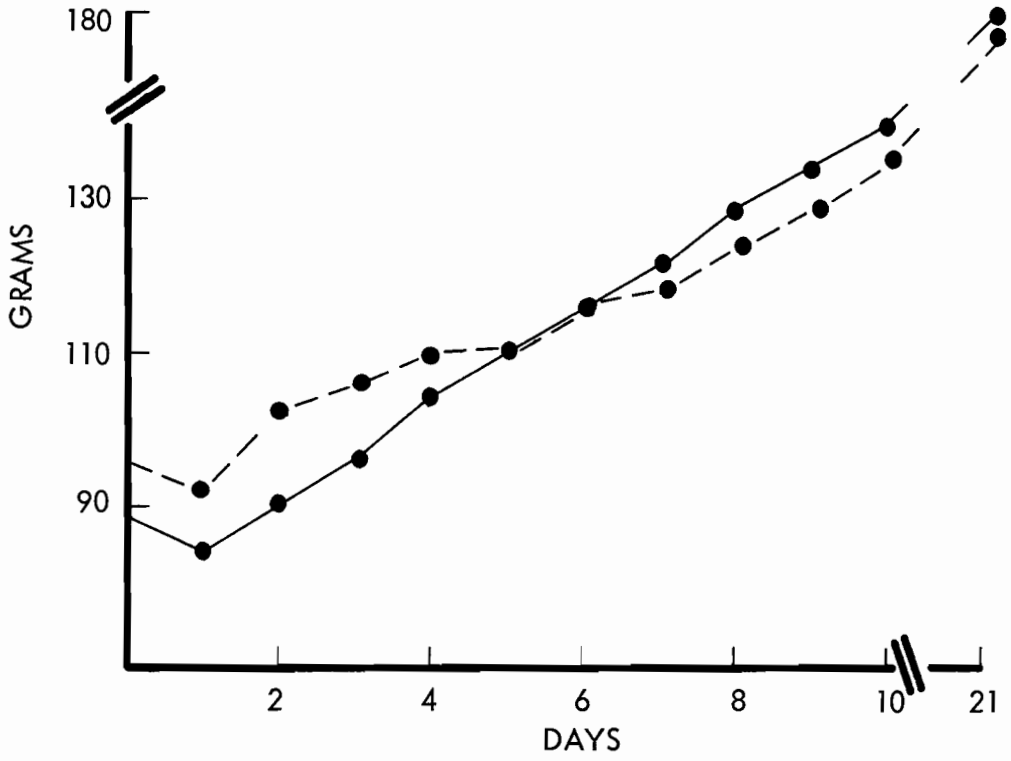
There was a sharp decrease in the body weight of salivarectomized animals in contrast to control rats following administration of dicumarol. The food consumption, on the other hand, remained relatively unchanged throughout the experimental period as illustrated in Figure 4.

### (2) Post mortem observations

All animals showed a foamy, sometimes dried, bloody nasal discharge, and pale, dry mucous membranes. The subcutaneous tissue of the face and

Figure 3

Growth Rate of Salivarectomized and Control Groups




Salivarectomized group (●——●).

Control group (●— — —●).

Each point represents the mean of 100 values obtained in five experiments.

TABLE II

Observations on Animals Given Hemorrhagic Diet

| Day | Observation |
|---|---|
| 1 | Both groups appeared normal |
| 2 | The 'experimental' animals less active than controls. About 50 of the experimental rats showed epistaxis. |
| 3 | Stool of experimental animals tarry (dark colored); stool of controls, normal. |
| 4 | Experimental animals moribund; face and neck edematous; frothy, bloody nasal discharge; breathing irregular; little or no response in many to stimuli. During the day 40 of the animals died. Control animals apparently normal. |
| 5 | Remaining 60 of experimental animals succumbed. Two of the control group showed edema of face and neck and bloody nasal discharge. Remainder of control group normal. |

TABLE III

Mortality of Salivarectomized and Control Animals

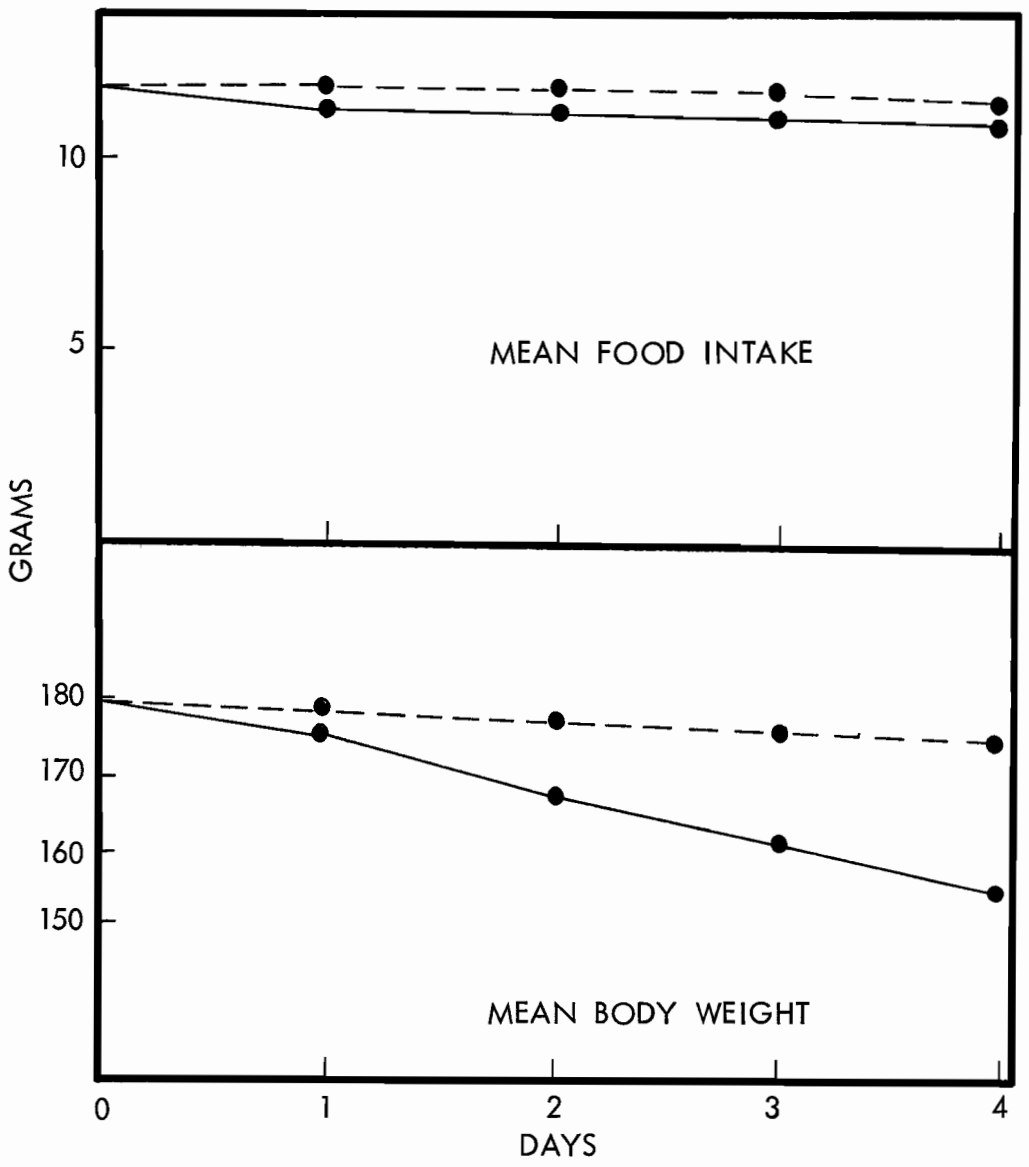
| Day | Group | |
|---|---|---|
| | Control | Experimental |
| | Number of deaths | |
| 1 | - | - |
| 2 | - | - |
| 3 | - | - |
| 4 | - | 40 |
| 5 | - | 60 |
| 6 | 2 | - |
| 7 | - | - |
| 8 | 8 | - |
| 9 | 8 | - |
| 10 | 18 | - |
| 11 | 24 | - |
| 12 | 12 | - |
| 13 | 12 | - |
| 14 | 16 | - |

Data represent five experiments.

Figure 4

Comparison of Food Intake and Weight Loss

in Salivarectomized and Control Groups




Salivarectomized group ( ●——● ).

Control group (●– – –●).

Each point represents the mean of 100 values, in five experiments.

Both groups were put on the dicumarol containing diet at day 0.

neck was edematous,with large ecchymoses. The skin was firm.

No sign of bleeding or infection could be detected in the superficial or deep tissues at the site of the incision. Multiple intercostal hemorrhages were present. Animals with hemorrhagic areas in the lung had a substantial quantity of (0.5 - 1.0 ml) yellow colored exudate in the pleural cavity. Rats with thymic hemorrhages showed hemothorax. The heart, trachea, oesophagus appeared patent.

The subcutaneous tissue and musculature of the abdominal wall showed numerous hemorrhagic lesions. Ten of the experimental animals had up to 5.0 ml of blood in the peritoneal cavity. The stomach in all the animals was full of food. The small intestine contained yellowish material,while the colon and sigmoid flexure contained black colored stool. The mucosa of the gastrointestinal tract, after washing with water, appeared intact. The serosal surface of the pyloric region of the stomach and cecum showed large confluent hemorrhagic areas. The epididymis and epididymal fat pads were edematous,with large hematomata.

The hemorrhagic areas varied in size from pinpoint to more extensive. An attempt was made to grade the hemorrhagic lesions as illustrated in Table IV.

Judged according to these criteria,the extent and site of bleeding at post mortem may be represented in a roughly quantitative manner as in Table V.

TABLE IV

Designation of Hemorrhage According to Size of Lesion

| Grade of lesion | Extent of bleeding |
|---|---|
| 0 | No lesion |
| 1 | Pinpoint hemorrhage |
| 2 | Well demarcated hemorrhagic area, 1-3 mm in diameter |
| 3 | Large confluent hemorrhage, more than 3 mm in diameter |

## CONCLUSIONS

The salivarectomized rat on dicumarol diet is a satisfactory subject for the production of experimental hemorrhage. The method is reproducible. The site and intensity of hemorrhages are uniform compared to that of sham operated controls receiving the same diet.

TABLE V

Post Mortem Assessment of the Relative Extent of Hemorrhage

| Organ | Group | | | |
|---|---|---|---|---|
| | Experimental | | Control | |
| | No. of animals | Mean grade of lesion | No. of animals | Mean grade of lesion |
| Stomach | 40 | 2.5 | 2 | 1.5 |
| Small intestine | 90 | 1.2 | 2 | 1.0 |
| Coecum | 50 | 1.5 | - | - |
| Lung | 45 | 3.0 | 3 | 3.0 |
| Liver | 5 | 1.0 | - | - |
| Kidney | 4 | 1.0 | - | - |
| Thymus | 54 | 3.0 | 1 | 3.0 |
| Epididymis | 85 | 3.0 | 62 | 2.0 |
| Testicle | 80 | 2.2 | - | - |
| Retroperitoneum | 60 | 2.0 | 10 | 2.0 |
| Abdominal muscle | 20 | 1.5 | - | - |
| Intercostal muscle | 60 | 2.2 | - | - |
| Buccal subcutaneum | 90 | 2.5 | 20 | 1.0 |
| Mesenteric lymph nodes | 95 | 1.0 | 20 | 1.0 |
| Cervical lymph nodes | 98 | 1.5 | - | - |
| Axillary lymph nodes | 80 | 1.0 | - | - |
| Pelvic lymph nodes | 90 | 1.0 | 10 | 1.0 |

Total number of animals 100 experimental and 100 control in five experiments.

## 2. HISTOLOGICAL EXAMINATION OF TISSUES

### INTRODUCTION

Having established that the feeding of a dicumarol containing Purina ration to salivarectomized rats affords a reproducible method for the experimental production of nontraumatic hemorrhage, the question of when and where bleeding first occurs was the first of importance to be answered.

The information could best be obtained through a detailed histopathological examination of the tissues at various time intervals during the feeding period.

An attempt was made also to correlate the blood clotting defect initiated by dicumarol (166, 167) with the state of the small vessel wall and the perivascular structures.

### EXPERIMENTAL

One hundred and twenty male albino rats with uniform body weight of 60 grams were divided into an 'experimental' and a 'control' group and prepared as previously described (see page 41).

Ten animals from each group were killed before the rats were placed on the dicumarol-containing diet and examined for detectable morphological changes that may have resulted from the removal of the submaxillary

salivary glands.

Daily, after the beginning of the feeding experiment, ten animals from each group were killed by instantaneous decapitation. The following organs were removed and fixed in 10 per cent formaldehyde (24 hours) for histological study: lung, thymus, stomach, intestinal tract, liver, spleen, prostate gland, bladder, epididymis, epididymal fat pads, testicles and the mesenteric, pelvic, axillary, cervical lymph nodes.

The fixed specimens were dehydrated in alcohol, embedded in paraffin and sectioned at 5 micron thickness. The sections were stained with Ehrlich's hematoxylin-eosin (168), Heidenhain's azan (169) for collagen, orceine-aniline-safranin method (170) for elastic fibers, and periodic acid-Schiff method (171), for ground substance.

Photomicrographs were taken with a Zeiss Photomicroscope and Adox KB 14 fine grain panchromatic film.

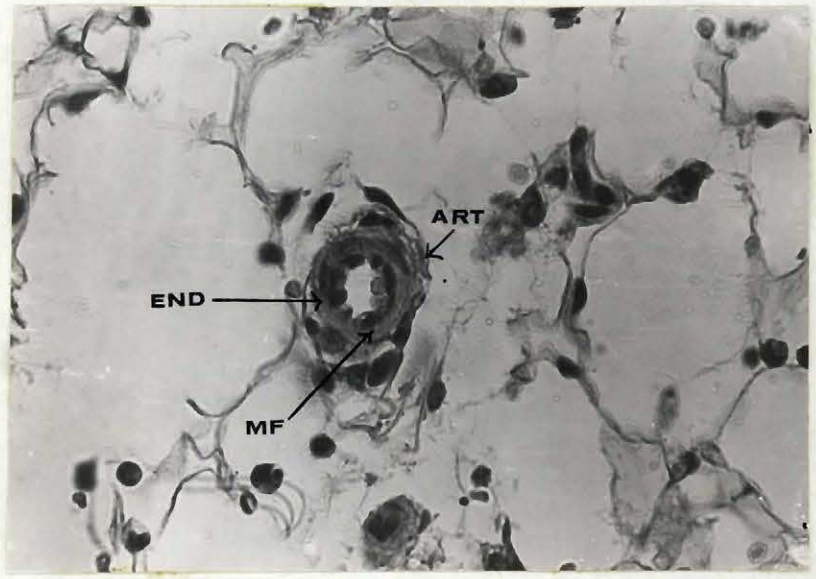
## RESULTS

### 1. Morphological changes after salivarectomy

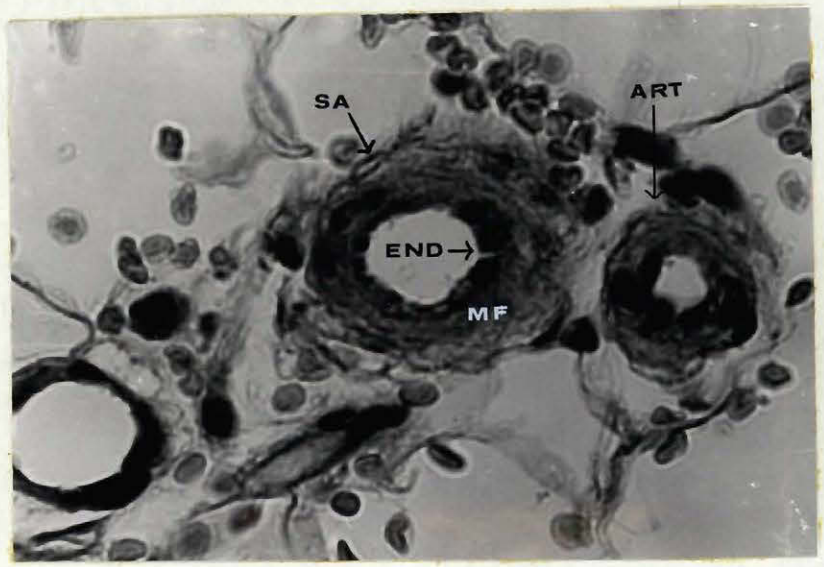
(1) The most apparent change in the experimental animals was the thickening of the arteriolar and small arterial wall, in all the organs examined (Figure 5B and 5C). There was swelling of the endothelium. In contrast to the oval shaped and peripherally situated nucleus of the endothelial cells in the control animals (Figure 5A), the nuclei in the experimental animals were round and centrally located. The endothelial

Figure 5

SMALL VESSELS



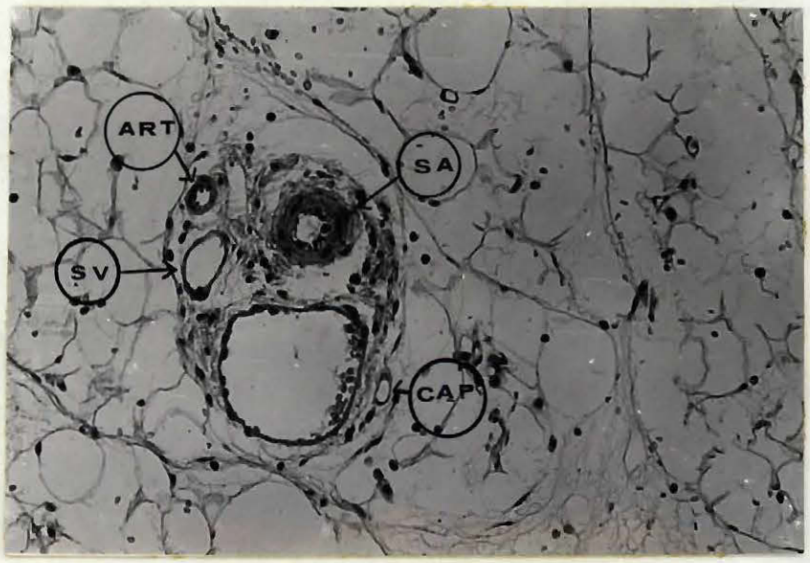

A. Arteriole of control (sham-operated) rat. Endothelium (END) and muscle fibers (MF) intact. Hematoxylin-eosin.




B. Small artery (SA) and arteriole (ART) of salivarectomized rat, 21 days after operation. Endothelial (END) and muscle fiber (MF) proliferation with narrowing of the lumen of the vessels. Hematoxylin-eosin.

Figure 5

SMALL VESSELS




C. Small vessels of salivarectomized rat. The wall of the small artery (SA) and arteriole (ART) show thickening. The capillary (CAP) and small vein (SV) show no structural alterations. Hematoxylin-eosin.

lining of the vessels were detached in many places from the lamina propria. The tunica muscularis showed hypertrophic muscle fibers with spacious intercellular channels. It is noteworthy that the capillary vessels showed no structural alterations.

(2) Of all the tissues examined the thyroid showed the most prominent histological response to removal of the submaxillary salivary glands. The illustration in Figure 6B shows the low cuboidal epithelial cells of the follicles with closely packed nuclei, basal in position. Moderate papillary epithelial proliferation; small follicular lumen; volume of colloid diminished, thin, pale and in some areas vacuolated at the periphery.

All the other organs examined were comparable to those of the control animals.

2. Morphological changes in tissues after administration of dicumarol

The details in Table VIA and B give a comparison of the progressive morphological changes observed in the control and the salivarectomized rats during the feeding of a diet containing dicumarol. The information gained from the histological examination of the tissues may be summarized as follows.

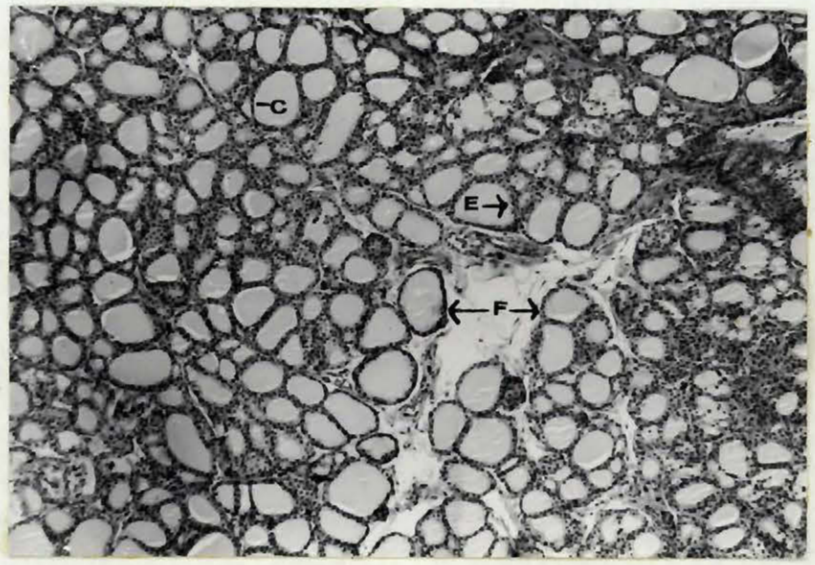
(1) Liver (Figure 7)

Experimental group

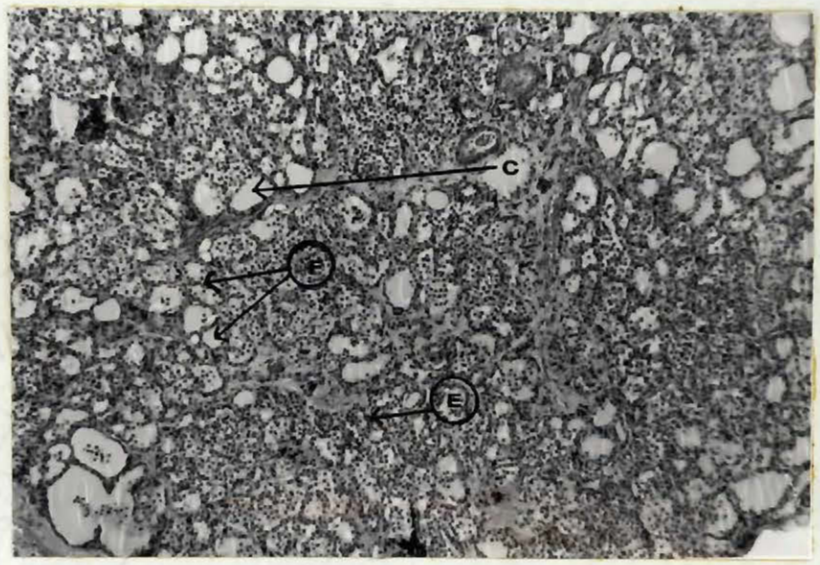
At the 48th hour the glycogen 'spaces' were absent in the parenchymal

Figure 6

THYROID




A. Flat epithelial (E) lining of the follicles (F) and colloid (C) in the thyroid gland of control rat. Hematoxylin-eosin.




B. Thyroid gland of salivarectomized rat. Size of follicles (F) reduced. Moderate epithelial (E) proliferation. The colloid (C) is pale, thin and vacuolated at the periphery. Hematoxylin-eosin.

TABLE VI A

Histological Findings

| Organ | Group | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Salivarectomized | | | | | Control | | | | |
| | Days | | | | | Days | | | | |
| | 1* | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| 1. Liver | | | | | | | | | | |
| Absence of glycogen | - | 8** | 10 | 10 | 10 | - | - | - | - | - |
| Central lobular necrosis | - | - | - | 5 | 10 | - | - | - | - | - |
| Parenchymal hemorrhage | - | - | - | - | - | - | - | - | - | 1 |
| 2. Spleen | | | | | | | | | | |
| Trabecular hemorrhage | - | - | 4 | 6 | 10 | - | - | - | - | - |
| 3. Pancreas | | | | | | | | | | |
| Interlobular connective tissue hem. | - | - | 6 | 8 | 7 | - | - | - | - | - |
| 4. Thymus | | | | | | | | | | |
| Interlobular connective tissue hem. | - | - | 2 | 4 | 4 | - | - | - | - | 3 |
| 5. Muscle (abdominal) | | | | | | | | | | |
| Hemorrhage | - | 2 | 5 | 4 | 8 | - | - | - | - | - |
| 6. Subcutaneous tissue of face | | | | | | | | | | |
| Hemorrhage | - | - | 6 | 8 | 10 | - | - | - | - | - |
| Inflammation | - | - | - | - | - | - | - | - | 2 | 1 |
| 7. Testicle | | | | | | | | | | |
| Hemorrhage | - | - | 2 | 9 | 8 | - | - | - | - | 3 |

TABLE VI B

Histological Findings

| Organ | Group | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Salivarectomized | | | | | Control | | | | |
| | Days | | | | | Days | | | | |
| | 1 | 2 | 3 | 4 | 5 | 1 | 2 | 3 | 4 | 5 |
| 8. Lymph nodes | | | | | | | | | | |
| a. axillary | - | - | 4 | 9 | 10 | - | - | - | - | 1 |
| b. cervical | - | - | 6 | 10 | 10 | - | - | - | 2 | 2 |
| c. mesenteric | - | 8 | 10 | 10 | 10 | - | - | - | 2 | 3 |
| d. pelvic | - | - | 8 | 9 | 10 | - | - | - | - | 4 |
| Accumulation of R.B.C. in sinusoids | | | | | | | | | | |
| 9. G.I. tract | | | | | | | | | | |
| a. stomach | - | 6 | 8 | 8 | 10 | - | - | - | - | 2 |
| b. cecum | - | 4 | 6 | 8 | 10 | - | - | - | - | - |
| Submucosal hemorrhage | | | | | | | | | | |
| 10. Epididymis | | | | | | | | | | |
| Connective tissue hemorrhage | - | - | 6 | 8 | 8 | - | - | - | - | 4 |
| 11. Bladder | | | | | | | | | | |
| Submucosal hemorrhage | - | - | 3 | 5 | 4 | - | - | - | - | - |
| 12. Lung | | | | | | | | | | |
| Hemorrhage | - | - | - | 6 | 7 | - | - | - | - | - |

* Days after the commencement of the dicumarol containing diet.

** Number of rats showing morphological change; each day 10 animals were examined.

cells of the central lobular zone (Figure 7B). Sinusoidal endothelium is patent. The hepatic cells of rats, killed in a state of shock, showed degenerative changes around the central vein. Cellular and nuclear detail unclear. Sinusoids dilated and congested. The necrotic area shows mild hemorrhage (Figure 7C).

Control group

As indicated in Table VI the liver in the controls was patent (Figure 7A) with the exception of one animal, in which the parenchyma below Glisson's capsule showed a small hemorrhagic area (Figure 7D).

(2) Spleen (Figure 8)

Experimental group

Thickening of the central arteriolar wall in the 'pulp'. Small hemorrhagic areas in the trabecular connective tissues, with wide separation of collagen fibers; no evidence of edema fluid; sinusoidal endothelium patent (Figure 8).

Control

No evident morphological change.

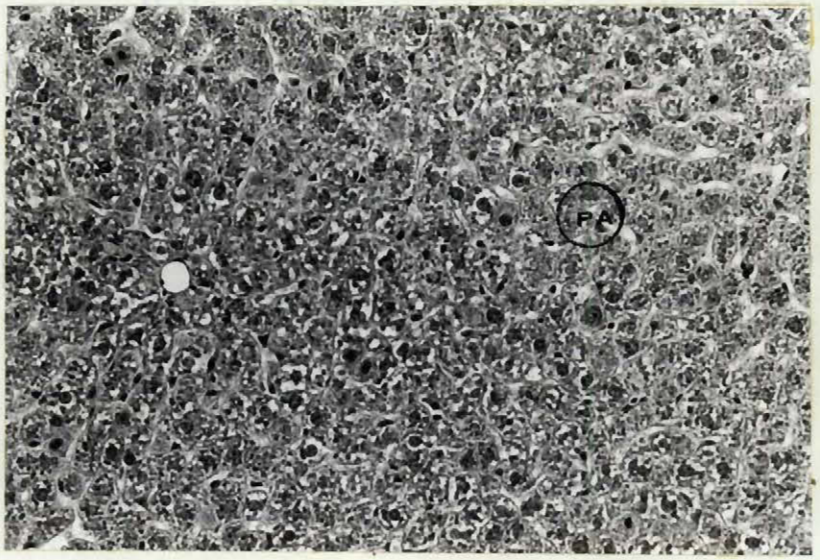
(3) Pancreas (Figure 9)

Experimental group

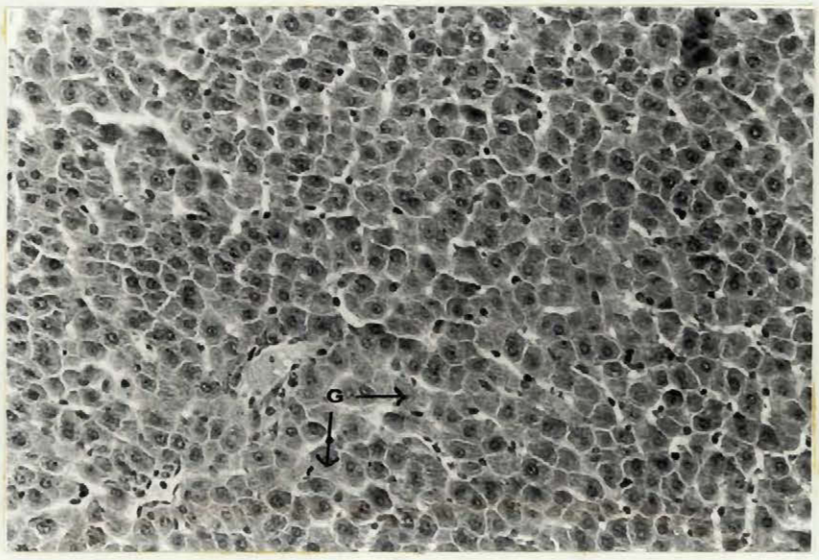
Parenchyma patent. Interlobular connective tissue fibers separated and spaces filled with red cells. No evidence of edema in hemorrhagic areas. Moderate leucocytic infiltration with edema around the hemorrhagic necrotic sites. Thickening of arteriolar and small arterial wall (Figure 9B).

Figure 7

LIVER



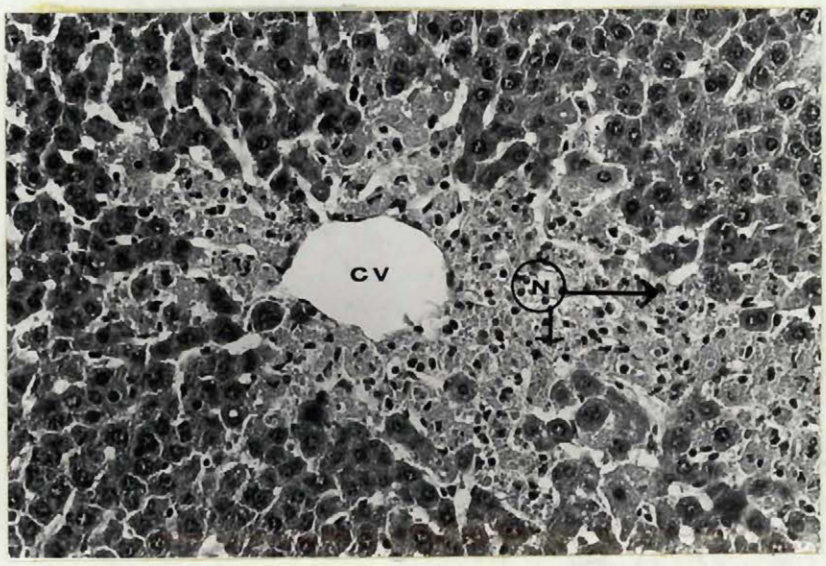

A. Liver of control rat, at the 48th hour on dicumarol containing diet. Parenchyma (PA) intact. Hematoxylin-eosin.



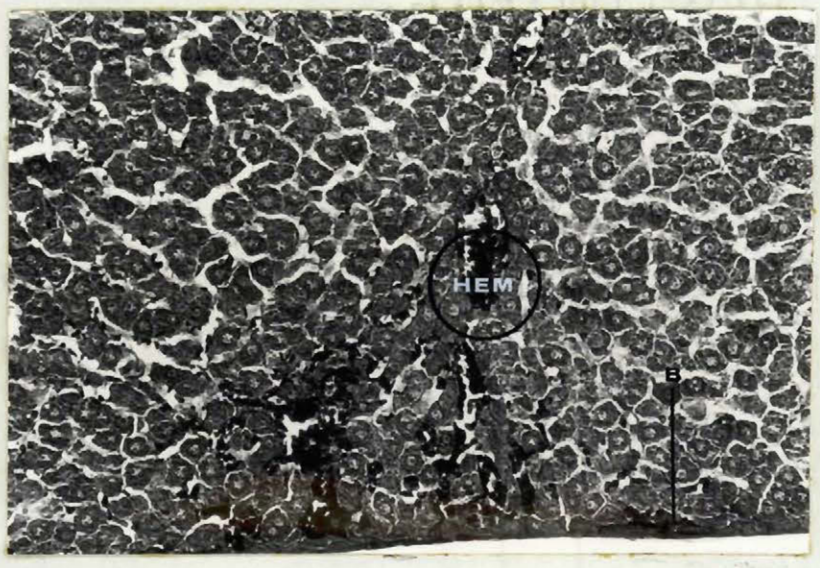

B. Liver of salivarectomized rat receiving dicumarol for 48 hours. Glycogen spaces (G) absent in the parenchymal cells. Hematoxylin-eosin.

Figure 7

LIVER



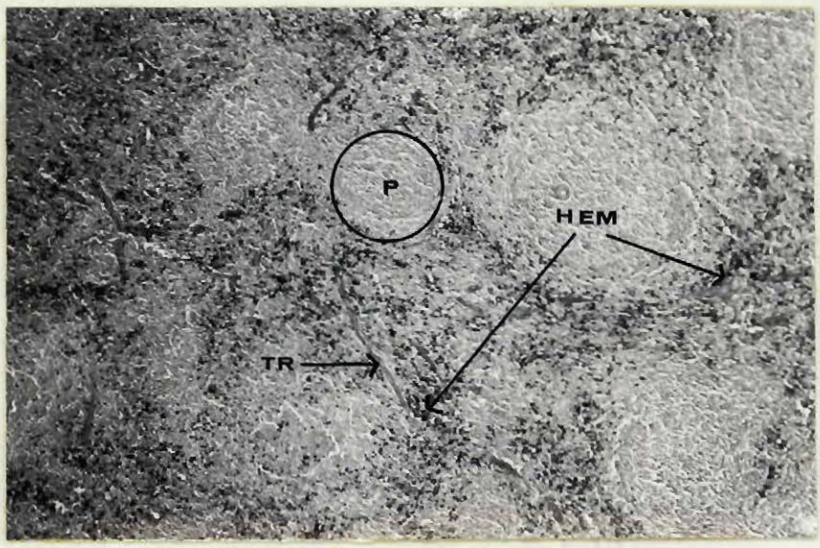

C. Hepatic cells of salivarectomixed rat show atrophy and necrosis (N) around the central vein (CV) 96 hours after dicumarol administration. Cytoplasmic detail undiscernible in the necrotic area. Hematoxylin-eosin.




D. Hemorrhage (HEM) in the liver parenchyma, below Glisson's capsule (B) of a control rat on the 5th day of the hemorrhagic diet. Azan.

Figure 8

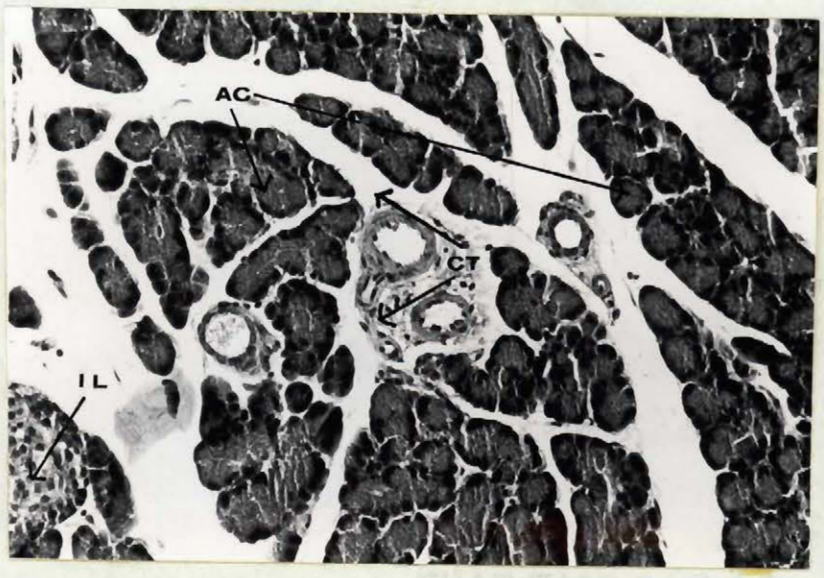
SPLEEN



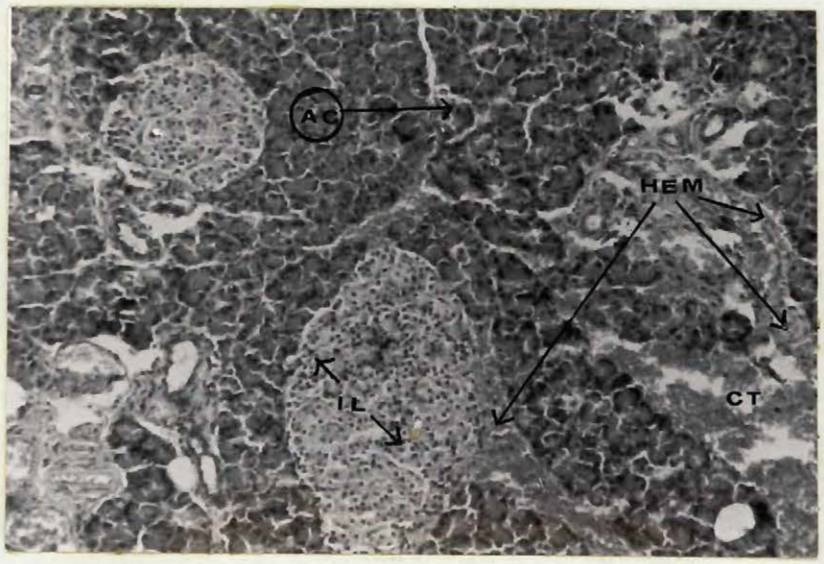

A. Spleen of salivarectomized rat, 72 hours after the commencement of the hemorrhagic diet. The 'pulp' (P) is intact. Hemorrhagic areas (HEM) in the trabecular (TR) connective tissue. Azan.

Figure 9

PANCREAS




A. Pancreas of control rat receiving dicumarol containing diet for 96 hours. Acinar tissue (AC), Islets of Langerhans (IL) and interlobular connective tissue (CT) are intact. Hematoxylin-eosin.




B. Numerous hemorrhagic areas (HEM) in the interlobular connective tissue (CT) spaces and around the Islets of Langerhans (IL) in the pancreas of salivarectomized rat 72 hours after feeding the hemorrhagic diet. Hematoxylin-eosin.

Control

Tissue patent (Figure 9A)

(4) Lymph nodes (Figure 10)

About 8 lymph nodes were examined in each of the rats studied.

Experimental group

Cortical subcapsular sinusoids dilated and filled with red blood cells in the mesenteric lymph nodes at the 48th hour. Sinusoidal endothelium is patent (Figure 10B). These changes extended to the medullary sinusoids as the extent of bleeding progressed in all the lymph nodes studied (Figure 10C).

Control

Three rats, killed on the fifth day, showed aggregations of red blood cells in the peripherial sinusoids of the pelvic lymph nodes. All other specimens were patent (Figure 10A).

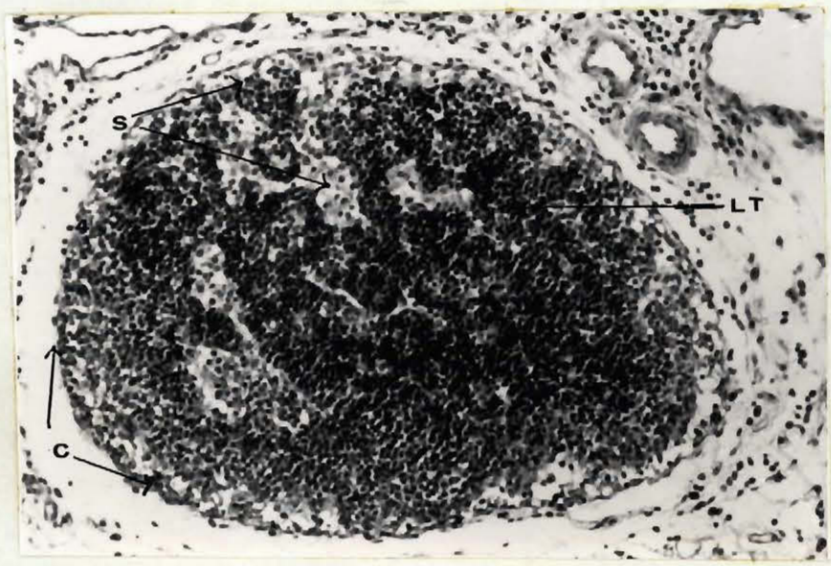
(5) Thymus (Figure 11)

Experimental group

Lymphoid tissue patent; no change evident in epithelial framework. Numerous Hassall's corpuscles visible in centre of lobules. Interlobular connective tissue showed separation of collagen fibers with evidence of massive hemorrhages (Figure 11B). Moderate inflammatory response in some sections around the hemorrhagic segments with perivascular edema and leucocytic infiltration. Marked thickening of the small arterial and arteriolar wall.

Figure 10

LYMPH NODE

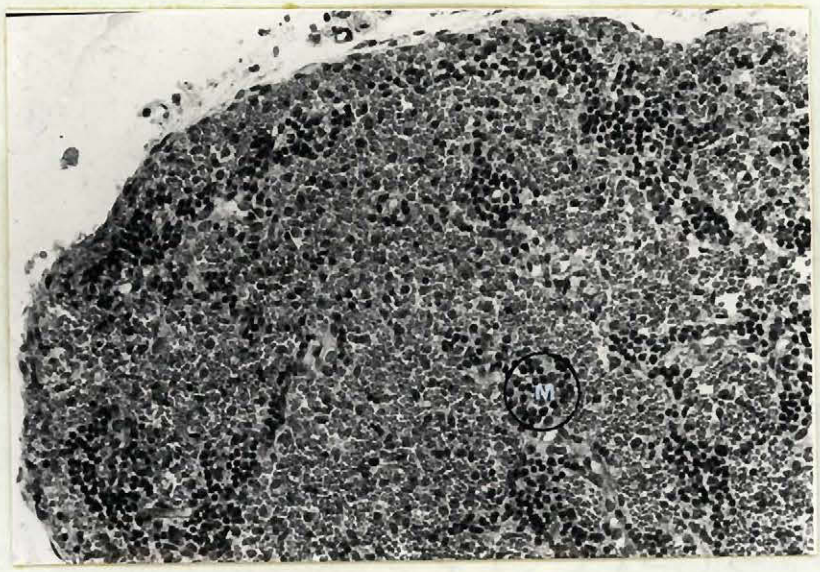



A. Pelvic lymph node of control rat, on hemorrhagic diet (96 hours). Lymphoid tissue (LT), cortical and medullary sinusoids (S), and connective tissue capsule (C) intact. Hematoxylin-eosin.


B. Mesenteric lymph node of salivarectomized rat 48 hours after feeding the hemorrhagic diet. Cortical sinusoids engorged with red blood cells (RBC). Hematoxylin-eosin.

Figure 10

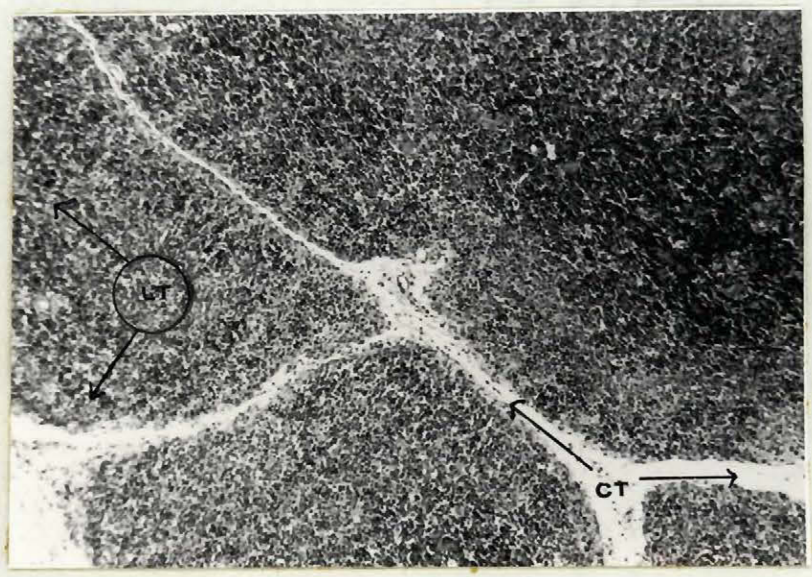
LYMPH NODE



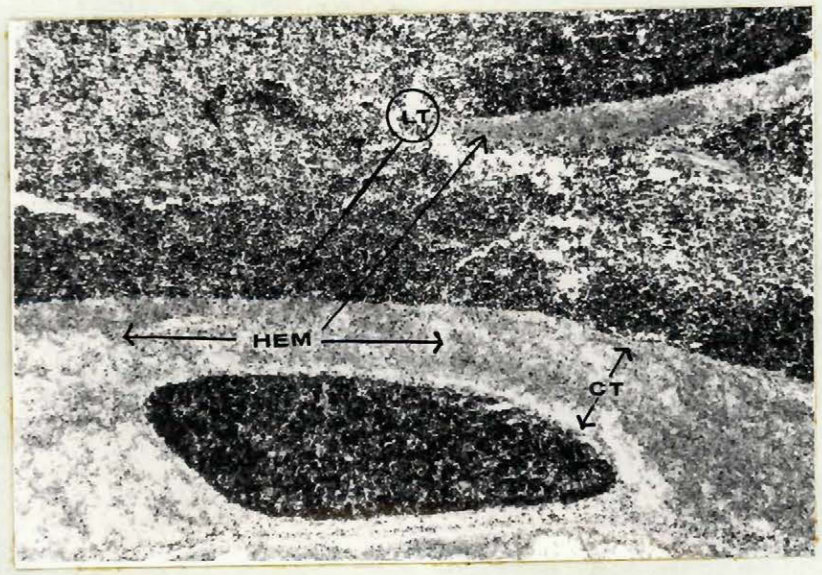

C. Mesenteric lymph node of salivarectomized rat, 72 hours on the hemorrhagic diet. Numerous red blood cells seen in the cortical and also in the medullary (M) sinusoids. Lymphatic tissue intact. Hematoxylin-eosin.

Figure 11

THYMUS




A. Thymus of control rat on dicumarol (96 hours). Lymphatic tissue (LT) with epithelial framework intact. Interlobular connective tissue (CT) well preserved. Hematoxylin-eosin.




B. Thymus of salivarectomized rat on dicumarol (72 hours). Lymphatic tissue (LT) well preserved. Massive hemorrhages (HEM) in the spacious interlobular connective tissue (CT). Hematoxylin-eosin.

Control

Animals with thymic hemorrhages showed changes as in experimental group except, that there was no thickening of the small vessel wall. Other sections examined were patent (Figure 11A).

(6) Muscle (Figure 12)

Experimental group

Specimens from abdominal muscle showed separation of muscle bundles, loss of fibrillar structure and karyolysis. Intercellular spaces filled with blood (Figure 12).

Control

All sections patent.

(7) Subcutaneous tissue of face (Figure 13)

Experimental group

Wide separation of connective tissue bundles of subcutaneum. Hemorrhages in some areas. Edema and venous stasis. Capillary network patent (Figure 13B).

Control

Buccal subcutaneum patent (Figure 13A).

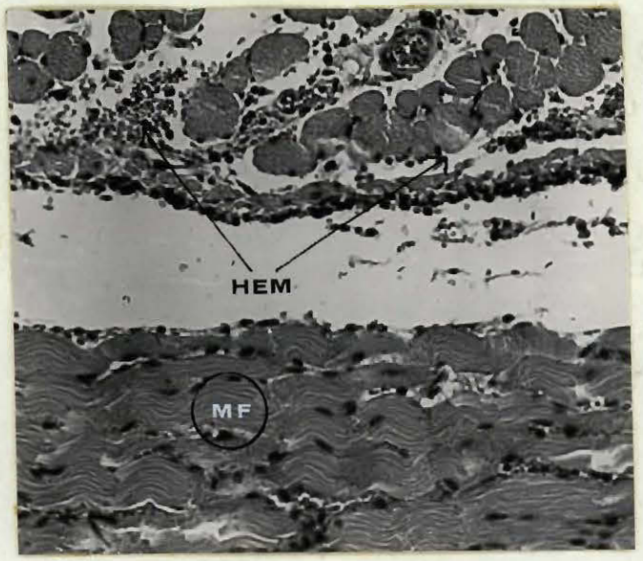
(8) Epididymis and epididymal fat pad (Figure 14)

Experimental group

Epithelial lining, basement membrane and connective tissue capsule

Figure 12

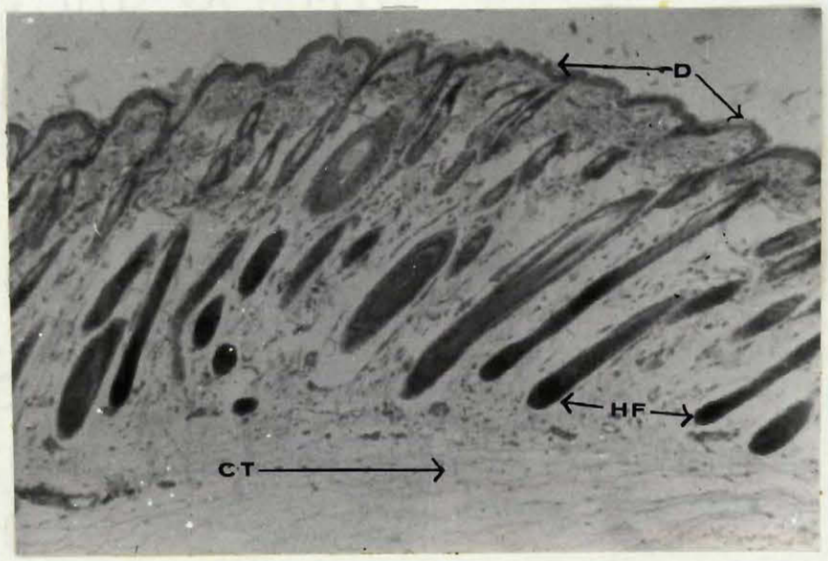
MUSCLE



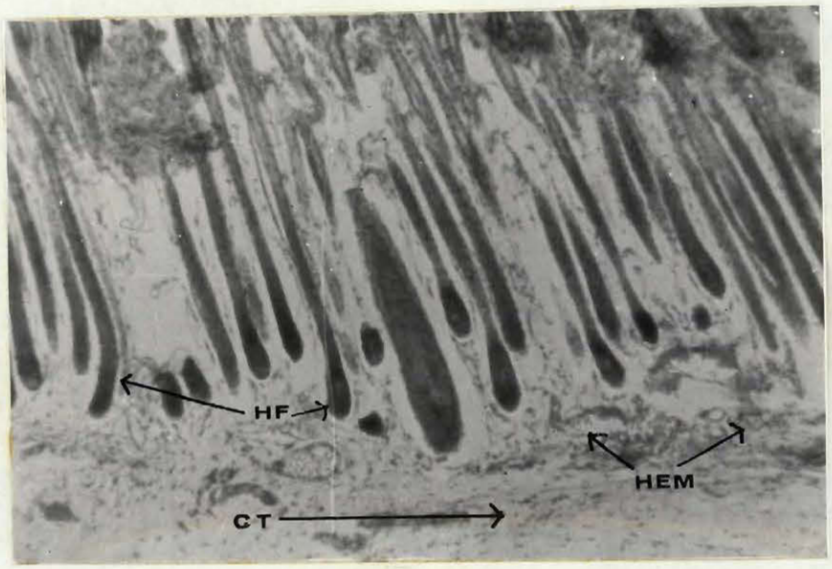

Abdominal muscle of salivarectomized rat on hemorrhagic diet (96 hours). Muscle fibers (MF) separated. Connective tissue between muscle bundles shows degeneration and hemorrhage (HEM). Hematoxylin-eosin.

Figure 13

SUBCUTANEOUS TISSUE




A. Subcutaneous tissue of control rat on dicumarol-containing diet (96 hours). Connective tissue fibers (CT) separated with edema. Hair follicles (HF) well preserved. Hematoxylin-eosin.




B. Subcutaneous tissue of salivarectomized rat on hemorrhagic diet (72 hours). Connective tissue fibers (CT) separated with scattered hemorrhages (HEM).

of ductuli efferentes, patent. Stereocilia well preserved. Arteriolar and small arterial vessel wall showed thickening. Connective tissue in many areas showed massive hemorrhages and separation of connective tissue fibers. Marked perivascular edema around hemorrhagic sites and infiltration by polymorphonuclear leucocytes. Absence of cellular detail in fatty loose connective tissue (Figure 14B). Capillary network patent.

Control

Some hemorrhagic areas visible in loose connective tissue. Other specimens patent (Figure 14A).

(9) Testicle (Figure 15)

Experimental group

Seminiferous tubules patent. No interference with spermatogenesis. Connective tissue network between seminiferous tubules showed separation of connective tissue fibers with bleeding. Hemorrhagic changes mostly below tunica vaginalis. Capillary network absent (Figure 15B).

Control

Similar changes visible in a few control animals. Other specimens are patent (Figure 15A).

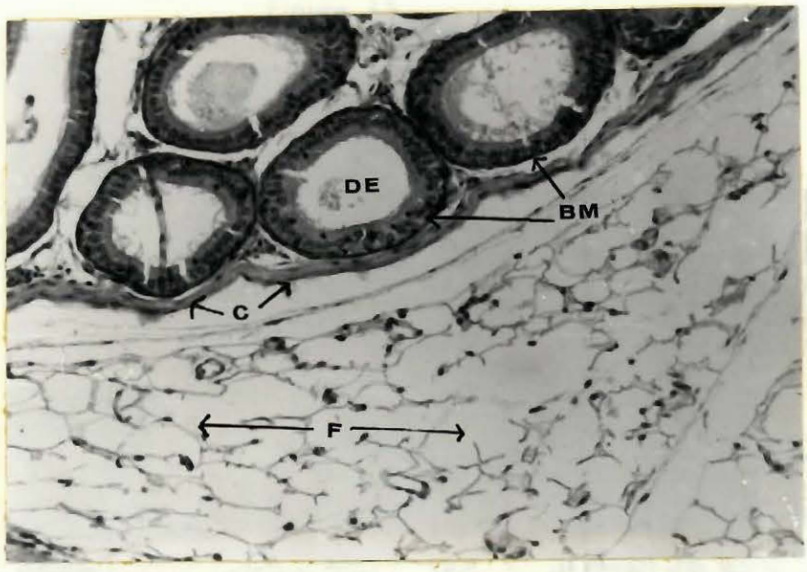
(10) Bladder (Figure 16)

Experimental group

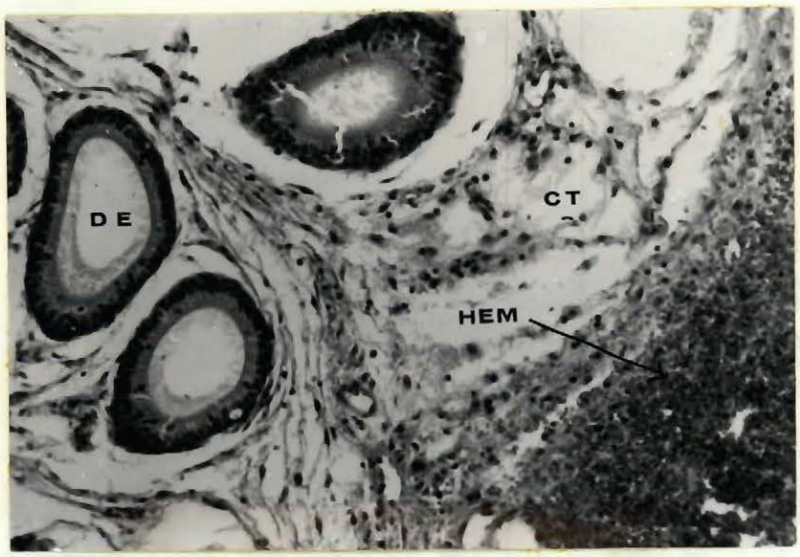
Mucosa intact; submucosal connective tissue fibers widely separated with massive hemorrhages in some areas (Figure 16A).

Figure 14

EPIDIDYMIS



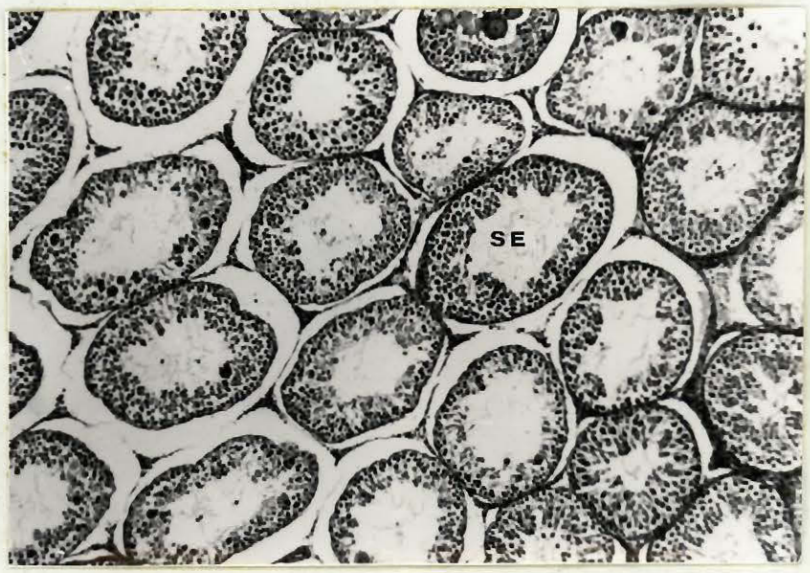

A. Epididymis of control rat on dicumarol (96 hours). Epithelial lining of ductuli efferentes (DE) well preserved. Basement membrane (BM) and connective tissue capsule (C) intact. Loose fatty connective tissue (F) shows no structural alteration. Hematoxylin-eosin.



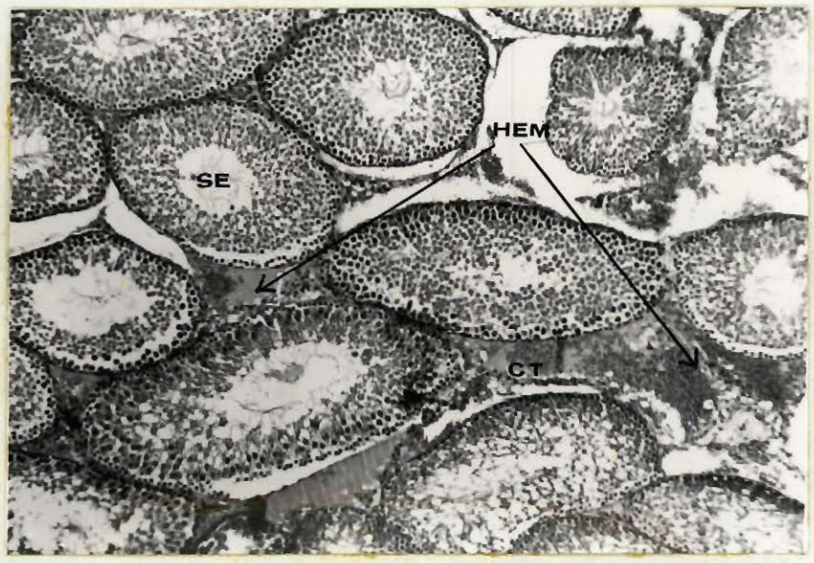

B. Epididymis of salivarectomized rat on hemorrhagic diet (72 hours). Connective tissue (CT) shows separation of fibers with massive hemorrhagic necrosis (HEM). The stereocilia of the epithelial cells of ductuli efferentes (DE) well preserved. Hematoxylin-eosin.

Figure 15

TESTICLE



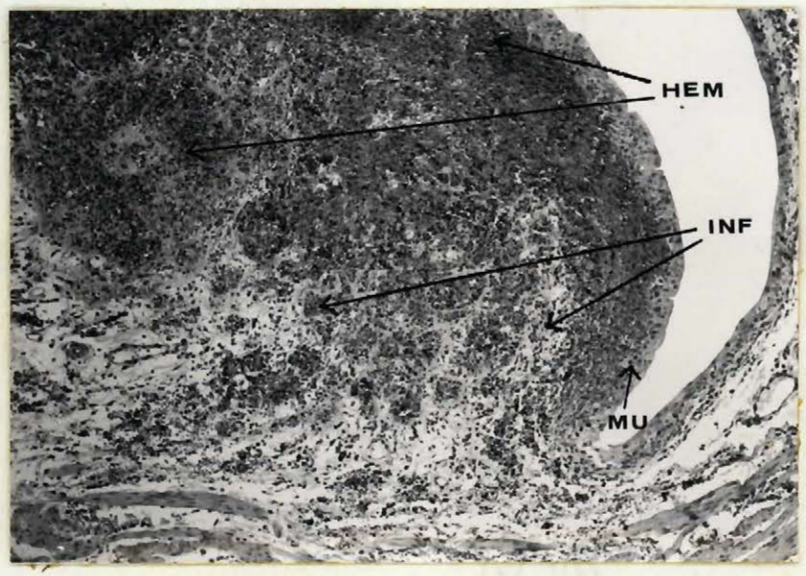

A. Testicular tissue of control rat on the hemorrhagic diet (96 hours). Seminiferous tubules (SE) and intertubular connective tissue well preserved. Hematoxylin-eosin.



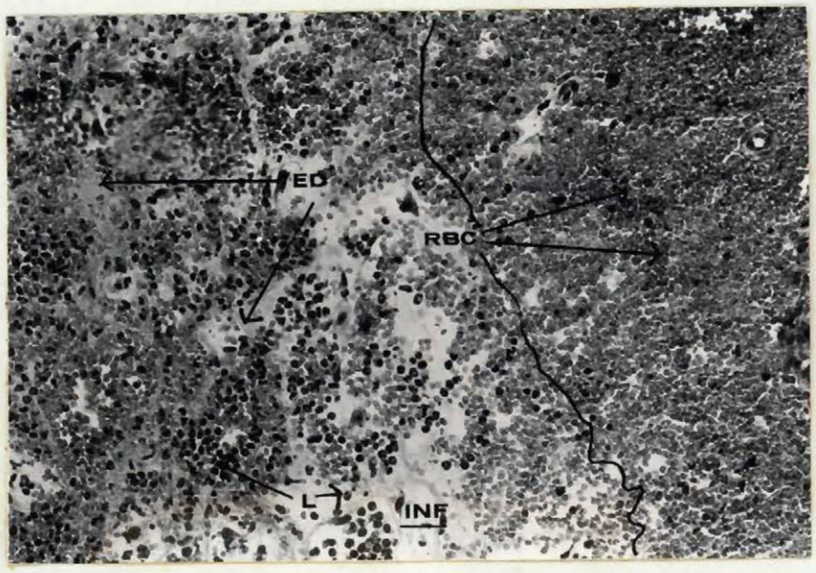

B. Testicular tissue of salivarectomized rat on the hemorrhagic diet (72 hours). Hemorrhages (HEM) in the intertubular connective tissue (CT). Seminiferous tubules intact. Hematoxylin-eosin.

Figure 16

BLADDER




A. Bladder of salivarectomized rat at the 96th hour on the hemorrhagic diet. Mucosa (MU) intact. Submucosal connective tissue shows hemorrhagic areas (HEM) surrounded by areas of inflammation (INF). Hematoxylin-eosin.




B. Larger magnification of specimen A. Hemorrhagic area (RBC) on the right of line shows massive accumulation of red blood cells without the presence of edema. Neighboring inflammation (INF) with edema (ED) separation of connective tissue fibers and moderate leucocytic infiltration (L). Hematoxylin-eosin.

Typical picture presenting the difference between vascular breakdown with massive hemorrhage and that of increased capillary permeability. The hemorrhagic area showed breakdown of connective tissue fibers, disappearance of ground substance by azan and P.A.S. stains, with extravasation of red blood cells, without any apparent edema. In the neighbourhood of the hemorrhagic necrotic region an inflammatory response by the tissues, initiated by the hemorrhage, shows venous stasis, massive edema, scattered leucocytic infiltration without escape of red blood cells from the capillaries, as shown in Figure 16B.

Control

Bladder tissue patent.

(11) Gastrointestinal tract (Figure 17)

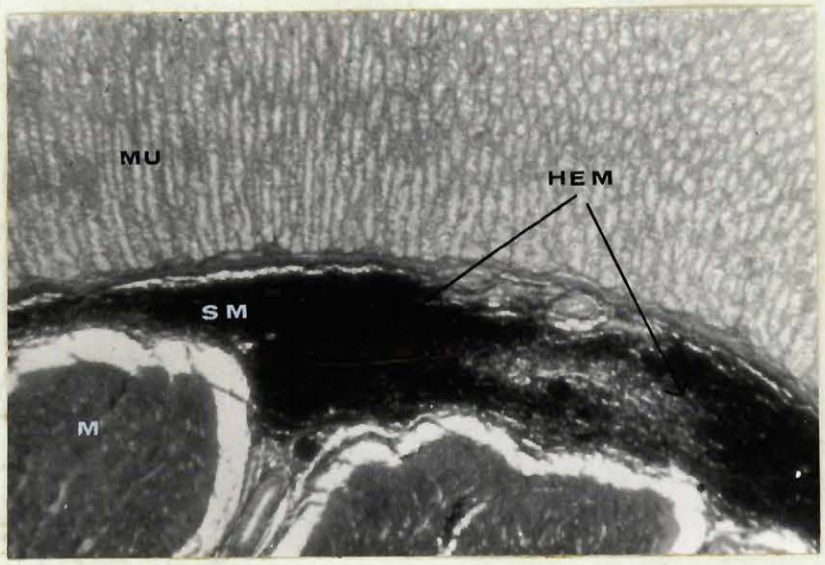
Experimental group

Small submucosal hemorrhages all along the gastrointestinal tract; most prominent in the pyloric region of the stomach and in the wall of the cecum. Mucosa in all specimens intact. Hemorrhagic areas prominent 48 hours after dicumarol administration. Wide separation of connective tissue fibers in hemorrhagic areas. Inflammatory changes in surrounding tissues. Submucosal region is greatly enlarged. In some sections the muscularis mucosa shows separation of muscle bundles with absence of cellular and nuclear detail (Figure 17).

Control

Figure 17

STOMACH




Stomach of salivarectomized rat on dicumarol containing diet for 72 hours. Mucosa (MU) intact, submucosal connective tissue (SM) shows massive hemorrhages (HEM). Muscle bundles (M) well preserved. Azan.

Only two of the animals showed gastric hemorrhages with typical accompanying changes five days after the feeding of the hemorrhagic diet.

(12) Lung (Figure 18)

Experimental group

Lower right lobe showed massive hemorrhages with moderate inflammatory response. Alveolar structure is absent in some sections, in others alveoli is filled with edema fluid and red blood cells (Figure 18B).

Control

Lungs of control rats intact (Figure 18A).

## CONCLUSIONS

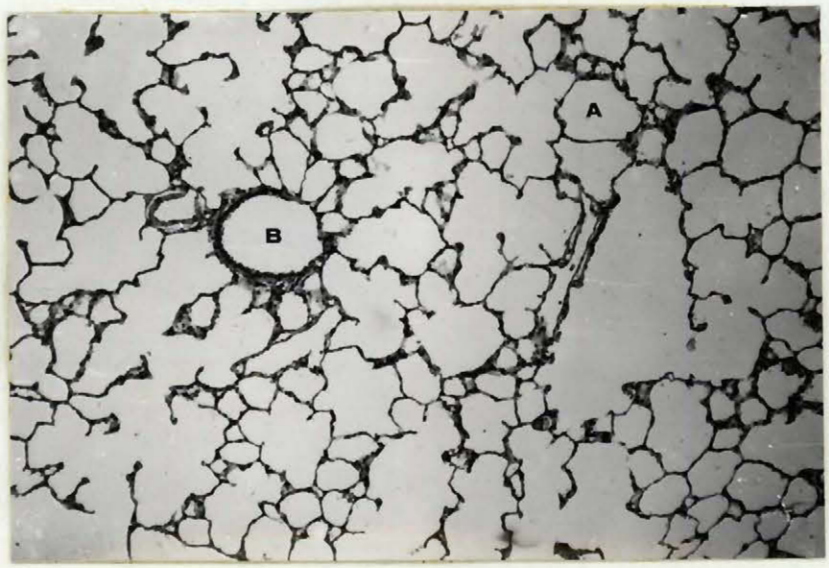
Removal of the submaxillary glands results in hyperplasia of the thyroid gland. To what extent this represents hyperactivity of the glands was investigated by measurement of the plasma bound iodine (P.B.I.) (see Table VII), organ weights and heart rate compared to the corresponding values of sham operated controls.

Endothelial and medial proliferation were evident in the small arteries and arterioles. These are characteristic of the early changes that accompany hypertension (172), which, if present, would increase the tendency toward bleeding.

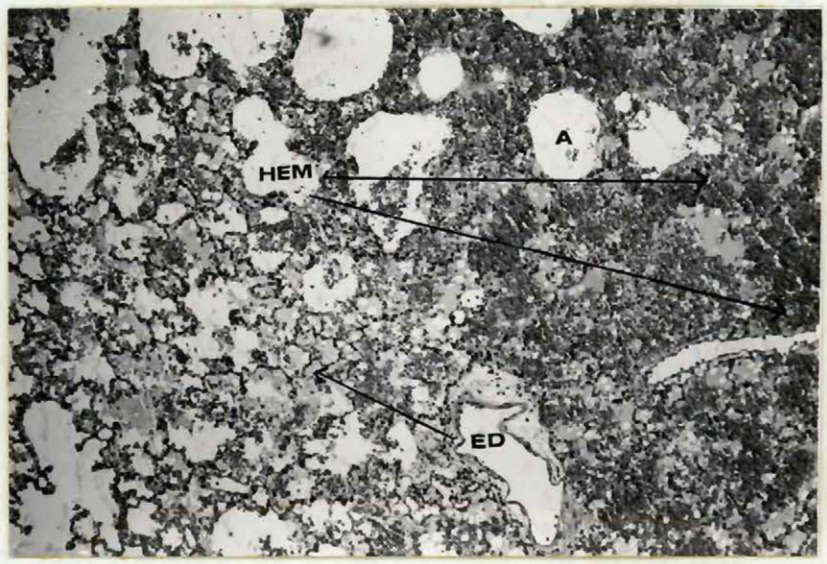
The observation that the parenchyma of the various organs, except

Figure 18

LUNG




A. Pulmonary tissue of control rat on the hemorrhagic diet (96 hours). Alveoli (A) capillary and bronchioli (B) well preserved. Hematoxylin-eosin.




B. Pulmonary tissue of salivarectomized rat on the hemorrhagic diet (72 hours) shows massive hemorrhages (HEM) and edema (ED) with scattered leucocytic infiltration. Hematoxylin-eosin.

in the liver and the lung was intact while the loose connective tissues showed degenerative changes with accompanying hemorrhages, supported our earlier impression, that spontaneous vascular breakdown is closely associated with the state of the perivascular tissues.

That the hemorrhages do not originate at the capillary level, but in the region proximal to the capillaries proper, is indicated by the absence of any capillary abnormality in the hemorrhagic areas as is seen with the light microscope.

The absence of evidence of edema in the hemorrhagic lesions lends further support to the view, that vascular breakdown and increased capillary 'permeability' are distinct phenomena (125).

The presence of edema and massive leucocytic infiltration without extravasation of red blood cells in the neighbourhood of the hemorrhagic areas, represents a true inflammatory response at the capillary level to hemorrhagic necrosis.

The occurrence of early hemorrhagic changes in the gastrointestinal tract and the accumulation of red blood cells in the mesenteric lymph nodes, were coincident with the loss of weight of the experimental rats and the disappearance of glycogen from the hepatic cells. These changes are indicative of impairment of intestinal absorption, thus causing virtual starvation of the animal, despite continued ingestion of food.

The central lobular necrosis of the liver lobules could be attributed to the direct toxic effect of dicumarol. However, considering that animals

that showed this type of change were in a state of shock, from the massive internal bleeding, the condition might be nonspecific, the result of anoxia.

# 3. ARTERIOGRAPHIC DEMONSTRATION OF THE PROGRESSION OF VASCULAR FRAGILITY

## INTRODUCTION

This study arose out of the repeated observations, that salivarectomized rats begin to loose weight about 48 hours after they are placed on the dicumarol containing Purina diet. This is illustrated in a typical experiment represented in Figure 4. The weight loss occurred despite little or no reduction in the food intake, as indicated in Figure 4, and with an extensive decrease in the glycogen content of the liver parenchymal cells, as revealed by histological examination (Figure 7B) and by chemical analysis (Figure 22). The fall in body weight attributed to alteration in the state of the wall of small vessels of the intestinal tract, with impairment in food adsorption, is suggested by histological evidence of the accumulation of red blood cells in the dilated cortical sinusoids of the mesenteric lymph nodes as shown in Figure 10B.

The idea occurred to the author, that radiography might be useful for showing the progressive increase in "fragility" or weakness of the vascular bed of the intestinal submucosa, in rats maintained on a dicumarol containing diet. Arteriography has been widely used for studying the vasculature of organs in the normal and in the pathological states (173, 174). By careful control of the infusion pressure, it is possible to fill the arterial network down to the small arterioles of diameter less than

40 microns. The radio-opaque fluid does not enter the capillary vessels. To our knowledge, our experiment represents the first effort to use the method for demonstrating progressive vascular fragility.

## METHOD

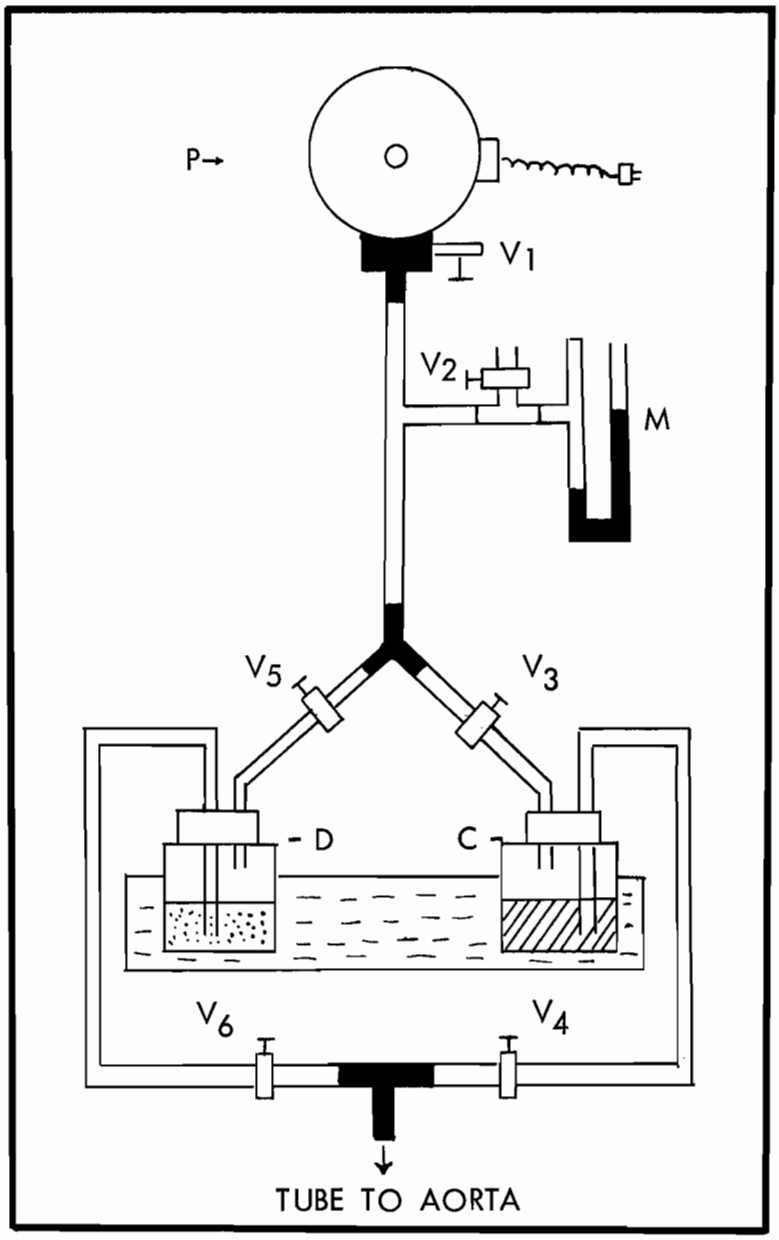
Eighty male albino rats were divided equally into a control (sham operated) and an experimental (salivarectomized) group, and prepared as previously described (page 41). The two groups were placed on the dicumarol containing Purina ration.

Just before the feeding of the hemorrhagic diet, and every day thereafter, throughout the experimental period of four days, 8 animals from each group were prepared for arteriographic examination.

Ether anesthesia was used. The thoracic cavity was opened and the aorta and inferior vena cava exposed. A polyethylene tube (Clay Adams, Size P.E. 160) filled with physiological saline at 37°C was inserted into the lumen of each vessel and secured with silk ties. The aortic tube was attached to a T-tube, T as shown in Figure 19, the two arms of which were connected to the reservoirs C and D respectively. The reservoir C contained physiological saline and D the radio-opaque medium, prepared according to the method of Schlesinger (175), were immersed in a water bath at 37°C. The pressure for infusion was applied by the pump P and was regulated by adjustment of valves $V_1$ (coarse adjustment) and $V_2$ (fine adjustment).

Figure 19

Apparatus Used for Arteriography




P - Electric pump.

$V_1$ - Coarse adjustment valve.

$V_2$ - Fine adjustment of pressure.

D - Reservoir containing contrast medium.

C - Reservoir containing physiological saline.

M - Manometer

The vascular tree first was washed free of blood with normal saline, by closing the clamps $V_5$ and $V_6$ and opening $V_3$ and $V_4$, with the pressure maintained at 100 mm Hg. Perfusion was continued until the fluid escaping from the inferior vena cava was almost colorless. The pressure was reduced to zero (manometric pressure) and clamps $V_3$ and $V_4$ were closed. Infusion of the contrast medium followed, by opening clamps $V_5$ and $V_6$, under a pressure of 110 mm Hg. The maximum filling of the vascular tree was indicated by cessation of the flow of the fluid in the aortic tube. The aorta then was clamped, the tubes removed and the carcass placed in the refrigerator at about 5°C for an hour, to permit solidification of the contrast medium (gelatin) in the vasculature.

Radiographs were taken with a Phillips Standard DLX instrument using 40 m.a. per sec. at 60 K.V. at a distance of 40 inches from the specimen.

## RESULTS

As illustrated in Figure 20A, there was no evidence of escape of medium from the arterial tree, in the control animals, throughout the experimental period.

In the experimental animals, as indicated in Figure 20B, there was evidence of extravasation of the contrast medium at the end of 48 hours. The radio-opaque fluid was confined to the perivascular areas of the

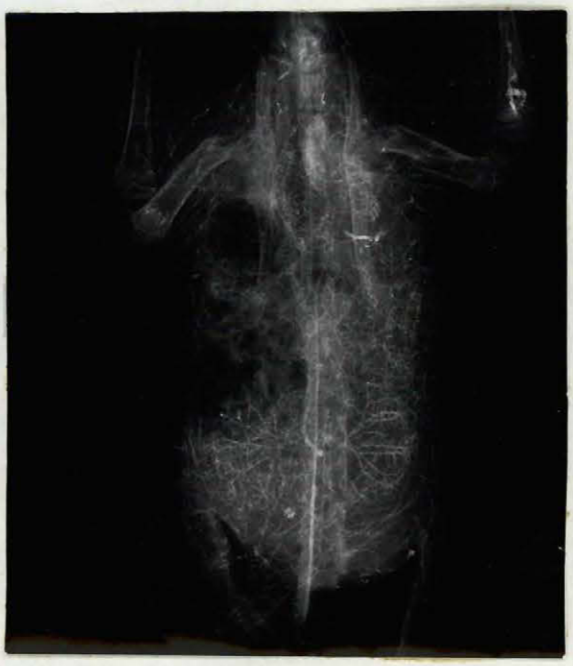
Figure 20

ARTERIOGRAPHIC DEMONSTRATION OF VASCULAR FRAGILITY

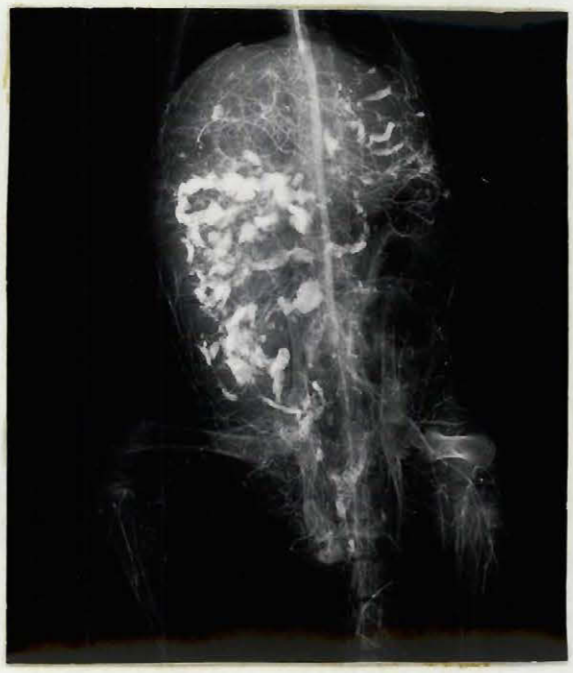
A. Arterial tree of control rat after 96 hours on the dicumarol containing diet.

B. Arteriogram of experimental rat 48 hours on the hemorrhagic diet shows leakage of contrast medium from the vessels of the intestinal tract.

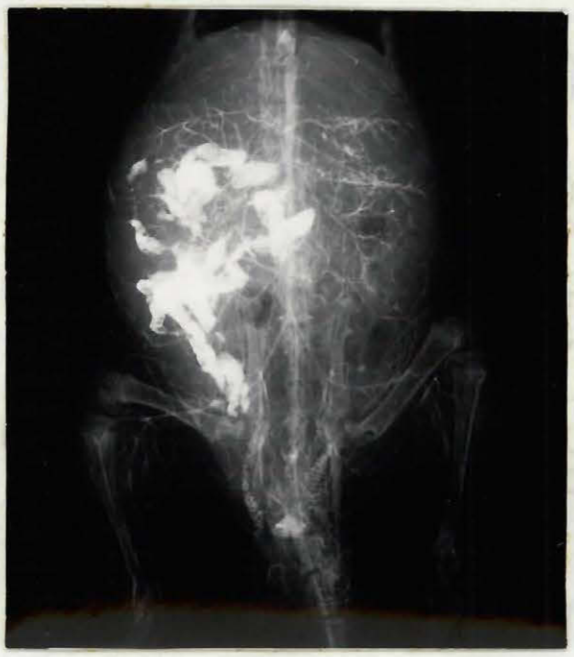
C. Massive escape of contrast medium from the vasculature of the intestinal tract of an experimental rat 72 hours on the hemorrhagic diet.



A



B



C

intestinal tract as shown in Figure 20C, at the end of 72 hours.

## CONCLUSIONS

Since the infusion pressure was maintained at 110 mm Hg, which was the mean systolic pressure of the control rats (Table VIII), the observed extravasation of the radio-opaque fluid from the arterial network in the salivarectomized animals, is clear evidence of the rapid progress of vascular fragility. The time, when vascular fragility becomes evident by arteriography, coincided with that of the first signs of accumulation of red blood cells in the mesenteric lymph nodes.

As the contrast medium does not enter the capillaries, the state of fragility seems to begin at the arteriolar or small arterial segment of the vascular network. This weakness of the vessel wall may result from the increased sensitivity of the vessel to the toxic effects of dicumarol, as evident in the pathological changes, indicated in the histological study (Figure 5C).

This author inclines to the view, that vascular fragility results from the direct toxic action of dicumarol on the small vessel wall.

## 4. INFLUENCE OF THE REMOVAL OF THE SUBMAXILLARY SALIVARY GLANDS ON THE BLOOD PRESSURE OF THE RAT

### INTRODUCTION

In a previous section (page 52) reference was made to the observation of a pronounced thickening of the wall of the small arteries and arterioles, in the salivarectomized group. This abnormality, when accompanied by pathological changes in the kidneys, heart and adrenal glands, could indicate the presence of hypertension. If fragility of the small vessels were to be associated with sustained elevation of the blood pressure, the condition would favor the occurrence of spontaneous internal bleeding.

### METHODS

(1) Preparation of animals

Twenty-four male albino rats of 60 g body weight were used. Twelve were salivarectomized, and the other twelve subjected to the sham operation. The animals were kept, 3 in a cage and were fed Purina rat Chow for 35 days. The body weights were recorded each day and the urinary output was measured daily from the 25th to the 35th day, when the animals were prepared for blood pressure measurement.

(2) Blood pressure measurement

The rats were anesthetized with Pentobarbital (50 mg per kg). The carotid artery was exposed and canulated with a polyethylene tube which was

connected through a pressure transducer to a San-born Recording apparatus. The blood pressure was measured for 15 minutes. The values recorded during the first 5 minutes were not used for the calculation of average systolic and diastolic pressure. The heart rate during this period also was recorded. At the end of the experiment the carotid artery was closed with a small clamp.

(3) Studies on the blood and the tissues

After the blood pressure was measured, about 5.0 ml of blood were taken by cardiac puncture from each animal. The blood was allowed to clot and centrifuged at 3,000 r.p.m. for 15 minutes. The serum was sent to the Reference Laboratory, North Hollywood, California, for estimation of the protein-bound iodine (PBI).

From each animal the heart, kidneys, adrenal and thyroid glands were removed, and the weight of each organ recorded. Specimens of the tissues were fixed in 10 per cent formaldehyde for 24 hours, and prepared for histological examination as previously described (page 52).

RESULTS

As noted earlier in figure 3, the gain in weight by the salivarectomized and sham operated rats was comparable up to the third week. Thereafter the growth rate of the experimental animals declined rapidly compared to that of the controls. At the end of the fifth week the difference between the average body weight of the two groups, as indicated in Table VII, was about

TABLE VII

Organ Weight, Urinary Output and Protein-Bound Iodine

| Estimation | Mean Values in the Two Groups | |
|---|---|---|
| | Salivarectomized | Control |
| Average body weight (g) | 258 ± 1.1* (12)** | 305 ± 1.56 (12) |
| Relative heart weight (mg/100 g body wt.) | 355 ± 1.57 (12) | 318 ± 1.25 (12) |
| Relative kidney weight (mg/100 g body wt.) | 382 ± 1.26 (24) | 359 ± 1.38 (24) |
| Average weight of adrenal (mg/100 g body wt.) | 17.2 ± 1.6 (24) | 18.3 ± 1.2 (24) |
| Thyroid weight (mg/100 g body wt.) | 7.55 ± 0.33 (24) | 6.35 ± 0.29 (24) |
| Protein-bound iodine*** (mg/100 ml serum) | 9.35 ± 1.25 (10) | 5.6 ± 0.11 (10) |
| Average urinary output | 6.0**** | 12.0 |

All rats were killed 35 days after removal of the submaxillary salivary glands. Controls were sham operated.

* Standard error of mean.

** Number of measurements.

*** Serum protein-bound iodine values were estimated, by the Reference Laboratory, North Hollywood, California.

**** Urinary output was measured daily for 10 days in the 12 salivarectomized and 12 control animals.

50 grams.

As indicated in the same Table, the weights of the heart, kidneys and thyroid glands were significantly increased in the salivarectomized animals.

The concentration of PBI of the experimental rats was higher than that of the control animals. This finding, with the hyperplasia of the thyroid gland, as indicated in Figure 6B, suggests the existence of a state of hyperthyroidism. As indicated in Table VII, the mean daily urinary output of the salivarectomized animals was only half that of the control group.

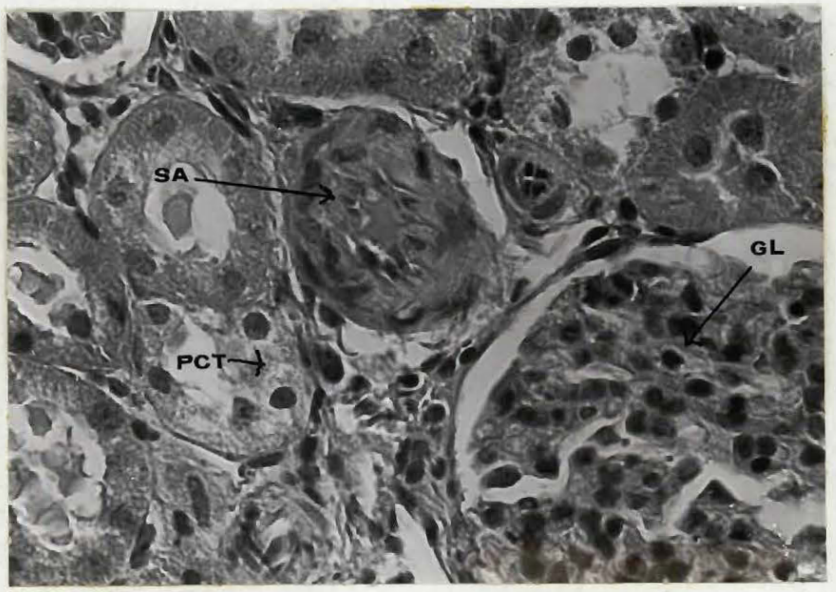
Histological examination of the heart, revealed hypertrophy of the muscle fibers in the left ventricle, as shown in Figure 21B.

No morphological changes were detectable in the kidneys, except a thickening of the small arterial and arteriolar wall as shown in Figure 21A. It is manifested by intimal (endothelial lining), and medial (muscle coat) proliferation. These interpretations were confirmed by Dr. H.C. Stary of the Department of Pathology of McGill University. There was no change in the mucopolysaccharide component (PAS staining), in the elastic fibers (elastin staining), nor in the collagen component (azan staining) of the vessel wall. These changes are characteristic of moderate hypertension (172).

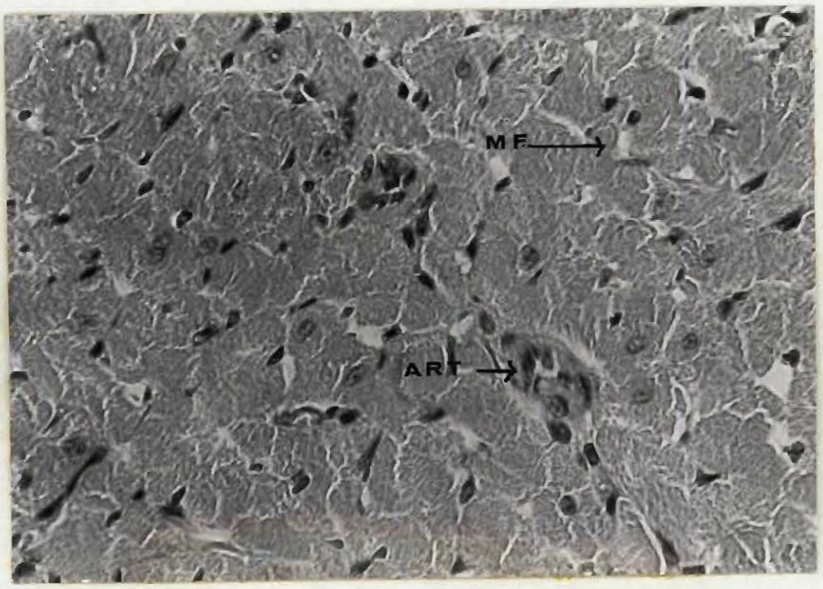
Further substantiation was indicated also, by the absence of pathological change in the glomeruli or in the tubules of the kidneys. (Cloudy swelling of the epithelium of the proximal convoluted tubules is evident in advanced cases of hypertension (176).

Figure 21

KIDNEY AND HEART




A. Kidney of salivarectomized rat, 35 days after operation. Glomerulus(GL) and proximal convoluted tubules (PCT) intact. Wall of a small artery near the glomerulus shows intimal and medial proliferation. Hematoxylin-eosin.




B. Heart muscle of salivarectomized rat 35 days after operation shows hypertrophy of left ventricular muscle fibers (MF). Wall of arteriole (ART) shows thickening. Hematoxylin-eosin.

No change occurred in the weight, or was evident in the histological appearance of the adrenal glands in the two groups.

As indicated in Table VIII, the blood pressure in the salivarectomized animals was significantly higher than that in the sham operated control group (under Nembutal anesthesia). Both the systolic and the diastolic pressure were increased. The pulse pressure likewise was elevated.

The heart rate in both groups remained the same.

## CONCLUSIONS

A search of the literature failed to reveal any reference to the influence of salivarectomy on the blood pressure, or to any connection between the physiology of the salivary glands and hypertension.

The significance of the elevated blood pressure in the experimental animals, along with the increased weight of the kidney and heart, strongly suggestive of a state of hypertension.

The increase in the pulse pressure, and the hypertrophy of the muscle fibers in the left ventricle of the heart are further evidence of the circulatory anomaly.

The elevated protein-bound iodine level, with an increase in the weight of the thyroid gland is in agreement with the hyperplasic changes found previously in the thyroid glands of the salivarectomized animals as indicated in Figure 6B. These changes are characteristic of hyperthyroidism. An elevation of the blood pressure may have resulted from hyperthyroidism.

TABLE VIII

Hemodynamic Changes in Salivarectomized Rats

| Measurement | Group | |
|---|---|---|
| | Salivarectomized | Control |
| Mean systolic pressure mm. Hg | 162 ± 1.7* (12)** | 113 ± 1.4 (11) |
| Mean diastolic pressure mm. Hg | 112 ± 1.2 (12) | 86 ± 1.2 (11) |
| Pulse pressure*** mm. Hg | 50.0 | 27.0 |
| Mean heart rate beats per minute | 390 ± 2.2 (12) | 387 ± 2.1 (11) |

* Standard error of mean

** Number of measurement

*** Pulse pressure is the difference between systolic and diastolic pressure.

The uniformity of the heart rates between the two groups however, makes this deduction unlikely.

The decreased urinary output from the salivarectomized rats, without any decrease in water intake, could indicate an accelerated reabsorption of water with a concomitant increase in blood volume. The increase in the circulating blood volume may then result in hypertension.

Unfortunately, lack of time did not permit investigation of other biochemical aspects of interest, such as the increased production of catecholamines (177), angiotensin (177), aldosterone (178), retention of sodium and its influence on the sensitivity of the sympathetic nerve endings to catecholamines (179).

The presence of hypertension in the salivarectomized rat, in addition to vascular fragility, predisposes the animal to spontaneous bleeding.

## 5. FOLLOW-UP BIOCHEMICAL STUDIES

### INTRODUCTION

The findings described in the preceding sections, in particular (1) the fall in the body weight of the salivarectomized animals despite the continued consumption of the dicumarol-containing diet, (2) histological evidence of the disappearance of glycogen from the liver parenchymal cells, after 48 hours of feeding the experimental ration, in the experimental group, (3) central lobular necrosis in the liver, and (4) the arteriographic and histological

evidence of the presence of vascular fragility in the submucosal vessel of the gastrointestinal tract as a result of dicumarol feeding, all point to a state of impaired intestinal absorption, and deprivation of the liver and tissues of nutriment. The animal thus is virtually in a state of starvation, notwithstanding the continued food intake.

If the foregoing conclusions be correct, one may anticipate a variety of metabolic consequences in the salivarectomized animals. An effort was made biochemically to ascertain evidence of the following: alteration in the respiratory activity of the liver; fall in the concentration of liver glycogen; increase in urinary nitrogen excretion; rise in the concentration of dicumarol in the blood and the tissues and increased excretion in the urine; alteration in the level of prothrombin; increase in serum glutamic-oxaloacetic transaminase (SGOT); and increased urinary excretion of carbohydrate, reflecting breakdown of mucopolysaccharides.

For convenience to the reader the description of each method and the results will be treated together in this section.

## EXPERIMENTAL

### (1) General procedure

Two dozen male white rats of uniform body weight (60 g) were used in each of 4 repeated studies. In each experiment the animals were equally divided into an experimental and a control group and prepared as previously described (page 41). The animals were kept, two in a metabolic cage, and

each day the body weight, the food intake were recorded, and the combined urine output collected.

Each day two animals from each group were removed, blood samples were taken by heart puncture by means of a silicone-coated syringe.

Two ml of the blood from each animal were transferred to a silicone-treated tube and the specimens centrifuged. The plasma transferred to a tube containing 1.34 per cent (w/v) sodium oxalate solution (9 parts of plasma to 1 part of oxalate). These specimens were kept in the cold until used (with a minimum of delay) for estimation of the prothrombin time.

The remainder of the blood was permitted to clot, the specimen centrifuged at 3,000 r.p.m. for 15 minutes and the serum removed for the estimation of dicumarol and SGOT.

The animals then were killed by decapitation, the liver quickly removed and divided into two portions. One portion was used for the estimation of glycogen and dicumarol, and the other washed with saline, chilled immediately and used for measurement of the respiratory activity.

Dicumarol was estimated also in a specimen of the abdominal muscle, intestine and of epididymal fat pad.

(2) Analytical methods and results

(a) Respiratory activity of liver

Four slices from each liver were prepared with a precooled Stadie microtome, weighed and placed in Warburg flasks containing 2.5 ml cold Krebs-Ringer-phosphate buffer, pH 7.4 (180) and a filter paper strip, saturated

with 10 per cent KOH, in the center well. In two of the vessels (duplicates) 0.5 ml of the substrate (0.035 M glucose) was placed in the side arm. The substrate was omitted from the other two vessels and the volumes were made up with the buffer solution. The vessels were equilibrated in the bath at 37°C for 10 minutes. The substrate was then added and the oxygen consumption was measured at 15 minute intervals during the experimental period of 75 minutes. The results were expressed as microliters of $O_2$ consumed per mg (wet weight) of tissue per hour. The results are given in Table IX.

The oxygen consumption of the liver slices from the salivarectomized and the control rats was not altered during the five-day period of feeding the hemorrhagic diet.

(b) Liver glycogen

Glycogen was determined colorimetrically with the anthrone method (181). Portions of the liver were frozen on dry ice, weighed and then digested in 30 per cent KOH solution at 100°C. The glycogen was precipitated with 95 per cent ethanol, washed and finally redissolved. The anthrone reagent was added and the color density measured with a Beckman Junior spectrophotometer at 620 mμ. Each estimation was run in duplicate.

The glycogen standard solution contained 50 mg per litre and was stored in the cold. A standard curve was prepared with each run. The final results were expressed as mg of glycogen per gram wet weight of liver tissue.

As was expected from the histological evidence (Figure 6B) the liver glycogen level in the experimental group fell off rapidly as shown in Figure 22.

TABLE IX

Respiratory Activity of Liver of Salivarectomized and Control Rats

| Time of sampling | Group | | | |
|---|---|---|---|---|
| | Salivarectomized | | Control | |
| | Without substrate | With substrate | Without substrate | With substrate |
| (days)* | (microlitre $O_2$ consumed per mg tissue per hour) | | | |
| 0 | 1.2[a] ± 0.5** | 1.4 ± 0.2 | 1.4 ± 0.2 | 1.5 ± 0.2 |
| 1 | 1.2 ± 0.3 | 1.4 ± 0.1 | 1.5 ± 0.1 | 1.5 ± 0.1 |
| 2 | 1.1 ± 0.2 | 1.5 ± 0.2 | 1.4 ± 0.2 | 1.5 ± 0.2 |
| 3 | 1.3 ± 0.2 | 1.5 ± 0.1 | 1.3 ± 0.2 | 1.4 ± 0.2 |
| 4 | 1.0 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.2 | 1.4 ± 0.1 |
| 5 | 1.2[b] ± 0.1 | 1.3[b] ± 0.2 | 1.4 ± 0.1 | 1.4 ± 0.1 |

* Day after the commencement of the dicumarol (0.5%) containing diet.

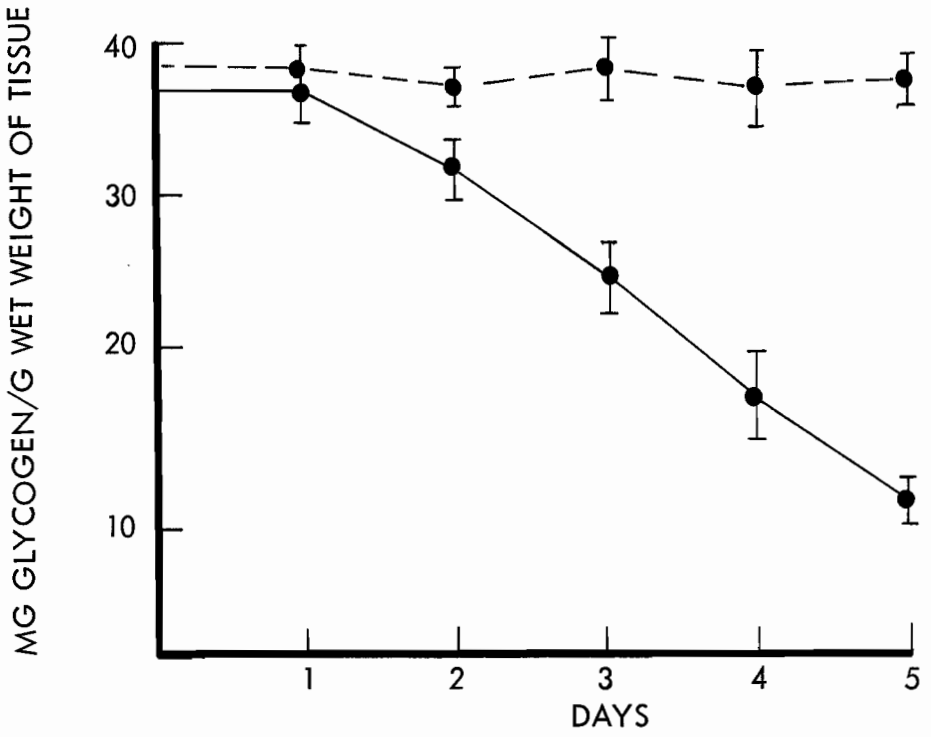
** Standard error of mean.

(a) Each value represents the mean of 16 determinations, except (b) on the fifth day. By the fifth day only 3 animals remained, hence 6 measurements.

Conditions:
Rat liver slices incubated in 3.0 ml of Krebs-Ringer phosphate buffer, pH 7.4, designated as "without substrate", or in the presence of 0.035 M glucose in 0.5 ml, added to 2.5 ml of the same buffer (with substrate). Incubations at 37°C.

Figure 22

Liver Glycogen Content of Salivarectomized and Control Rats on Hemorrhagic Diet




Salivarectomized group (●——●).

Control group (●– – –●).

Each point is the mean of 16 determinations, except that of the salivarectomized rats on the fifth day (*), which is the mean of 6 measurements.

Standard deviation ( I ).

It also became evident that the low level of liver glycogen had no effect on the respiratory activity of the tissue slices.

The sharp decrease in the glycogen concentration, however, further indicated the existence of a state of starvation in the salivarectomized animals.

(c) Urinary nitrogen excretion

The urinary nitrogen was estimated by the micro-Kjeldahl method (182). The results, expressed as mg of nitrogen excreted per animal per day, are indicated in Figure 23.

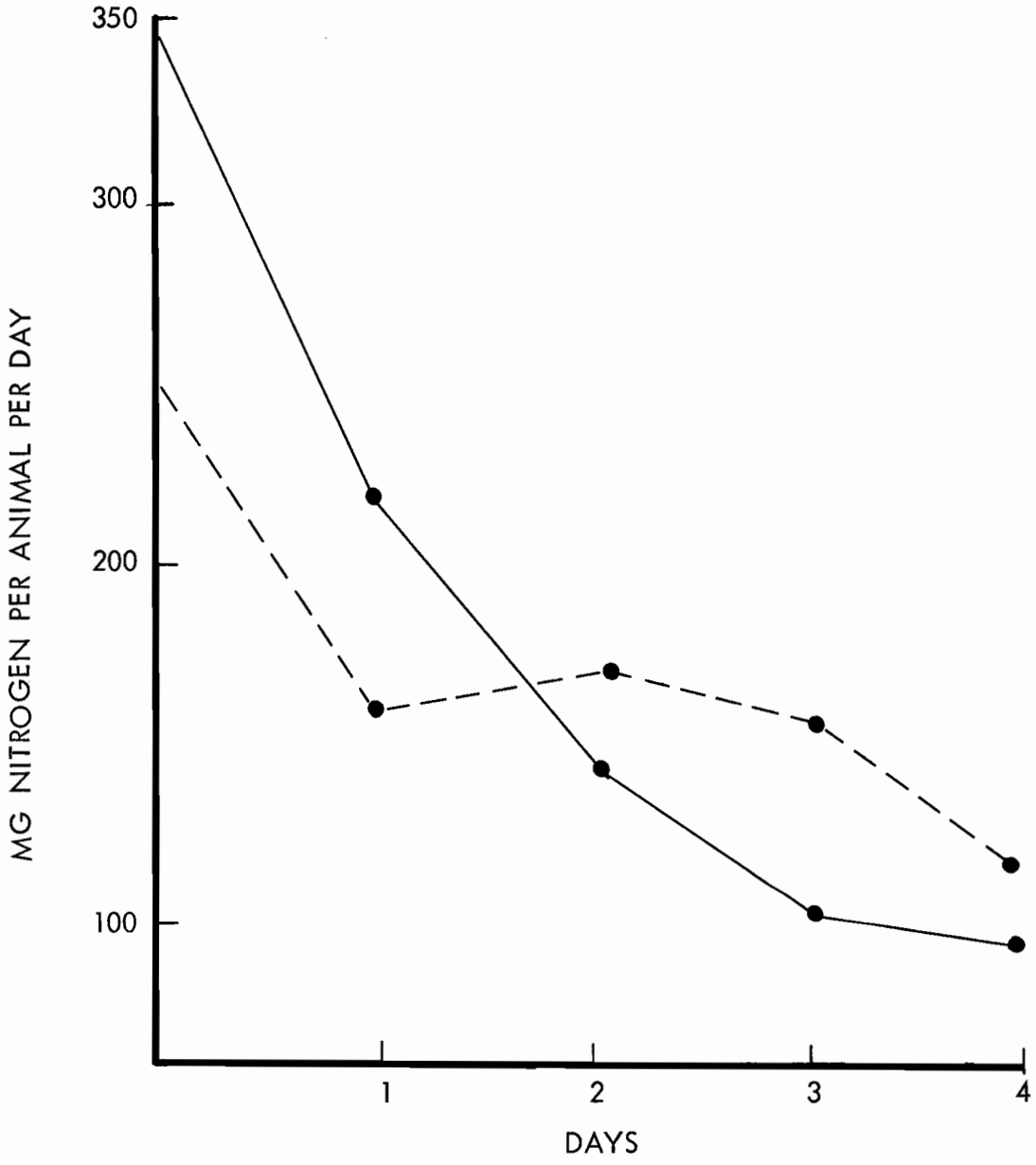
The rate of nitrogen excretion in the control group, as well as in the salivarectomized group, fell off sharply following dicumarol administration. The salivarectomized animals, before the beginning of the feeding of the experimental ration, excreted about a hundred mg more nitrogen than the controls. This increase may reflect a greater protein turnover resulting from a state of hyperthyroidism as evidenced by the elevated PBI values (Table VII) and by the morphological appearance of the thyroid gland (Figure 6B).

(d) Concentration of dicumarol in the tissues

Serum and urine specimens were used without further treatment. The tissues (liver, muscle, epididymal fat pad, small intestine) were placed on dry ice, weighed and held in the frozen state. When required for analysis they were thawed and disintegrated with an Elvehjem-Potter hand homogenizer in physiological saline to give a final concentration of 10 per cent (w/v).

Figure 23

Urinary Nitrogen Excretion




Salivarectomized group (●——●).

Control group (●– – –●).

All values are the mean of 8 determinations.

Dicumarol was extracted from the specimens by the method of Axelrod and Cooper (183), with heptane-water (acidified with 3N HCl). The two reagents were used in the proportion 20:3.5 (v/v). At room temperature, about 95 per cent of the dicumarol in the homogenate is extractable into the heptane phase. The dicumarol was removed from the latter by shaking with 2.5 N NaOH. The absorption at 315 mμ was then measured with a Beckman DU spectrophotometer.

The serum and urine samples were extracted in the same manner as the tissues.

A 'blank', with water substituted for the specimen (homogenate, serum or urine), was run through the described procedure and used for setting the zero point of the spectrophotometer.

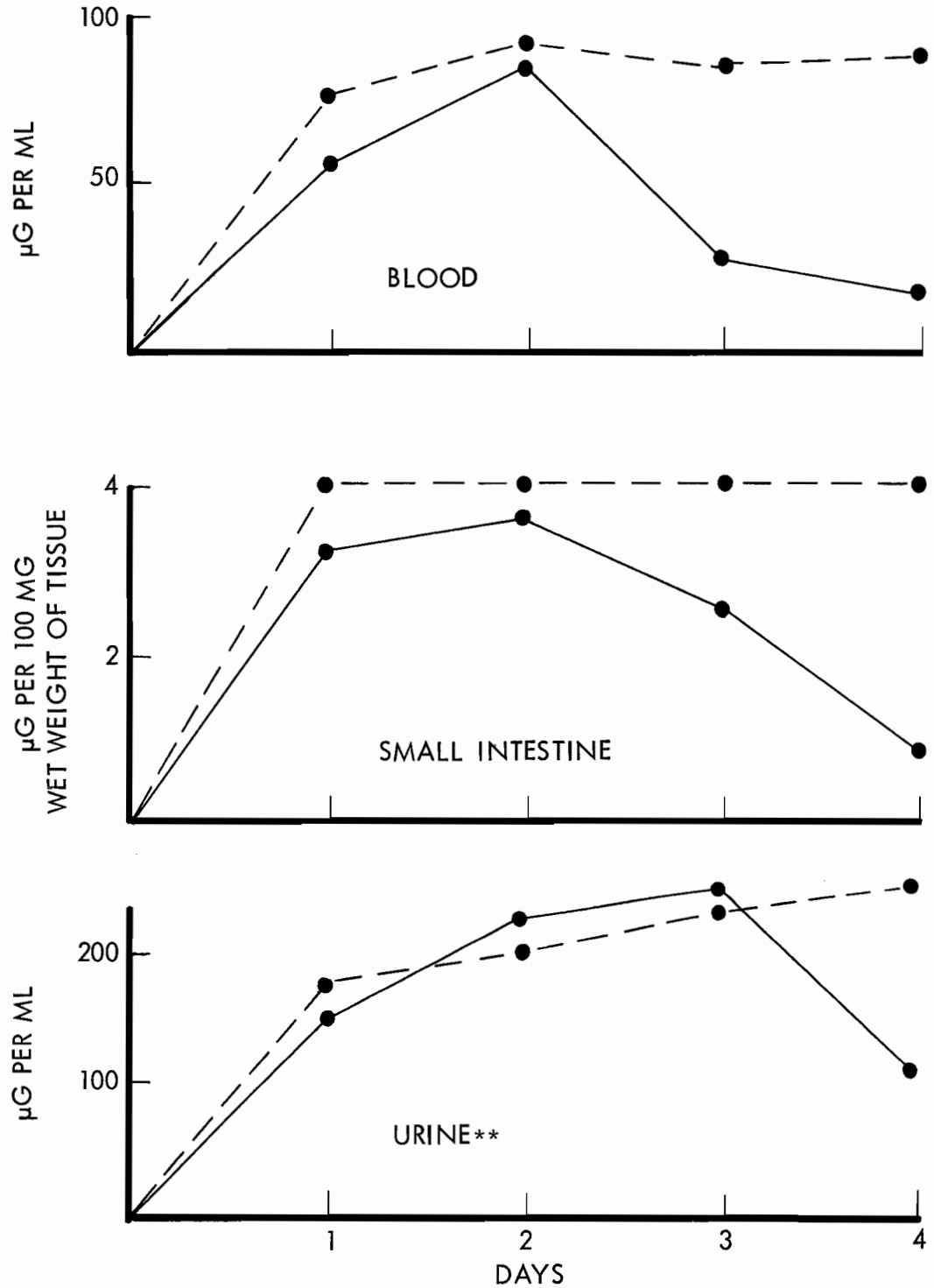
The concentration of dicumarol in the standard solution was 100 mg per litre in 0.1 N NaOH. The solution when kept at 4°C remained unchanged for at least a month. A standard curve was prepared each day and all the analyses were done in duplicate.

The results of the analyses are represented in Figure 24 and Figure 25.

It is of interest that in the control (sham operated) animals the concentration of dicumarol in the liver, muscle, blood and small intestine (ileum, jejunum) tended to reach a maximum about the end of the first day and thereafter remained virtually constant. In the salivarectomized animals, on the other hand, the maximum was reached in the blood and the intestine by the end of 24 hours and in the liver and the muscle, by the end of the

Figure 24

Concentration of Dicumarol*



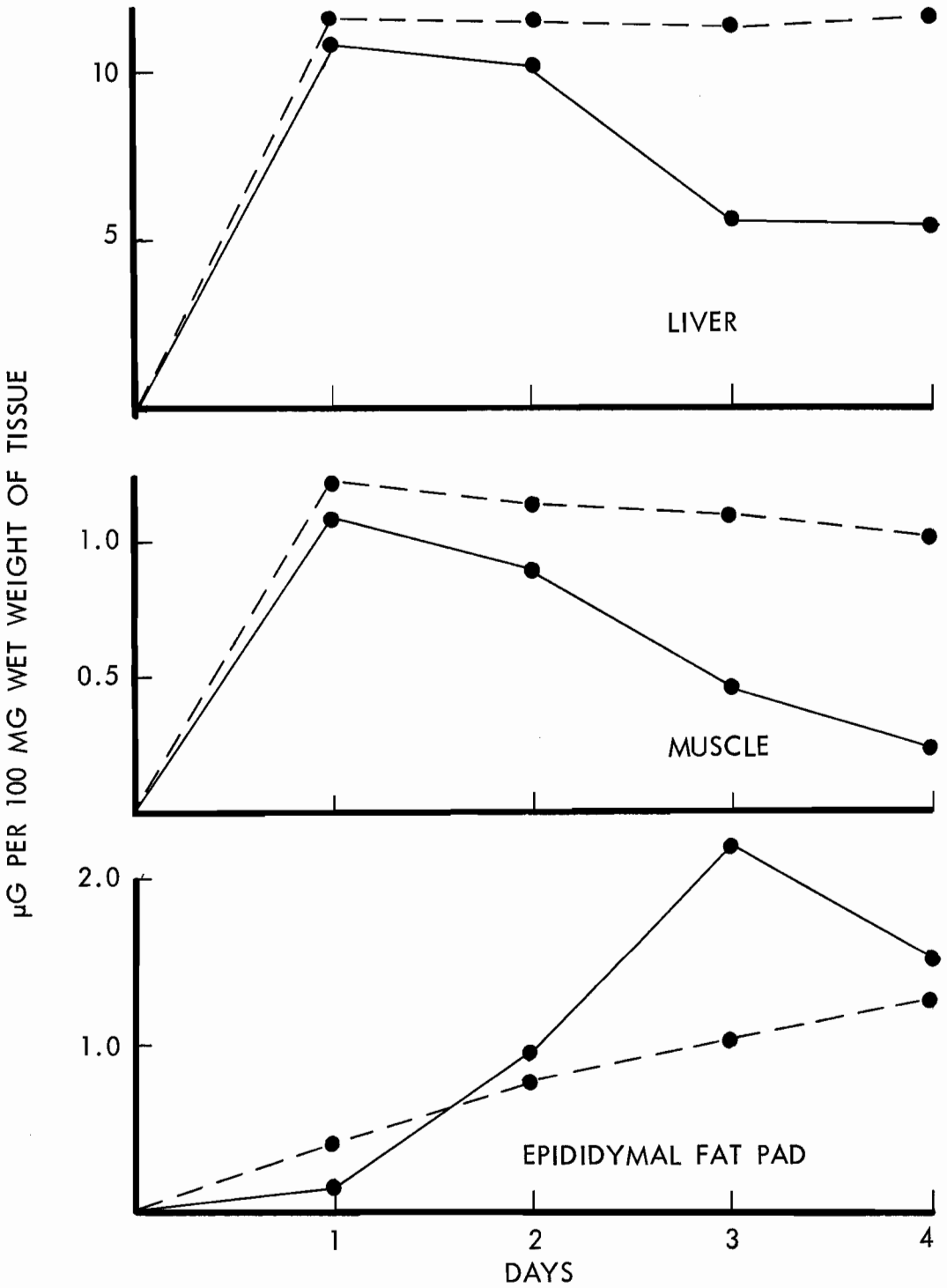

* Dicumarol was given in the daily food (0.5% w/w).

** Values for urine correspond to the mean of 8 measurements.

All plots represent the mean of 16 determinations.

Figure 25

Concentration of Dicumarol in Tissues




Salivarectomized group (●——●).

Control group (●– – –●).

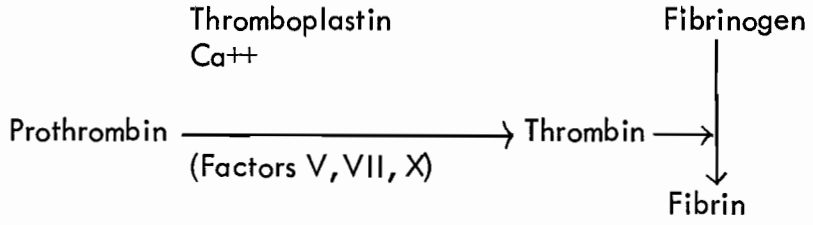
Each plot represents the mean of 16 determinations.

second day. Thereafter the concentration of the drug fell off rapidly with a greatly increased rate of urinary excretion.

The drug was excreted in the urine of the control group at a relatively steady rate.

(e) Prothrombin time

Dicumarol, ingested or otherwise administered, is known to interfere with the production of the components of the prothrombin complex (prothrombin and factors V, VII and X) in the liver (153). The participation of these proteins in the coagulation mechanism may be represented as follows:




The 'one-stage prothrombin time' determination (184) is based on the principle that when all the clotting factors required for the conversion of prothrombin to thrombin are present in excess, except one, the clotting time of the system affords a quantitative measure of the deficient component. To estimate prothrombin in a plasma specimen in terms of the clotting time, the other factors (V, VII and X) are provided in excess as follows:

To 8 parts of the plasma specimen are added: 1 part of 'aged' serum from a normal rat (this serum contains factors VII and X but no prothrombin) and 1 part of $Ca_3(PO_4)_2$-treated rat plasma which contains no factor VII, X

or prothrombin, but contains factor V.

In the test, 0.1 ml of the preparation is pipetted to 0.1 ml of a thromboplastin reagent (a solution of activated thromboplastin prepared from rabbit brain, and sold by Dade Reagents, Inc. Miami, Florida). The contents of the tube are mixed and placed in a water bath at 37°C. To the mixture 0.1 ml of $CaCl_2$ (0.025M) is added rapidly. The interval from the time of addition of calcium, to the instant a clot is formed, represents the prothrombin time.

The results of the test are presented in Table X. It is evident that the formation of prothrombin was progressively impaired in both groups as a result of the ingestion of dicumarol. The values indicated by the triple asterisks reveal further that all, or some of the factors (V, VII and X) of the prothrombin complex also were lacking, at least in part, in the blood. There was no difference in the prothrombin time between the two groups up the third day of dicumarol feeding. Thereafter, prothrombin time of the salivarectomized rats was more prolonged than that of the control group. The effect of the addition of an excess of the factors however indicates that the difference in prothrombin time was mainly due to the deficiency of one or more of the factor(s), but not of prothrombin itself. The low glycogen content of the liver in the salivarectomized group may account for a decreased synthesis of these factors.

(f) Serum glutamic-oxaloacetic transaminase

Glutamic-oxaloacetic transaminase (GOT) is an intracellular enzyme.

TABLE X

Prothrombin Time

| Time after dicumarol administration | Group | |
|---|---|---|
| | Salivarectomized | Control |
| | Prothrombin time | |
| (days)* | (sec) | (sec) |
| 0 | 22.0** | 21.0 |
| 1 | 23.0 | 24.0 |
| 2 | 34.2 | 38.4 |
| 3 | 53.1<br>(122.0)*** | 50.3<br>(150.0) |
| 4 | 73.1<br>(217.0) | 70.1<br>(132.0) |

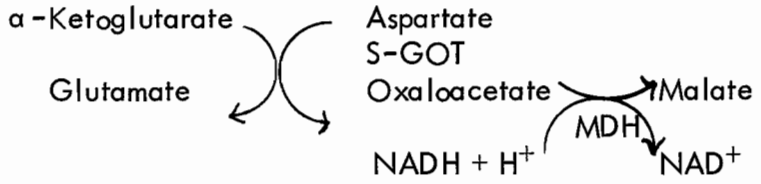
* Dicumarol was administered in the daily food in the concentration of 0.5 per cent.

** Each value is the mean of 16 determinations.

*** Prothrombin time without the addition of aged serum and $Ca_2(PO_4)_2$ adsorbed plasma (i.e. without addition of excess factors V, VII and X).

Under normal physiological conditions little, if any of it occurs in the blood or the tissue fluid. In pathological conditions such as hypoxia, infarction, infection, toxic states or other form of impairment, however, the enzyme along with others, glutamic pyruvic transaminase (GPT) and lactic dehydrogenase (LDH), tend to escape from the injured cell cytoplasm into the blood. The presence of any considerable amount of GOT in the blood serum is indicative of tissue distress, and the degree of activity of the enzyme affords an index of the intensity of injury.

The GOT activity of the serum (S-GOT) in our study was estimated by the method of Karmen (185). The principle of the method is indicated in the following reaction sequence:




The amount of oxaloacetate formed in the transamination reaction between α-ketoglutarate and aspartate, catalyzed by the transaminase, is estimated by measuring the decrease in the absorption at 340 mμ (resulting from oxidation of NADH) in the presence of added malic dehydrogenase (MDH).

The composition of our assay system and method of calculation are given in Table XI.

TABLE XI

Composition of the S-GOT Assay System

| | | |
|---|---|---|
| System: | NADH (1 mg/ml) | 0.2 ml |
| | Na aspartate (Stock soln.) | 0.5 " |
| | Malic dehydrogenase | 0.1 " |
| | Water | 1.8 " |
| | Serum (S-GOT) | 0.2 " |
| | Na α-ketoglutarate (Stock soln.) | 0.2 " |

The α-ketoglutarate is added last, and the optical density of the medium measured at 1 min. intervals for 15 minutes.

Calculation of S-GOT activity:

$$\text{S-GOT (units)} = \frac{\text{Optical density} \times 1000}{\text{Time required (minutes)} \times \text{Quantity of serum (ml)} \times \text{Temp. coeff't} \times \text{Light path (mm)}}$$

TABLE XII

Serum Glutamic-oxaloacetic Transaminase Activity

| Time after dicumarol administration | Group | |
|---|---|---|
| | Salivarectomized | Control |
| | S-GOT units per ml serum | |
| (days) | | |
| 1 | 75* | 79 |
| 2 | 92 | 104 |
| 3 | 107 | 94 |
| 4 | 132 | 75 |
| 5 | 1398** | 98 |

* Each value represents the mean of 16 determinations, except **, which represents the mean of 6 measurements.

The daily values of the S-GOT of the control (sham operated) group fluctuated about the initail level and may be considered as having remained unchanged during the 5-day period. The enzyme activity in the salivarectomized group increased steeply after the fourth day as bleeding became extensive.

(g) Urinary excretion of carbohydrate

The histological evidence in our studies confirms the observation of many workers that vascular fragility in spontaneous hemorrhage is attained by a dissolution of the ground substance of the connective tissues. Since bleeding in the salivarectomized animal becomes general in distribution by the third day, we surmised that carbohydrate fragments from the breakdown of ground substance might be excreted in the urine in these animals. Tests for mucopolysaccharide in the urine, therefore, were carried out daily with the anthrone method using glucose as the standard. Each estimation was done in duplicate. The results are presented in Figure 26.

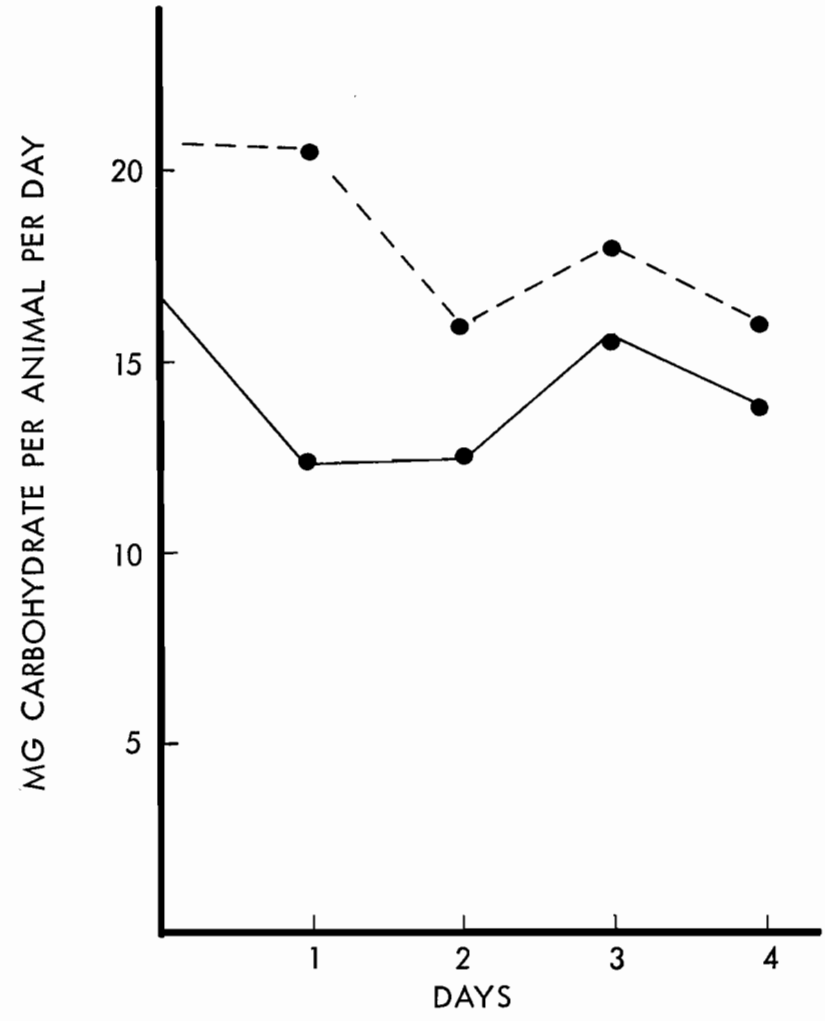
Anthrone-reactive material was excreted by the animals of both groups. However, as the quantity did not increase with the development of severe fragility, it was considered to be of little significance and therefore no attempt was made to identify the carbohydrate.

## DISCUSSION AND CONCLUSIONS

The findings presented in this section should be interpreted in the light of the histological, radiographic and other studies described in the pre-

Figure 26

Urinary Excretion of Carbohydrate




Salivarectomized group (●——●).

Control group (●– – –●).

Each plot represents the mean of 8 determinations.

ceding sections. The usefulness and importance of these investigations as a basis for biochemical follow-up studies will be appreciated.

As already shown, the salivarectomized animals when placed on the dicumarol containing diet continued to eat, but judged from the decrease in body weight, the fall in the liver glycogen, the fall in the concentration of dicumarol in the blood and tissues and the decrease in the urinary nitrogen excretion, the animals evidently were not able to absorb the nutrients, at least at the normal rate.

Most of the changes described appeared 48 hours after the commencement of the hemorrhagic diet. This time coincides with the onset of widespread hemorrhages in the gastrointestinal tract. Thus, the animals are apparently in a state of starvation despite the continued, though diminishing, food intake.

Authorities on the subjects of shock and toxicity of drugs are well aware of the vulnerability and diminished survival capacity of the fasted or malnourished animal. Studies in our own laboratory by Loiselle (186) and by Rubinstein (187), have shown that when the liver glycogen approaches depletion, the liver becomes vulnerable to damage and the ability to detoxify drugs is impaired. One obtains the clear impression that when the liver reaches this state, other body functions also suffer impairment.

This picture is seen also in our studies, but with one important difference, namely, that the state of starvation itself is a consequence of the hemorrhagic effect of dicumarol on the vasculature of the gastrointestinal tract.

The manner in which glycogen depletion in the liver causes this organ and other tissues to become vulnerable to the hemorrhagic action of dicumarol, is still not understood.

It is evident from our findings that there is no direct relation between the concentration of dicumarol and the time of onset of hemorrhage in any of the organs or tissues.

It is well known that a severe deficiency of any of the factors necessary for blood coagulation, or a severe inhibition of the action of any one of them, gives rise to internal bleeding. In the case of dicumarol poisoning, the impairment of prothrombin production in the liver is usually considered as being the initiating factor in the production of vascular fragility. It is particularly noteworthy in our study that bleeding had begun before there was any change in the prothrombin time. This observation suggests that the impaired state of coagulability of the blood is a secondary effect, and not a primary cause of hemorrhage in the salivarectomized rat.

Dicumarol, being a fat soluble substance like other substances of this class - ether, cyclopropane estrogen, etc., - when administered to the animal tends to be taken up in greater concentration in the liver and in the adipose tissues of which the epididymal fat pads are representative. The liver acquires a high concentration within a short time. Much of the drug then is shifted to the adipose tissues while some of it is excreted in the urine. It may be expected that the concentration in any part of the adipose tissue (e.g. epididymal fat pad) would be relatively small, but the overall amount

in the total fat depots doubtless is greater than that in the liver.

The abrupt fall in the concentration of dicumarol in the liver, fat depots and other tissues in the salivarectomized rat, suggests that this may result from the loss of the drug from the cells into the interstitial fluid because of the collapsed circulation (shock). The overall tissue breakdown is clearly reflected in the steep (10 fold) rise in the serum GOT by the fifth day in the experimental group.

The respiratory activity of the liver slices reflects the state of the aerobic energy metabolism. It is of interest that the oxygen consumption of the liver slices from both the experimental and the control animals remained unchanged during the 5 day period. These results, therefore, do not support the view that dicumarol acts predominantly as an 'uncoupler' of oxidative phosphorylation. However, the absence of endogenous substrate (glycogen and hence glucose) could account for the decreased $Q_{O_2}$.

As mentioned in the survey of the literature of this thesis, and has been confirmed further in our morphological studies, vascular fragility is attended by a dissolution of ground substance in the perivascular sheath and the surrounding supporting connective tissue. Bleeding usually occurs early in the loose connective tissues, e.g. epididymal fat pad. This process of breakdown points to the action of hydrolases such as hyaluronidase, beta-glucuronidase, proteinases and others. An effort to demonstrate the excretion in the urine of carbohydrate breakdown-products of mucopolysaccharide was inconclusive. Only a small concentration of anthrone-reactive

material was measurable. Nevertheless, the histological evidence points to a disintegration of the connective tissue and we are still seeking to confirm this by biochemical means.

The breakdown of connective tissue doubtless reflects a generalized autolytic trend, especially during the advanced stages of intoxication. The effect of dicumarol on the release of lysosomal hydrolases is discussed in the following section.

## 6. THE ROLE OF HYDROLYTIC ENZYMES IN DICUMAROL-INDUCED HEMORRHAGE

### INTRODUCTION

The histological evidence of connective tissue breakdown in dicumarol-induced hemorrhage (see page 79) prompted us to seek further confirmation of the development of vascular fragility by means of histochemical methods and by studying the behaviour of various tissue and blood hydrolases.

Breakdown of the mucoprotein (or glycoprotein) basement membrane of the wall of the small vessels and/or the surrounding connective tissue, could be the consequence of hydrolytic enzyme digestion originating (a) from the blood (by the activation of enzymes, or destruction of their inhibitors) and (b) from the cellular elements of the vessel wall and/or perivascular cellular elements (mast cells, fibroblasts, etc.).

De Duve and co-workers (188, 189, 190) have described a new group of cytoplasmic particles, the "lysosomes", which are located in the region of the Golgi Apparatus. The particles contain a variety of enzymes, with an acid pH optimum, including many types of hydrolases: phosphatases, beta-glucuronidase, proteinases, peptidases and others. In conditions leading to severe tissue damage as in hypoxia, chemical poisoning by $CCl_4$, endotoxin shock, the enzymes are released into the cytoplasm and into the extracellular spaces (191, 192, 193). These enzymes are considered to be responsible for the breakdown of cells and tissues in autolysis.

It is reasonable to surmise, that the release and activation of various hydrolases from the lysosomes may contribute also to the breakdown of the peri-endothelial sheath of the small vessels and neighbouring connective tissue in dicumarol poisoning. Since it is not practicable to isolate lysosomes from endothelial cells or from pericapillary mast cells and fibroblasts, we endeavoured to study the behaviour of the particles from liver cells.

## PROCEDURES

The study included four repeated experiments. In each, 24 male albino rats of uniform body weight (60 grams) were divided into two equal groups. One was salivarectomized and the other sham operated as in the previously described studies.

On the day before, and each day after the animals were placed on the dicumarol containing ration, two animals were removed, blood was

taken by heart puncture and permitted to clot and the serum removed. One portion (about half) was used for assay of the trypsin, chymotrypsin and hyaluronidase activity. The other was reserved for the assay of the activity of the respective inhibitors (antitrypsin, antichymotrypsin, and antihyaluronidase).

The abdominal cavity was then opened and specimens of mesentery removed (4 pieces from each animal) and fixed immediately in chilled acetone for histochemical studies.

From eight of the experimental animals and eight of the controls (before the feeding of the dicumarol diet), the liver was removed and used for study of the lysosomes.

In addition to the above mentioned, the liver was removed from 12 normal rats (additional controls) to ascertain the effect of salivarectomy and of dicumarol on the lysosomes.

(1) Estimation of lysosomal enzymes

The lysosomes from the livers were isolated according to the method of Petti, Cartoni and Ruggiu (194). The organ was weighed, cut into small fragments and disintegrated in 15 ml of 0.25 M sucrose solution with a mechanically driven Potter-Elvehjem homogenizer with three up-and-down runs of the pestle rotating at 780 r.p.m. The homogenate was then made up to volume with 0.25 M sucrose solution so as to give a final concentration of 10 per cent of tissue (wet weight/v).

The homogenate was divided into three portions. One was kept as the control. To portion two normal rat serum was added in the proportion of 1 part of serum to 4 of homogenate. To portion three, normal rat serum, saturated with dicumarol (0.9 mg dicumarol per ml of serum), was added in the same ratio as in portion two. The three preparations were incubated at 29°C for 15 minutes.

The samples were then centrifuged at 40,000 r.p.m. in a preparative ultracentrifuge, Spinco Model L, No. 40 rotor for 30 minutes ($144 \times 10^3 \times g$) at 4°C.

The lysosomal particles were found by de Duve to have a sedimentation constant between that of the mitochondria and the microsomes.

Sedimentation of the homogenate at 40,000 r.p.m. for 30 minutes ($144 \times 10^3 \times g$), as used in our study, yielded a supernatant devoid of granules (lysosomes, etc.) and hence with little hydrolase activity. However, if the homogenate is subjected to any treatment that will rupture the granules, the supernatant after high speed centrifugation will contain hydrolase activity. Measurement of acid-phosphatase and beta-glucuronidase activity in the supernatant therefore was used instead of attempting to isolate the particles.

(a) Acid phosphatase

The acid phosphatase activity of the supernatant was measured as described by Gianetto and de Duve (195). 1 ml of supernatant (enzyme source) was incubated for 15 minutes with an equal volume of substrate

solution (ß-glycerophosphate, (Eastman Kodak Company, Rochester), 0.1 M in acetate buffer, pH 5.0), at 37°C. The reaction was stopped by the addition of 8 per cent TCA and the phosphate liberated was measured by the method of Fiske and Subbarow (196).

The phosphatase activity was expressed as mg inorganic phosphate liberated by the end of 15 minutes, per 100 mg wet weight of tissue.

(b) ß-glucuronidase

The ß-glucuronidase activity was assayed according to the method of Billett (197). The reaction mixture containing 0.5 ml of enzyme preparation (supernatant), 1.0 ml 1.0 M potassium acetate buffer (pH 4.3), 1.0 ml of the substrate solution (8-hydroxyquinoline glucuronide, 32 mg/50 ml) and 0.5 ml of water, was incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 5% TCA and the protein removed by centrifugation. The amount of 8-hydroxyquinoline liberated in the supernatant was determined by diazotization and the colour density measured at 470 mμ in a Coleman Junior spectrophotometer. The enzyme activity was calculated as the total chromogen found after the enzyme-substrate mixture had been incubated for 30 minutes. Each estimation was done in duplicate.

(c) Histochemical examination

The histochemical demonstration of the presence of lysosomes in the endothelial cells of the small vessels and in the pericapillary mast cells was performed according to the procedure of Gomori as modified by

Novikoff (198, 199), taking the acid phosphatase activity as the indicator of the presence of the lysosomes.

Small pieces of mesentery along with peritoneal smears were taken according to procedures recommended by Clermont (200). The specimens were fixed in cold acetone for 24 hours. They were then washed with distilled water (4 washings) and incubated in acetate buffer at pH 5.0 in the presence of 0.01 M ß-glycerophosphate as the substrate. The inorganic phosphate liberated into the cytoplasm of the cells was converted to lead phosphate by the addition of lead nitrate (0.04 M). The final reaction step in the procedure involved the conversion of lead phosphate to the brown sulphide by treatment of the specimen with a dilute solution of ammonium sulfide.

After washing the specimens of mesentery in tap water, they were spread on microscopic slides, mounted in Canada balsam, covered and photographed as shown on page 127.

(2) Studies on the hydrolytic enzymes and their inhibitors in the blood

(a) Hyaluronidase

The hyaluronidase activity was assayed by the method of Cobbin and Dicker (201):

Serum was diluted 1:60, with Veronal-acetate buffer, pH 4.6. One part of the diluted serum was added to 5 parts of hyaluronic acid solution (4% H.A. in Veronal-acetate buffer) in an Oswald-Fenske viscosimeter (No. 100), at 37°C. The flow-time measurement of the reaction mixture was

repeated at 5 minutes intervals during a period of 30 minutes. In the calculation of the results the final value obtained at the end of 30 min. incubation was used.

The relative viscosity (U) was obtained from the relation:

$$U = \frac{T_u}{T_e}$$

where $T_u$ represents the average of the flow-time values obtained in four runs, and $T_e$, the corresponding values with the buffer alone.

(b) Assay of antihyaluronidase

Antihyaluronidase is present in animal as well as in human serum. The serum was used as the inhibitor preparation without further treatment.

The activity of the inhibitor was assayed by the method of Haas (202) which relates the rate of decrease in viscosity of a hyaluronic acid solution to the concentration of hyaluronidase.

The test system is described in Table XIII. The medium designated in the Table as "A" was pre-incubated for ten minutes and added to medium "B" in the Oswald-Fenske viscosimeter. The flow-times of the mixed enzyme substrate and enzyme-substrate-inhibitor solutions were recorded at various intervals. In this assay the flow-times undergo a progressive decrease and give a measure of the rate of hydrolysis of the hyaluronic acid by the enzyme. The viscosity of the solution was calculated and the time necessary for the value to reach one half of the original viscosity (half viscosity time) was used as an index of the antihyaluronidase activity.

TABLE XIII

Composition of the System Used for Assay of the Hyaluronidase - Inhibitor in Serum

| Ingredients of Medium A | Flask Number 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| | (ml) | | | | |
| Borate Buffer, 0.2 M; pH 6.7 | 0.6 | 0.6 | -- | 0.6 | -- |
| Water | 0.8 | 0.8 | 0.8 | 0.6 | 0.6 |
| Serum | -- | -- | -- | 0.2 | 0.2 |
| Hyaluronidase*, 0.8 mg/ml | -- | -- | 0.6 | -- | 0.6 |
| Magnesium Chloride, 0.11 M | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Ingredients of Medium B | 1' | 2' | 3' | 4' | 5' |
| Phosphate Buffer, 0.5 M; pH 7.1 | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Sodium Chloride, 2.0 M | 0.4 | 0.4 | 0.4 | 0.4 | 0.4 |
| Sodium Acetate, 0.2 M | 1.0 | -- | -- | -- | -- |
| Hyaluronic Acid*, 6.0 mg/ml | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 |
| Tubes containing Medium A and Medium B are pre-incubated for 10 minutes at 29°C, then contents are mixed and the viscosity measured at various intervals. | | | | | |
| Viscosity | | | | | |
| Flow time (sec.) | 53.0 | 104.0 | -- | 106.0 | -- |
| Half viscosity time | -- | | 655 ($R_o$) | | 4000 (R) |

Method of calculation. Example:

$$\text{Activity of Antihyaluronidase A} = \frac{R - R_o}{R_o} = \frac{4000 - 655}{655} = 5.7$$

$$\text{Per cent Inhibition I} = \frac{A \times 100}{A + 1} = \frac{5.7 \times 100}{6.7} = 85 \text{ per cent}$$

* Obtained from Nutritional Biochemical Corp., Cleveland.

Thus, the activity of the inhibitor is represented by the relationship:

$$A \text{ (activity)} = \frac{R-R_o}{R_o}$$

where $R_o$ = half-viscosity time before reaction of hyaluronidase with the serum (inhibitor),

and $R$ = half-viscosity time after reaction of hyaluronidase with the serum.

The degree of inhibition by the serum $I = \frac{A}{A+1} \times 100$ per cent.

(c) Tryptase and inhibitor

Serum trypsin and trypsin inhibitor were assayed according to the method of Kallos, Kahn and Rizok (203).

The synthetic substrate, N-benzoyl-l-lysine hydrazide, was synthetized and kindly supplied by Dr. J. Kallos of the Research Laboratory of St. Mary's Memorial Hospital, Montreal. It is readily hydrolyzed by trypsin. Serum was used as the source of enzyme. The liberated hydrazine was converted to hydrazone by the addition of Ehrlich's reagent (p-dimethylaminobenzaldehyde) and the optical density was read at 455 mμ in a Coleman Junior spectrophotometer. The optical density is proportional to the concentration of the enzyme. The enzyme activity of serum was compared with that of a standard of crystalline trypsin (Sigma Chemical Company) as indicated in Table XVIII. Each determination was done in duplicate.

The antitryptase activity of the serum was estimated by incubating a given amount of pure trypsin with a definite amount of the serum and then determining the tryptic activity as described in the preceding paragraph.

The decrease in enzyme activity reflects, with time, the amount of inhibitor present in the serum. The inhibitor activity was expressed as per cent inhibition.

(d) Chymotryptase and inhibitor

Serum chymotrypsin and chymotrypsin inhibitor were determined according to the method of Kallos, Arthur, Rizok and Kahn (204). The specific substrate, N-acetyl-l-tyrosine hydrazide, also was synthetized and kindly supplied by Dr. J. Kallos. The hydrazide liberated by the enzyme was estimated as described in the preceding paragraph.

Antichymotryptase was estimated by a procedure analogous to that described above for antitryptase.

## RESULTS AND DISCUSSION

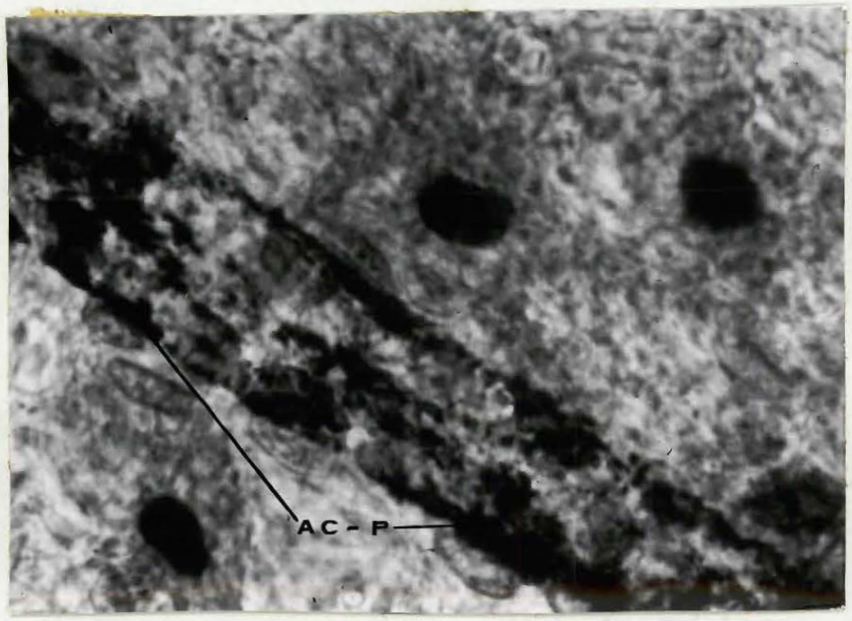
### (1) Lysosomal studies

As shown in Figure 27A acid phosphatase-containing granular structures were observed in abundance in the cytoplasm of the endothelial cells of the capillaries, the venules and the arterioles of the rat mesentery. There was no difference in the appearance or in the number of the granules between the salivarectomized and the sham operated control animals before administration of the hemorrhagic diet.

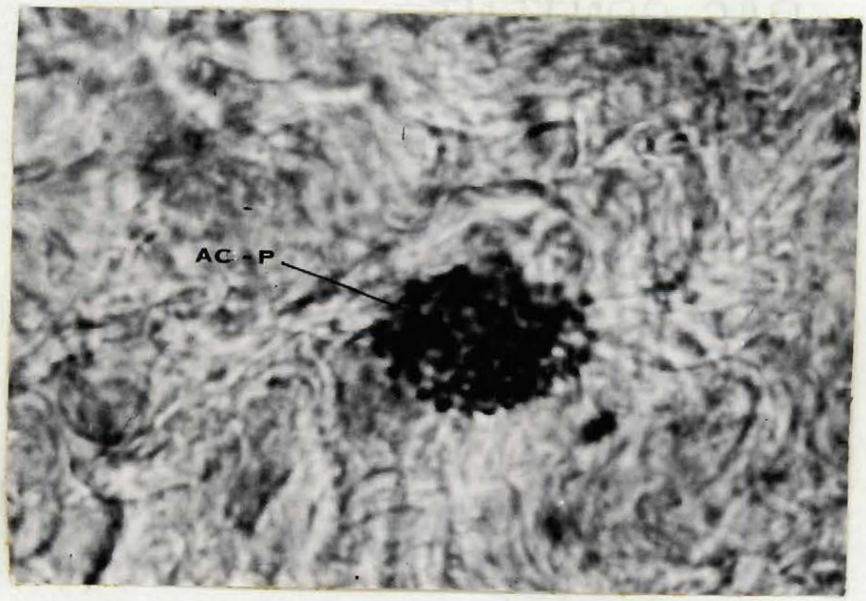
The pericapillary connective tissue and the peritoneal mast cells also contained an abundance of acid phosphatase positive granules which were localized in the cytoplasm as indicated in Figure 27B. In some of the specimens examined, which were counter-stained with toluidine blue, a dense granular coat was observed also in the perinuclear region.

Figure 27

HISTOCHEMICAL DEMONSTRATION OF ACID PHOSPHATASE CONTAINING GRANULES IN THE ENDOTHELIUM OF THE SMALL VESSEL AND IN THE PERICAPILLARY MAST CELLS.




A. Capillary endothelium of salivarectomized rat, showing numerous brown-granules indicating acid-phosphatase activity (AC-P). Gomori.




B. Pericapillary mast cell filled with acid phosphatase granules (AC-P). Gomori.

An effort to follow the progressive degranulation of the endothelial or of the mast cells during the course of the bleeding (48-96 hours after the feeding of the hemorrhagic diet) was unsuccessful, because the massive hemorrhages obscured the fine details. Furthermore, the leucocytes which are known to be rich in acid phosphatase containing granules were extremely numerous on the sections examined. However, while we were not successful in demonstrating the 'liberation' of the 'lysosomes' in our morphological study, nevertheless we demonstrated the presence of such structures in the cytoplasm of the endothelial cells of the small vessels and that in the mast cells.

Our results were contrary to that reported by Noback and Paff (205). According to these authors, acid phosphatase activity is confined to the nucleus of the endothelial and the mast cells. Montagna and Noback (206) also reported that the granules could be demonstrated in the cytoplasm. A careful comparison of the procedure used by these investigators and that of the present author revealed differences in the method, with respect to the kind of fixative used and in the duration of incubation of the tissues with the substrate (ß-glycerophosphate).

The present author has investigated the conditions of the method by examining the tissues after various intervals of incubation with the substrate. Prolonged incubation (16 hours) of the preparations with the substrate was found to increase the intensity of the nuclear staining. Novikoff (130) considers nuclear staining an artifact, attributable to the adsorption of

phosphate ions on the nuclear membrane and diffusion of the ions into the nucleus. Incubation for less than 6 hours or more than 24 hours tends to show the granules localized in the cytoplasm. Thus, the duration of incubation appears to be the condition mainly responsible for the variability. For this reason one hesitates to ascribe a specific locale or distribution to the granules. The demonstration of phosphatase activity in the presence of substrate or the absence of the activity on addition of a phosphatase inhibitor such as sodium fluoride (207) and in the absence of substrate, are sufficient evidence of the presence of the enzyme.

On the chemical side of the picture, the results presented in Tables XIV and XV indicate a substantial release of acid phosphatase and beta-glucuronidase from the lysosomes of the liver cells in vitro. It is reasonable to surmise that the uptake of dicumarol by the cell could be a factor causing the breakdown of the membrane of the granules. This action may be favoured by the high lipid solubility of the drug. It became evident also from the study of the release of the two enzymes into the nonsedimentable supernatant fraction of our preparation, that salivarectomy may adversely influence the stability of the membrane of the lysosomes.

It is to be noted however, that serum saturated with dicumarol (preparation 3) takes up 9 times the maximum concentration ever found in the plasma in vivo. This concentration was used so that when the serum was diluted with the homogenate, the concentration of dicumarol would be comparable to the highest level attained by ingestion of the drug.

TABLE XIV

Acid Phosphatase Activity of Rat Liver Lysosomes

| Type of Preparation | Liberation of $P_i$ (mg/100 mg wet weight of tissue) |
|---|---|
| Sham-operated rat liver supernatant* | 3.9 ± 1.2** (8)*** |
| Salivarectomized rat liver supernatant* | 7.0 ± 0.9 (8) |
| Normal rat liver supernatant | |
| Preparation 1. | 4.1 ± 1.1 (12) |
| Preparation 2. | 3.9 ± 0.8 (12) |
| Preparation 3. | 14.0 ± 1.4 (12) |

* Homogenate, without pre-incubation, centrifuged at 36,000 r.p.m. for 30 min. at 15°C. Acid phosphatase activity of the supernatant then assayed on basis of the quantity of inorganic phosphate (calculated as elemental phosphorus, $P_i$) liberated from the substrate, ß-glycerophosphate (0.1 M in acetate buffer at pH 5.0).

** Standard error of mean.

*** Numbers in bracket correspond to the number of determinations.

Preparation 1. Homogenate pre-incubated at 29°C for 30 minutes, centrifuged and assayed for $P_i$ liberated.

Preparation 2. Homogenate pre-incubated with normal rat serum at 29°C for 30 minutes. Serum:homogenate = 1:4. Centrifuged and assayed.

Preparation 3. Homogenate pre-incubated with normal rat serum containing 0.9 mg per ml dicumarol at 29°C for 30 minutes. Serum: homogenate = 1:4.

TABLE XV

ß-Glucuronidase Activity of Rat Liver

| Preparation | 8-Hydroxyquinoline liberated* (µg per 50 mg wet weight of tissue) |
|---|---|
| Sham-operated rat liver supernatant | 52 ± 4.2** (8)*** |
| Salivarectomized rat liver supernatant | 118 ± 6.4 (8) |
| Normal rat liver supernatant | |
| Preparation 1. | 64 ± 8.0 (12) |
| Preparation 2. | 59 ± 4.0 (12) |
| Preparation 3. | 210 ± 14.0 (12) |

* Homogenate treated as described under Table
All values correspond to the amount of 8-hydroxyquinoline liberated from the glucuronide after 30 minutes incubation of supernatant (enzyme source) with the substrate at 37°C.

** Standard error of mean.

*** Number of determinations.

Preparation 1. Homogenate pre-incubated at 29°C for 30 minutes and supernatant assayed.

Preparation 2. Homogenate pre-incubated with normal rat serum at 29°C for 30 minutes. Serum:homogenate = 1:4.

Preparation 3. Homogenate pre-incubated with normal rat serum containing dicumarol (0.9 mg/ml) at 29°C for 30 minutes. Serum: homogenate = 1:4.

(2) Hydrolytic enzymes of the blood and their inhibitors

(a) Hyaluronidase and hyaluronidase inhibitor

The results from the 4 experiments summarized in Tables XVI and XVII indicate no significant difference between the serum hyaluronidase activity of the salivarectomized and the sham operated (control) groups during the five day experimental period. These data are in agreement with our concept that the initiation of the breakdown of the basement membrane does not originate within the blood vessel itself. The slight elevation of the antihyaluronidase activity in the serum of the salivarectomized rats during the development of spontaneous hemorrhage in all probability reflects a defence reaction by the organism against the massive liberation of hydrolytic enzymes in the necrotic hemorrhagic areas.

(b) Trypsin, chymotrypsin and their inhibitors

The data represented in Tables XVIII and XIX show that there was no increase in the tryptic or the chymotryptic activity in the serum of the salivarectomized or the control rats during the experimental period. The values correspond to those found for hyaluronidase. The respective inhibitors in the salivarectomized group, however, showed an increase in concentration of about 10 per cent as indicated in Table XX during the hemorrhagic period.

The increase in the antiproteinase activity paralleled that of the antihyaluronidase, thus indicating further that the inhibitor is nonspecific in action (208).

TABLE XVI

Hyaluronidase Activity of Serum

| Duration of feeding dicumarol | Group | |
|---|---|---|
| | Salivarectomized | Control |
| (days) | (relative viscosity*) | |
| 0 | 1.18** | 1.20 |
| 1 | 1.20 | 1.22 |
| 2 | 1.19 | 1.18 |
| 3 | 1.20 | 1.19 |
| 4 | 1.21 | 1.20 |
| 5 | 1.17*** | 1.21 |
| Dicumarol added, 0.9 mg/ml normal rat serum | 1.22**** | |

* Relative viscosity $= \frac{\text{Flow time of reaction mixture}}{\text{Flow time of buffer}}$

Reaction mixture contained: Five parts of hyaluronic acid (4%) in veronal-acetate buffer at pH 4.6, incubated with one part of serum diluted (1:60) with the same buffer, in an Oswald-Fenske viscometer, at 37°C.

** Each value represents the mean of 16 measurements (duplicate measurements on each of two animals in each of four repeated experiments) at the end of 30 minutes incubation at 37°C.

*** This value represents the mean of 6 determinations as the majority of the salivarectomized rats were dead on the fifth day.

**** Value corresponds to the mean of 8 measurements.

TABLE XVII

Hyaluronidase Inhibitor Activity of Serum

| Duration of feeding dicumarol | Group | |
|---|---|---|
| | Salivarectomized | Control |
| (days) | (inhibition %) | |
| 0 | 80* | 79 |
| 1 | 81 | 78 |
| 2 | 83 | 80 |
| 3 | 88 | 79 |
| 4 | 87 | 80 |
| 5 | 89** | 79 |
| Dicumarol added, 0.9 mg/ml normal rat serum | 80*** | |

* Each value is the mean of 16 determinations (as in Table XVI)

** The value is the mean of 6 determinations (as in Table XVI)

*** Mean of 8 measurements.

TABLE XVIII

Tryptic Activity of Serum

| Duration of feeding dicumarol | Group | |
|---|---|---|
| | Salivarectomized | Control |
| (days) | (μg trypsin per test sample*) | |
| 0 | 7.0** | 6.5 |
| 1 | 6.0 | 6.8 |
| 2 | 8.0 | 7.8 |
| 3 | 7.4 | 7.1 |
| 4 | 6.9 | 7.1 |
| 5 | 7.4*** | 7.0 |
| Dicumarol added, 0.9 mg/ml normal rat serum | 7.2**** | |

* μg trypsin, based on the activity of crystalline trypsin standard (Sigma Chemical Co.). The test preparation contained 0.25 ml serum (enzyme source), 0.25 ml substrate (α-N-benzoyl-L-lysine hydrazide) in Tris buffer, 0.1 M at pH 8.1. Incubation for 30 minutes at 37°C.

** All values are the mean of 16 determinations (as in Table XVI)

*** Mean of 6 determinations (as in Table XVI)

**** Mean of 8 measurements.

TABLE XIX

Chymotryptic Activity of Serum

| Duration of feeding dicumarol | Group | |
|---|---|---|
| | Salivarectomized | Control |
| (days) | (µg chymotrypsin per test sample*) | |
| 0 | 3.5** | 4.2 |
| 1 | 3.8 | 3.7 |
| 2 | 3.9 | 3.8 |
| 3 | 4.0 | 3.6 |
| 4 | 3.6 | 4.1 |
| 5 | 3.9*** | 4.0 |
| Dicumarol added, 0.9 mg/ml normal rat serum | 3.6**** | |

* µg chymotrypsin, based on the activity of crystalline chymotrypsin standard (Sigma Chemical Co.). The test preparation contained 0.25 ml serum (enzyme source) 0.25 ml substrate (N-acetyl-L-tyrosine hydrazide) in Tris buffer, 0.05 M, at pH 7.8. Incubation for 30 minutes at 37°C.

** All values are the mean of 16 determinations (as in Table XVI)

*** Mean of 6 determinations (as in Table XVI)

**** Mean of 8 measurements

TABLE XX

Antitryptic and Antichymotryptic Activity of Serum

| Duration of feeding dicumarol | Group | | | |
|---|---|---|---|---|
| | Salivarectomized | | Control | |
| | Trypsin* | Chymotrypsin** | Trypsin | Chymotrypsin |
| (days) | (inhibition %) | | | |
| 0 | 79*** | 81 | 80 | 81 |
| 1 | 81 | 80 | 82 | 84 |
| 2 | 87 | 84 | 79 | 81 |
| 3 | 89 | 86 | 80 | 80 |
| 4 | 90 | 89 | 81 | 79 |
| 5 | 89**** | 90**** | 78 | 82 |
| Dicumarol added, 0.9 mg/ml, to normal rat serum | 80 α | 78 α | — | — |

* 10 μg of crystalline trypsin in 0.15 ml Tris buffer, 0.1 M at pH 8.1 is mixed with 0.1 ml of buffer-diluted serum (containing 0.02 ml of serum) and kept for 15 minutes at room temperature. The enzyme activity is then measured as described in the text.

** 18 μg of crystalline chymotrypsin in 0.15 ml Tris buffer, 0.05 M at pH 7.8 is mixed with 0.1 ml buffer-diluted serum (containing 0.02 ml of serum) and kept for 15 minutes at room temperature. The enzyme activity was then measured as described in text.

*** All values are the mean of 16 determinations (as in Table XVI)

**** Values are the mean of 6 determinations (as in Table XVI)

α Values are the mean of 8 measurements.

## CONCLUSIONS

The assay results of the proteinase and hyaluronidase activity of the blood serum remained unchanged in both the salivarectomized and the control animals during the 5 day period of dicumarol feeding. This observation further supports the author's view, that the breakdown of the small vessel wall is initiated from the tissues around the vessel and not from the circulating blood as Sells supposed (1).

The slow but significant elevation of the activity of the antihyaluronidase and the antiproteinase during the onset of hemorrhage is suggestive of the operation of a chemical defence mechanism against the disintegrating process.

The failure of dicumarol added to serum samples in high concentration to elicit any hydrolytic enzyme activity, is also contrary to the postulation of Sells (1).

Salivarectomy, in some manner as yet not understood, leads to 'fragility' of the lysosomal membrane. Dicumarol, possibly by virtue of its lipid solubility, appears also to weaken the lysosomal membrane and cause release of the acid hydrolases. This was evident from the hydrolase activity of the supernatant fraction after high-speed centrifugation of the dicumarol-treated homogenates.

The presence of acid phosphatase-containing granular bodies was demonstrated in the endothelial cells of the small vessels and in the pericapillary connective tissue mast cells.

The data obtained in this study further support the view of numerous workers (103, 104, 106), that vascular fragility is perivascular in origin.

## 7. STUDIES ON HEMOPHILIC BLOOD

### INTRODUCTION

Sells (1), in our laboratory in 1957, studying the problem of vascular fragility, concluded that the weakening and ultimate breakdown of the small vessels is the result of enzymatic dissolution of the mucopolysaccharide components of the capillary wall. The ground substance was thought to become liable to attack by hyaluronidase and proteinases when the concentration of inhibitors - antihyaluronidase and antiproteinases - in the serum was decreased. To test the validity of this hypothesis he assayed the inhibitor level in the serum of a few hemophilic children in the hospital and was puzzled to find that the inhibitor activity was higher, instead of lower, than normal. Study of this apparent anomaly was not pursued further.

The present author, unaware of the above mentioned experience of Sells, became interested in the phenomenon of inhibition from an observation made in a former study, namely, that bleeding animals show a high activity of serum antiproteinase and antihyaluronidase (page 138). Two blood specimens from hemophilic patients were kindly provided in 1963 by Dr. Cecil Harris, Director of Hematology at the St. Mary's Hospital, Montreal. Both

samples were tested and the levels of antihyaluronidase and antiproteinase were found to be greatly elevated. In addition, the antithrombin activity also was found to be very high. The latter finding, tied up with a number of observations as described in the following paragraphs, opened up the present study.

Johnson and Seegers (209), reported that incubation of platelet cofactor I with normal or hemophilic plasma resulted in the inactivation of the cofactor. If the above preparation was extracted with ether, the activity of the cofactor was regenerated.

Ether extraction of serum (or plasma) is commonly used to remove the thrombin inhibitor which Seegers calls 'antithrombin III' (210). The present author therefore surmised a possible inverse relationship between the co-factor I and antithrombin III. This inference was further substantiated by the finding of Cooperberg and Teitelbaum (211) that the concentration of the cofactor in the serum of patients with coronary disease is increased. Teitelbaum et al. (212) found furthermore, that an inverse relationship develops in the rat when fed a thrombogenic diet.

The observation that platelet cofactor I disappears from the plasma during coagulation led Seegers to suggest that the cofactor may function as an acceptor of an inhibitor.

The present author's observation suggests the possible relationship between the conditions that lead to spontaneous hemorrhage, on the one hand, and thrombosis on the other. In the former condition the $\frac{\text{antithrombin}}{\text{platelet cofactor I}}$ ratio is high and in the latter it is low.

Numerous workers (213, 214) have shown that a minute quantity of thrombin is necessary for the activation of platelet cofactor I. It is reasonable to suppose that an increase in the concentration of antithrombin in the circulation might neutralize a small amount of thrombin (or autoprothrombin C) formed initially, and thus retard the process of coagulation.

Still further evidence of the significance of the aforementioned ratio or relationship is indicated in clinical reports on the treatment of hemophilia. The most widely used therapy is infusion of citrated fresh plasma or, if available, a solution of cofactor I concentrate (Cohn fraction I, consisting mainly of fibrinogen and platelet cofactor I, which is precipitated along with fibrinogen in the Cohn procedure). The effect of these preparations is to increase the cofactor 'activity' in the circulation of the hemophiliac either by raising the concentration of the cofactor, or by neutralizing an inhibitor (215). The relatively large amounts of the concentrate ( 10 to 12 grams) required to arrest bleeding suggests that the cofactor performes a neutralizing role (215).

To obtain further confirmation of the postulated inverse relationship between platelet cofactor I and antithrombin, assays were carried out on a number of normal and hemophiliac subjects as described in the following section.

## METHODS

### (1) Serum antithrombin

Serum antithrombin was assayed by a modification of the method of Brown et al. (216). The method is based on the principle that when known amounts of thrombin (enzyme) are incubated with serum (inhibitor), the decrease in enzyme activity with time reflects the amount of inhibitor in the serum.

In the test serum was used as the inhibitor preparation without further purification.

The inhibition tests were carried out at 37°C with the aid of an "Adams Thrombitron" (Fisher Scientific Company) as follows:

To 0.45 ml of serum 0.05 ml of thrombin solution (10 N.U. of thrombin in physiological saline) was added and the preparation incubated at 37°C for 10 minutes. At 3,5 and 10 minute intervals a 0.1 ml aliquot was removed and added to 0.2 ml of $BaSO_4$-treated plasma (fibrinogen source) and the clotting time of the specimen was measured.

In the control sample saline (0.85%) was substituted for serum.

The unit of enzyme activity was taken as the activity when 10 N. Units of thrombin (Parke Davis Topical Thrombin) in 0.1 ml of physiological saline clotted 0.2 ml $BaSO_4$-treated plasma in 5.0 seconds (fibrinogen clotting time).

Thus the activity expressed as percentage would be

$$A = \frac{T_o}{T_1} \times 100$$

where $T_1$ represents the clotting time (sec.) of the test sample containing

fibrinogen (substrate), thrombin (enzyme) and serum (inhibitor)

and $T_o$ represents the clotting time of the control sample containing fibrinogen, thrombin and saline.

The per cent inhibition would be $I = 100 - A$.

(2) Plasma antithrombin

Plasma antithrombin was assayed by the method of Biggs and Macfarlane (217) as indicated in Figure 33.

Human platelet cofactor I concentrate was kindly supplied by Dr. Albert Fisher of Connaught Laboratories, University of Toronto. Assays were carried out on blood samples taken from 100 normal subjects and from 17 hemophilic patients. The specimens were kindly provided by Dr. Cecil Harris, Director of Hematology at the St. Mary's Memorial Hospital, Montreal.

## RESULTS AND DISCUSSION

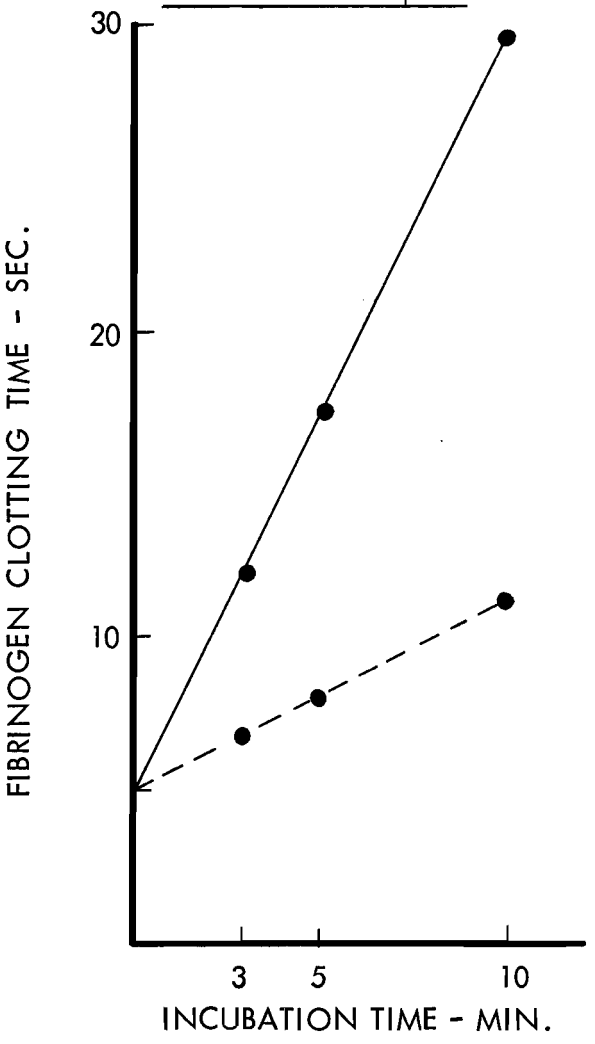
(1) Antithrombin

A comparison of the thrombin neutralizing capacity of the normal serum and of the hemophilic specimens is indicated in Figure 28. In contrast to the mean inhibition of 56.9 per cent obtained with the normal specimens, the inhibition with the specimens taken from hemophilic subjects at the height of a bleeding episode, before treatment, was 82.7 per cent as shown in Table XXI. Thus the antithrombin level in the hemophiliac is significantly elevated.

As shown in Table XXI, adsorption of hemophilic or normal serum with protamine sulphate and with barium sulphate did not change the degree

Figure 28

Antithrombin Activity in Serum of Hemophilic and Normal Subjects




Test system:
10 N.U. of thrombin (Topical Thrombin, Parke Davis Co.) in 0.05 ml of saline is added to 0.45 ml of test serum and incubated at 37°C. At intervals of 3,5 and 10 minutes, 0.1 ml aliquot of serum (inhibitor) and thrombin (enzyme) solution was removed and added to 0.2 ml fibrinogen solution ($BaSO_4$ - treated normal plasma), the clotting time of which was determined.

Hemophilic serum (●———●), each point is the mean of duplicate measurements of 17 specimens.

Normal serum (●– – –●), each point is the mean of duplicate measurements of 100 specimens.

TABLE XXI

Serum Thrombin Inhibitor Activity of Normal and Hemophilic Blood

| Serum preparation | Inhibition (%) |
|---|---|
| Normal serum (control) (a) | 56.9* ± 0.5** (100)*** |
| Hemophilic serum | 82.7 ± 0.5 (17) |
| Hemophilic serum heated at 56°C (b) | 70.8 ± 0.2 (6) |
| Normal serum heated at 56°C | 50.3 ± 0.2 (6) |
| Hemophilic serum treated with $BaSO_4$ (c) | 82.7 ± 0.5 (8) |
| Normal serum treated with $BaSO_4$ | 56.2 ± 0.3 (6) |
| Hemophilic serum dialyzed against saline (d) | 70.8 ± 0.2 (6) |
| Normal serum dialyzed against saline | 48.4 ± 0.3 (8) |
| Hemophilic serum treated with protamine sulfate (e) | 82.2 ± 0.4 (8) |
| Normal serum treated with protamine sulfate | 55.8 ± 0.4 (6) |
| Hemophilic serum + Cofactor I concentrate (4.0 mg/ml) (f) | 66.0 ± 0.4 (8) |
| Normal serum + Cofactor I concentrate (4.0 mg/ml) | 37.5 ± 0.24 (24) |
| Ether extracted hemophilic serum (g) | none (8) |
| Ether extracted normal serum | none (6) |

* and (a) to (g): For explanation see following page.

*All calculations are based on the values obtained after 10 minutes incubation of thrombin with serum at 37°C. In all tests a standard of 10 N.U. of thrombin (Parke Davis, Topical Thrombin) was used.

**Standard error of mean.

***Number of specimens tested in duplicate.

(a) The number (100) of normal specimens included 19 of cord blood.

(b) Heated at 56°C for 3 min. Sample cooled and assayed for antithrombin activity.

(c) Serum mixed with barium sulphate (0.1 g/ml) for 15 min. in a mechanical mixer, centrifuged at 1500 r.p.m. for 15 min. Supernatant assayed for antithrombin activity.

(d) Serum dialyzed against physiological saline, for 20 hrs. at 4°C. Serum: saline = 1:60.

(e) Serum mixed with protamine sulphate (3.0 mg/ml) for 15 min. in a mechanical mixer, centrifuged at 1500 r.p.m. for 15 min. Supernatant assayed for antithrombin activity.

(f) Platelet Cofactor I concentrate (4.0 mg/ml) added to serum and pre-incubated at 37°C for 15 min. Antithrombin activity determined immediately.

(g) Ether extraction carried out as described by Seegers et al. (209).

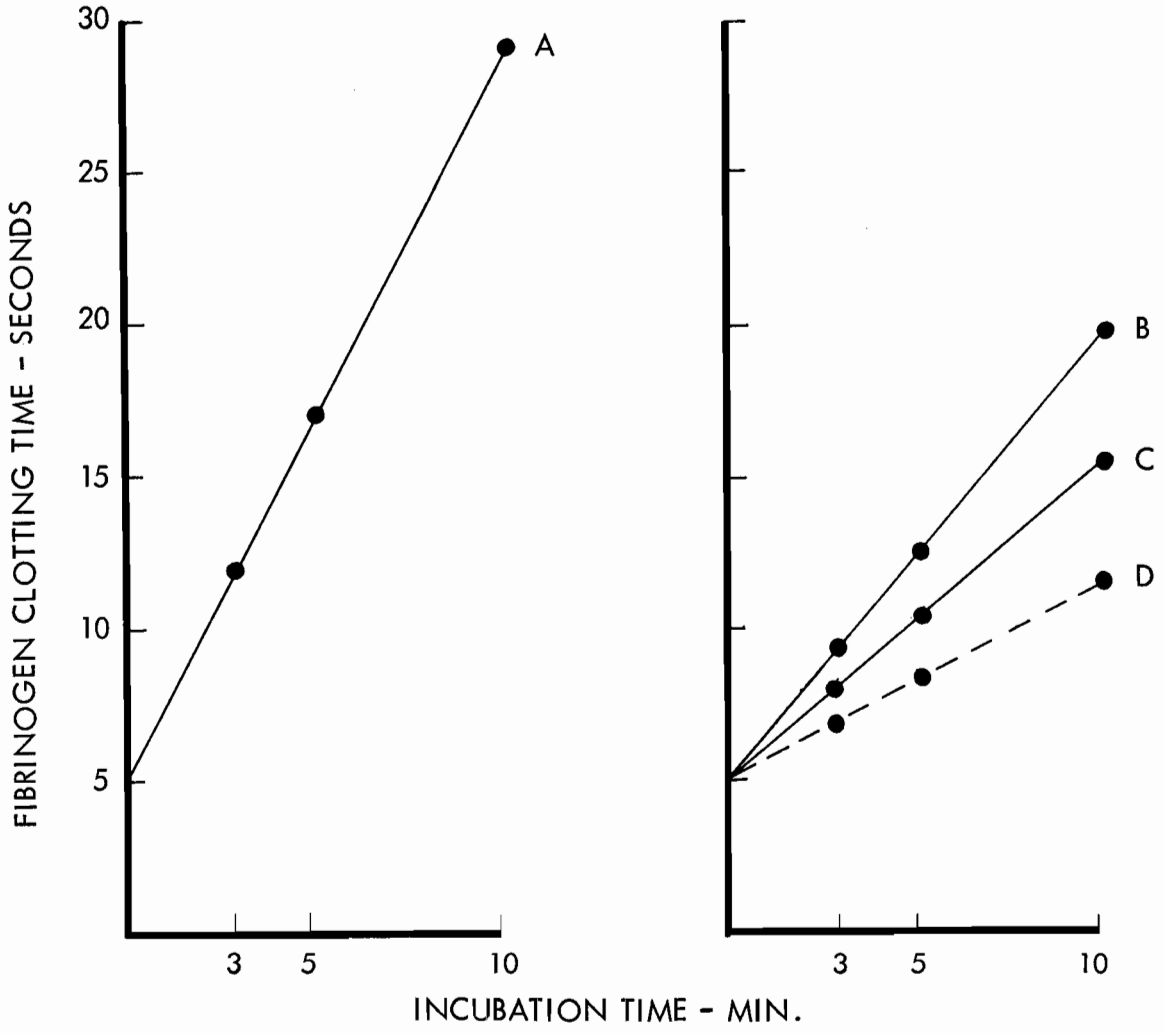
of inhibition. Ether extraction of the serum, however, resulted in a complete loss of inhibitor activity in the hemophilic as well as in the control (normal) specimens. Heating of the hemophilic serum at 56°C for 5 minutes; or dialysis at 4°C for 20 hours against 60 volumes of physiological saline, resulted in a partial decrease in the inhibition. Both treatments gave the same degree (11.9 per cent loss) of inhibition. Control serum when subjected to the same heat treatment showed a loss of 6.6 per cent of the inhibitory activity and 8.5 per cent after dialysis. These observations suggest (a) that serum contains more than one substance with thrombin neutralizing property, or (b) that the molecule is complex and that some part of the inhibitor is comparatively labile. The difference between the inhibitory activity of the hemophilic and the control sera after these treatments, suggests that the hemophilic serum has a higher concentration of the "heat-labile-and-dialysable" substance(s).

As shown in Figure 29, the addition of platelet cofactor I concentrate, in concentrations of 3 and 5 mg per ml of hemophilic serum, resulted in a pronounced decrease in the antithrombin activity. The concentrations used are comparable to that used clinically for the arrest of bleeding in hemophiliacs.

When 4.0 mg of concentrate were added to 1 ml of hemophilic serum, the degree of inhibition fell from 82.7 per cent to 66.0 per cent, which is close to the value obtained with normal serum. The concentrate had a similar effect on normal serum as indicated in Table XXI.

Figure 29

The Influence of Platelet Cofactor I Concentrate on the Antithrombin Activity of Hemophilic Serum




Test system:
10 N.U. of thrombin in 0.05 ml of physiol. saline is added to 4.5 ml of serum and incubated at 37°C. At intervals of 3, 5 and 10 minutes 0.1 ml aliquot is taken and added to 0.2 ml of fibrinogen solution, and the clotting time is recorded.

A - Hemophilic serum

B - Hemophilic serum pre-incubated with 3.0 mg/ml of Platelet Cofactor I concentrate at 37°C for 15 min.

C - Hemophilic serum pre-incubated with 5.0 mg/ml of Platelet Cofactor I concentrate at 37°C for 15 min.

D - Normal (control) serum.

Each point represents the mean of 6 measurements.

TABLE XXII

Inhibition of Thrombin by Hemophilic Serum

| Subject No. | Condition | Inhibition (mean %) |
|---|---|---|
| 1 | Hemophiliac (bleeding) before treatment | 81.5 |
| | Same subject (1) after treatment with Platelet Cofactor I concentrate* | 56.5 |
| 2 | Hemophiliac in remission phase | 82.2 |
| | Hemophiliac (2) received plasma for 48 hours** | 76.7 |
| 3 | Normal subject - Control*** | 56.5 |

* Patient (1) was admitted to the Emergency ward. He then received 8.0 grams of Platelet Cofactor I concentrate. Serum antithrombin was determined at admission and 24 hours later.

** Patient (2) was admitted at the height of a bleeding episode. Serum antithrombin was measured at admission, and 48 hours later; he had received 8 units (about 1300 ml) of fresh frozen plasma.

*** The author served as control.

All tests were done in duplicate.

Blood samples and laboratory space for this study were kindly provided by Dr. C. Harris at St. Mary's Hospital, Montreal, Quebec.

Serum specimens obtained from hemophilic subjects at the height of the bleeding episode and at various times after the administration of platelet cofactor I concentrate, showed also a fall in thrombin neutralizing power as indicated in Table XXII.

As $BaSO_4$ adsorption failed to alter the thrombin inhibitory activity of hemophilic serum before the addition of the concentrate, the likelihood of the presence of some residual prothrombin was ruled out, since $BaSO_4$ is known to adsorb prothrombin. The platelet cofactor preparation in saline solution showed no thrombin activity when added to a fibrinogen source (e.g. plasma). The concentrate was found also to be free of prothrombin contamination since the addition of 0.1 ml of $CaCl_2$ (0.025 M) failed to produce clot formation even up to the 6th hour. The results thus indicate that some substance in the platelet cofactor concentrate either combines with, or becomes adsorbed on the antithrombin. The substance presumably is the cofactor I itself. However, the possibility that the concentrate may have proteolytic activity or activate a proteolytic enzyme in hemophilic serum, which enzyme in turn might destroy the antithrombin, has also been considered.

No tryptic, chymotryptic, esterase or hyaluronidase activity was detectable in the cofactor I concentrate, nor any increase in proteolytic activity of the serum after the addition of platelet cofactor I.

To determine whether the fall in the thrombin inhibition in hemophilic serum after the addition of cofactor concentrate is due to inactivation,

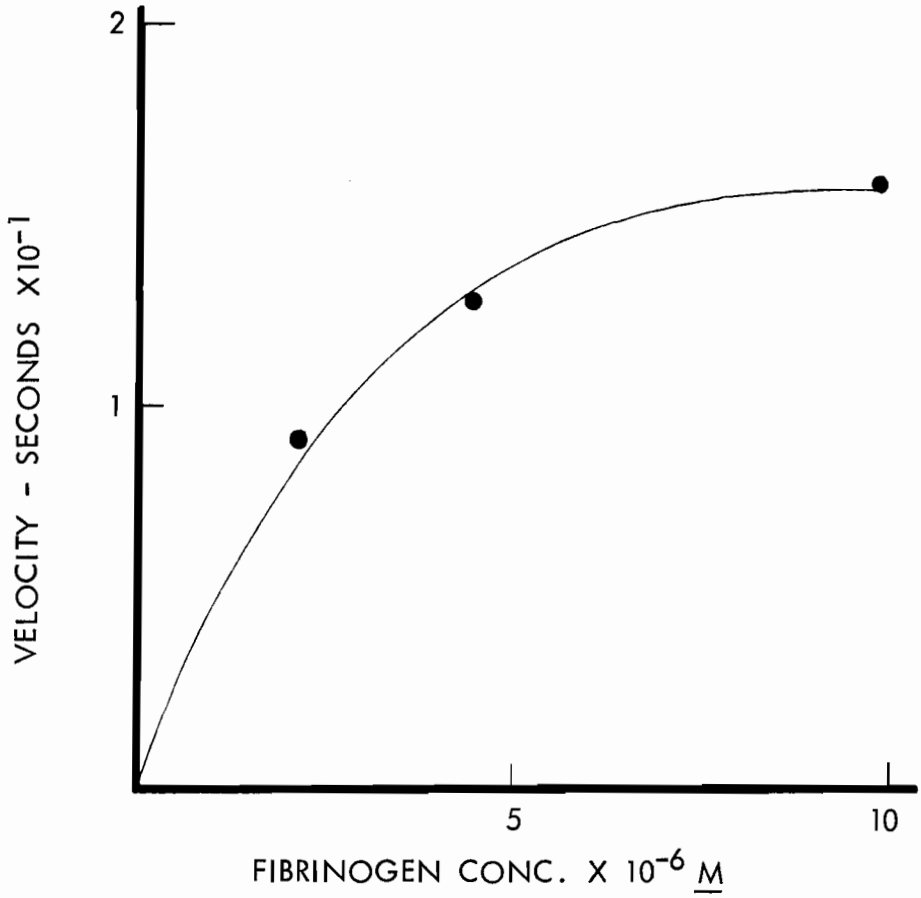
adsorption or destruction of the inhibitor, the kinetics of the thrombin-fibrinogen-inhibitor system and the thrombin-fibrinogen-inhibitor-cofactor concentrate system were studied.

The substrate (fibrinogen) concentration of the barium sulphate-treated plasma preparation was found to be 340 mg per cent (218). Taking a value of 300,000 as the average molecular weight of fibrinogen (219), the concentration in the plasma would be $0.88 \times 10^{-5}$ M. Dilutions of the fibrinogen specimen were made with physiological saline. In all these studies, 10 N.U. of thrombin were used as the enzyme concentration (E). The thrombin catalyzed conversion of fibrinogen to fibrin, as a function of substrate concentration is indicated in Figure 30 . The initial velocity is represented by a linear relationship up to a substrate concentration of $0.88 \times 10^{-5}$ M (Figure 30). Using the approach of Taylor et al.(220), for the study of the kinetics of the thrombin-antithrombin system, we assigned a serum concentration of 0.05 g/100 ml and a molecular weight of 300,000 - (mean molecular weight of alfa-2-globulins) - to the inhibitor.

The linear Lineweaver-Burk plot obtained by plotting the reciprocal of the velocity as a function of the reciprocal of substrate concentration, is shown in Figure 31. The difference between competitive and non-competitive inhibition is best demonstrated by studying the degree of inhibition with various concentrations of substrate and of inhibitor. As the maximum velocity for the uninhibited system and that of the inhibited system are different, while the intercept on the 1/S axis is independent

Figure 30

Thrombin-Fibrinogen Reaction as a Function of Fibrinogen Concentration




Each plot is the mean of 3 measurements.

Enzyme (thrombin) concentration 10 N.U.

Figure 31

Lineweaver - Burk Plot of Thrombin - Fibrinogen

and Thrombin - Fibrinogen - Inhibitor Systems

The reciprocal of the velocity as a function of the reciprocal of substrate concentration is plotted, giving a straight line relationship at different inhibitor concentrations.

A - Thrombin/fibrinogen system (E + S). [ E ] = 10 N. Units.

B - Thrombin/fibrinogen/normal serum (E + S + I).
Concentration of inhibitor, $1.67 \times 10^{-6}$ M.

B' - Same system as B. Concentration of inhibitor, $0.83 \times 10^{-6}$ M.

C - Thrombin/fibrinogen/hemophilic serum. Concentration of inhibitor unknown.

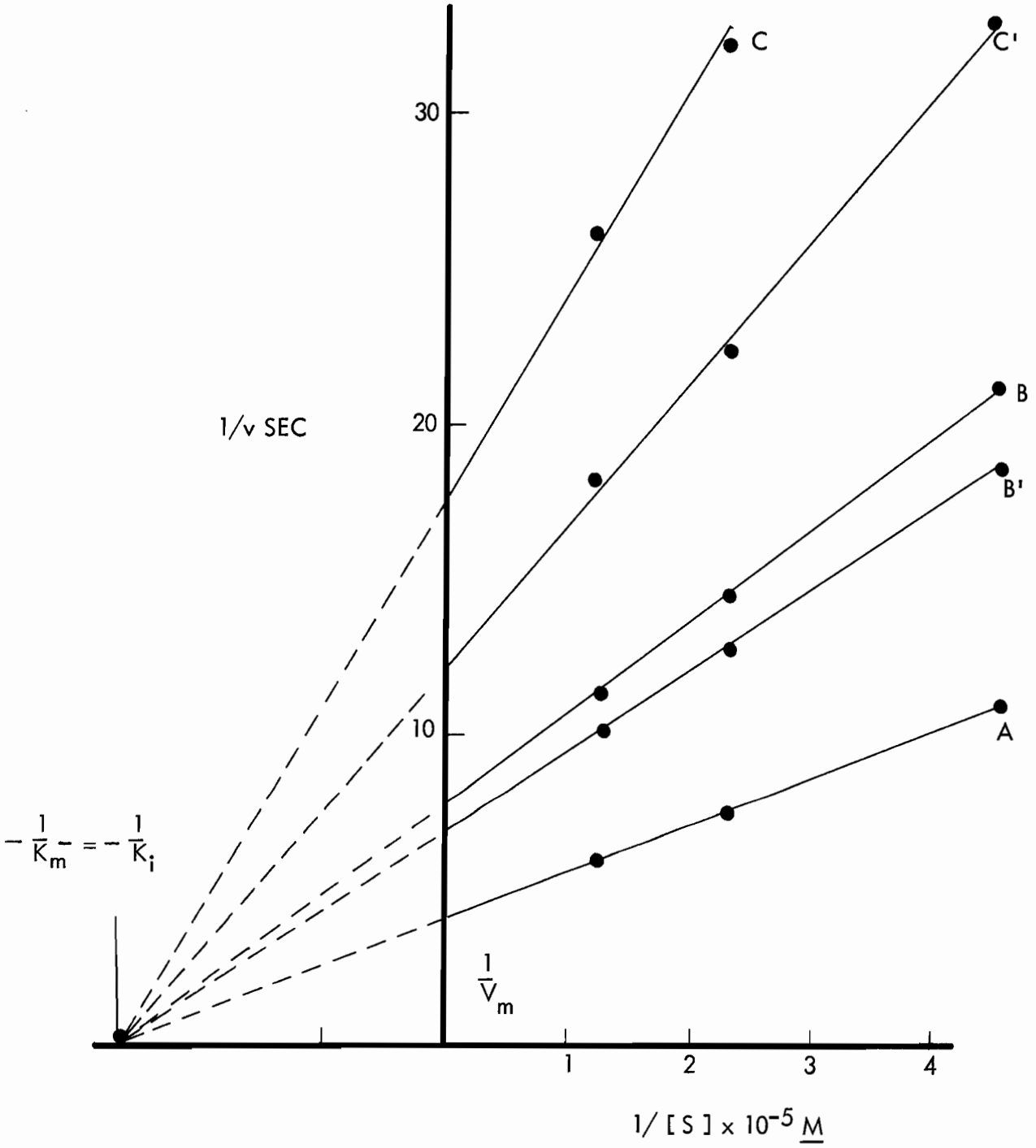
C' - Thrombin/fibrinogen/hemophilic serum which contains 4.0 mg/ml of Platelet Cofactor I concentrate.

As the intercept on the 1/S axis is independent of inhibitor concentration, with normal as well as with hemophilic serum, the inhibition is non-competetive in nature.

All points represent the mean of 6 measurements.

Figure 31

Lineweaver - Burk Plot of Thrombin - Fibrinogen and Thrombin - Fibrinogen - Inhibitor Systems



of the inhibitor concentration (Figure 31), one may conclude that the thrombin-antithrombin reaction is noncompetitive in nature. It is evident also, that the hemophilic sera differed only in the concentration of inhibitor in the specimens and that the effect of the added cofactor I concentrate was merely one of dilution of the sera. By taking advantage of the Dixon Expansion (Fig.32), in which the reciprocal of the velocity as a function of inhibitor concentration is plotted at various substrate concentrations, as shown in Figure 32, the hemophilic serum was found to contain $9.25 \times 10^{-6}$ M of inhibitor. On addition of platelet cofactor I concentrate, the concentration of the inhibitor was decreased to $5.0 \times 10^{-6}$ M, which is still 3 times greater than that of normal serum ($1.67 \times 10^{-6}$ M). These results clearly demonstrate the antagonistic effect of the cofactor toward the inhibitor. Our results further support the finding that high concentrations of certain noncompetitive inhibitors may not completely inhibit the activity of an enzyme, because unlike the competitive inhibitors, they do not attach to the active center but at some distance from it. The affinity of the enzyme for the inhibitor $1/K_i$ and for the substrate $1/K_s$, was calculated as follows:

If $K_m$ is essentially an equilibrium constant (221),

$$\frac{1}{K_m} = \frac{v}{[S](V_m - v)}$$

where $K_m$ = equilibrium constant of the uninhibited system

$v$ = velocity at a given substrate concentration

Figure 32

Dixon Expansion of Thrombin - Fibrinogen - Inhibitor System

The reciprocal of the velocity as a function of inhibitor concentration is plotted giving a strait line relationship, at different substrate concentrations (A, B, C).

A - Substrate concentration (fibrinogen), $0.88 \times 10^{-5}$ M.

B - Substrate concentration, $0.44 \times 10^{-5}$ M.

C - Substrate concentration, $0.22 \times 10^{-5}$ M.

H - Hemophilic serum used as inhibitor source, with substrate concentration of $0.88 \times 10^{-5}$ M.

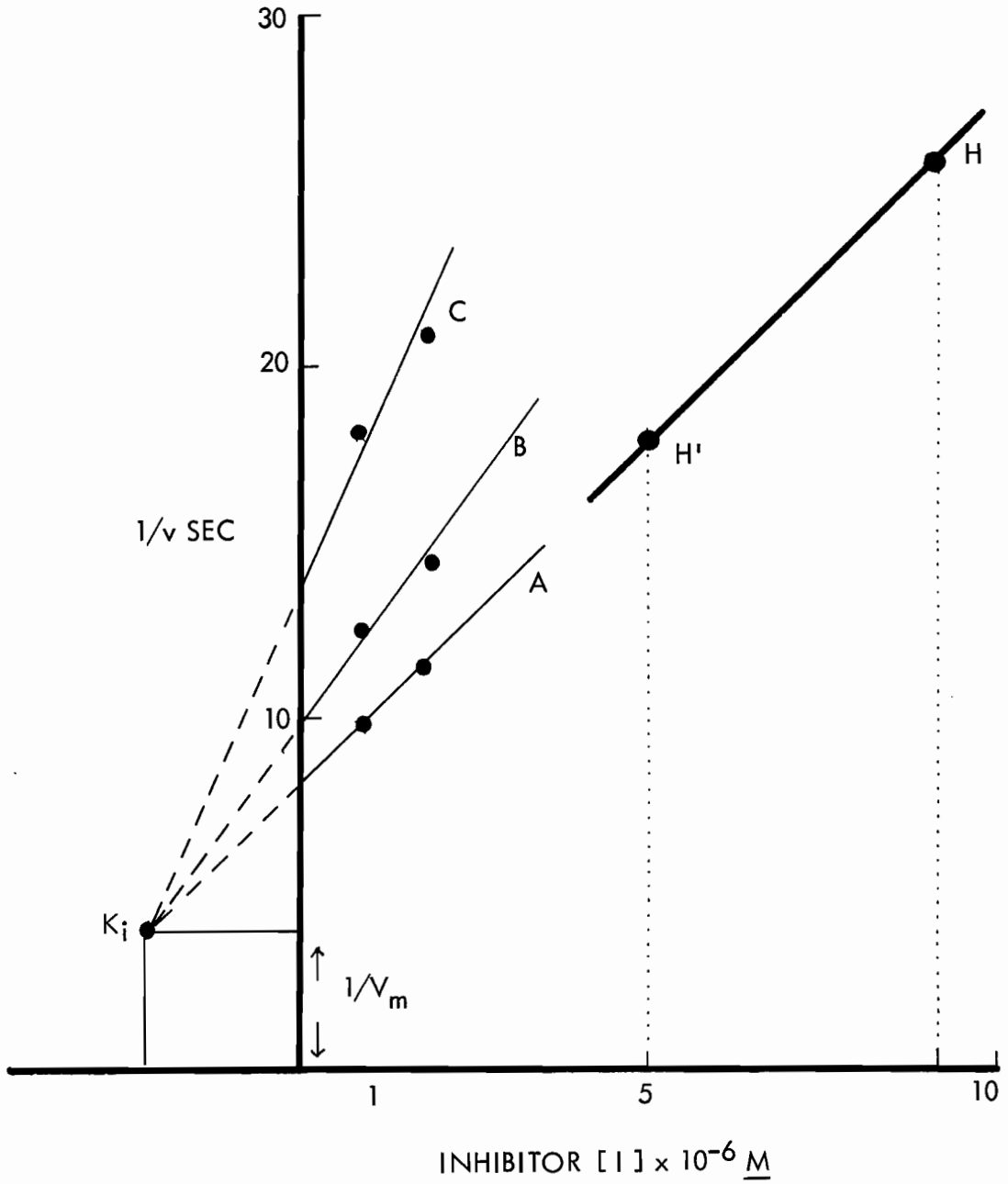
H' - Same system as H, except that hemophilic serum was pre-incubated with Platelet Cofactor I concentrate (4.0 mg/ml) for 15 minutes, at 37°C.

The enzyme concentration in all experiments was 10 N.U. of thrombin, in 0.05 ml of saline.

All points are the mean of 6 measurements.

Figure 32

Dixon Expansion of Thrombin - Fibrinogen - Inhibitor System



$$v = \frac{1}{\text{clotting time}}$$

$V_m$ = maximum velocity of the uninhibited system

[S] = substrate concentration in moles per litre.

Thus, by the use of Lineweaver-Burk plot and the above mentioned equation, the affinity of thrombin for fibrinogen may be represented by

$$\frac{1}{K_m} = 4.55 \times 10^5 \underline{M}.$$

The affinity of the inhibitor for the enzyme in our reaction system is represented by the following:

$$\frac{1}{K_i} = \frac{(V_m/V_p) - 1}{[I]}$$

where $K_i$ = inhibitor constant

$V_m$ = maximum velocity uninhibited system

$V_p$ = maximum velocity of inhibited system

[I] = inhibitor concentration in moles per litre

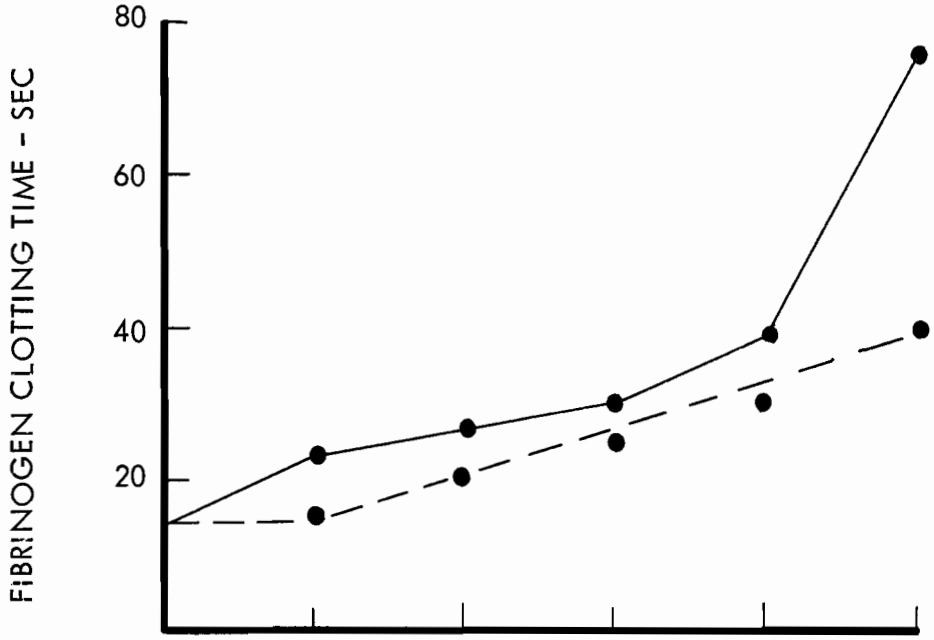
$$\frac{1}{K_i} = 4.72 \times 10^5 \underline{M}.$$

Thus, the affinity of the enzyme (thrombin) for the inhibitor (anti-thrombin) is practically the same as that of the enzyme for the substrate (fibrinogen).

As represented in Figure 33, the concentration of the thrombin

Figure 33

Plasma Antithrombin Test of Biggs - Macfarlane




| | | | | | | |
|---|---|---|---|---|---|---|
| PLASMA* 1/5 (ml) | 0.00 | 0.05 | 0.10 | 0.15 | 0.25 | 0.30 |
| SALINE (ml) | 0.30 | 0.25 | 0.20 | 0.15 | 0.05 | 0.00 |
| THROMBIN (N.U.) | 10 | 10 | 10 | 10 | 10 | 10 |

Hemophiliac subject. Each point is the mean of duplicate measurements from four specimens. ●——●

Normal (control) subject. Each point is the mean of duplicate measurements from eight specimens. ●— — —●

* Plasma is diluted with physiological saline (P:S = 1:5).

inhibitor(s) in the plasma of hemophiliacs was also elevated.

(2) Hyaluronidase and antithrombin

As pointed out previously (page 3 ), the most puzzling single question in the problem of spontaneous internal bleeding is: what has the coagulibility of the circulating blood to do with the condition of the wall of the small blood vessels?

The development of vascular fragility, in this author's opinion, is a consequence of the breakdown of or some kind of physicochemical change in the ground substance of the perivascular sheath and surrounding connective tissue. It would appear that the action of hyaluronidase is involved in the process. The idea occurred to the author that hyaluronidase may be connected also with the inhibitory system in the plasma or serum. This was tested as follows:

Before adding the thrombin to the hemophilic or control serum specimens in the test for antithrombin, 3 and 5 mg respectively of hyaluronidase were added per ml of serum to two tubes and incubated for 15 minutes at 37°C. Thrombin then was added and the antithrombin activity was measured.

The results are indicated in Figure 34. With normal serum as the source of the inhibitor, and without the addition of hyaluronidase, the linear relationship designated as $A^1$ was obtained between the duration of incubation and the clotting time. The curves $A^2$ and $A^3$ were obtained in the presence of 3.0 and 5.0 mg hyaluronidase per ml serum.

The curves $B^1$, $B^2$ and $B^3$ represent the corresponding results with hemophilic serum.

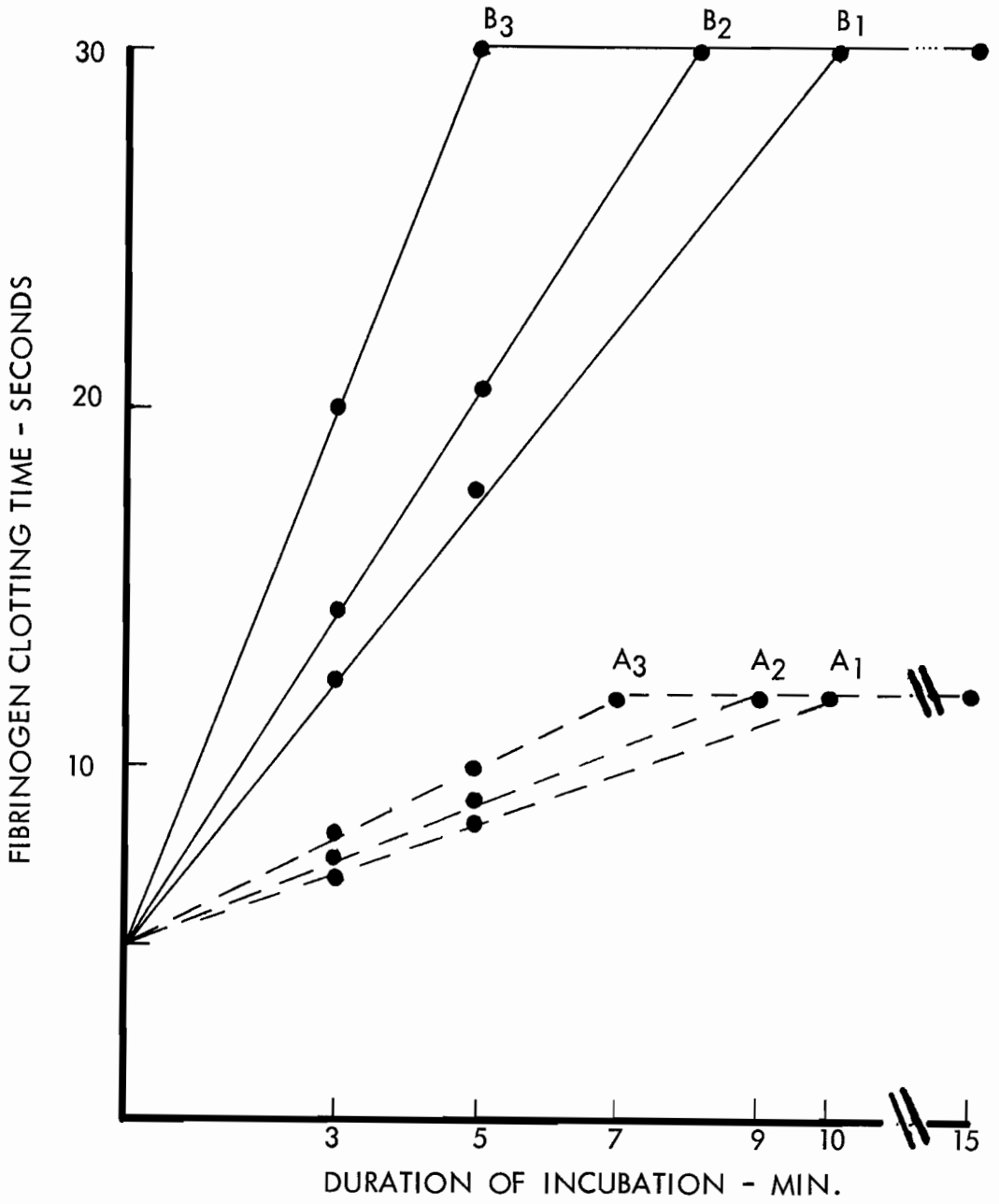
The results are of special interest since they may have a bearing upon the relationship between the coagulability of the blood and the state of the mucopolysaccharide in the vessel wall. The clotting time reached the same limiting value of 12 seconds for each of the control samples and similarly 30 seconds for those with the hemophilic serum. Thus, the degree of inhibition remained the same in the presence or in the absence of hyaluronidase. When the limiting degree of inhibition was reached, no further change took place with prolonged incubation. However, on the addition of hyaluronidase to the samples ($A^2$, $A^3$ in the control, and $B^2$, $B^3$ in the hemophilic serum), the maximum inhibition obtainable in the system was reached earlier.

According to the author's interpretation of the results, hyaluronidase appears to have an 'activating' effect on the antithrombin-thrombin system. It is evident that the thrombin and hyaluronidase inhibitors are not identical, since if they were components of some kind of nonspecific type of inhibitor, one would expect weaker inhibition of the thrombin if the latter were added the hyaluronidase. Theoretically, the reaction sites available to thrombin should be at least partially saturated. This question will be studied further.

Figure 34

'Activation' of Serum Thrombin-Inhibitor

by Hyaluronidase in Vitro




Test system as described in Figure

Hemophilic serum (●——●); $B_1$ - Hemophilic serum; $B_2$ - Hemophilic serum pre-incubated with 3.0 mg/ml Hyaluronidase (Testicular Hyaluronidase, Nutritional Biochemical Corp.); $B_3$ - Hemophilic serum pre-incubated with 5.0 mg/ml Hyaluronidase. All incubation at 37°C, for 15 minutes, then tested for anti-thrombin activity.

Normal serum (●– – –●); $A_1$ - Normal serum; $A_2$ - serum pre-incubated with 3.0 mg/ml hyaluronidase; $A_3$ - normal serum pre-incubated with 5.0 mg/ml hyaluronidase.

All values are the mean of 8 measurements.

TABLE XXIII

Antiprotease and Antihyaluronidase Activity of Hemophilic Serum

| Particulars | Hemophiliac | Normal |
|---|---|---|
| | per cent inhibition | |
| Antihyaluronidase | 82 | 60 |
| Antitrypsin | 90 | 70 |
| Antichymotrypsin | 87 | 72 |

## CONCLUSIONS

The dual action of hyaluronidase is indicative of a connection between the state of the blood coagulation system and the state of the walls of the small vessels. The presence of hyaluronidase in the perivascular connective tissue would tend to break down the supporting ground substance with accompanying hemorrhages (107). If hyaluronidase were to gain entrance into the blood it would also tend to inhibit thrombin, by the 'activation' of antithrombin (Figure 34). It is thus reasonable to suppose, that hyaluronidase is at least one of the factors which play a cardinal role in the onset of spontaneous hemorrhage.

The findings described in this study indicate also a common feature between the hemophiliac and the salivarectomized rat on dicumarol diet, when both are in a hemorrhagic state, namely, a high level of inhibitors (Table XXIII) in the blood (serum).

The addition of platelet cofactor I concentrate to hemophilic or normal serum results in lowering the antithrombin activity. Similarly the administration of fresh frozen plasma or platelet cofactor I concentrate to the hemophiliac lowers the antithrombin activity, at least as shown by the reduced concentration of the inhibitor in the serum. The interaction of platelet cofactor I (concentrate) with antithrombin (Figure 29) indicates further that (a) antithrombin is the platelet cofactor I inhibitor, or (b) that the platelet cofactor I inhibitor, which is thought to be increased in hemophilia (122), has also antithrombin activity.

## GENERAL DISCUSSION

The chemical approach to vascular fragility and to hemostasis is a relatively new development as a result of the numerous biochemical, physiological and instrumental advances of the past two decades.

The study presented herein has been done mainly on the rat as the experimental animal. The question may be asked: to what extent do the findings have application also to other species? Our limited experience with the guinea pig, and the observations of other workers on other animals, along with clinical experience with dicumarol, warrant the expectation that the findings apply also to other species.

The first major advance in the study was to develop a procedure for the production of uniform hemorrhage (page 49). The nonexistence of such a method has been largely responsible for the relative lack of chemical study on vascular fragility (1).

Salivarectomy in the rat greatly decreases the resistance to the toxic action of dicumarol. Resistance is a prominent characteristic of older animals (223); young animals succumb much sooner than older ones when fed dicumarol containing diet. The difference in behaviour apparently is not attributable only to the relatively larger dose of the drug per unit weight in the small animals when the two groups are fed the same diet. A somewhat similar phenomenon is seen among hemophilic individuals in that the young hemophiliacs show a greater tendency to bleed than the older.

It is evident from our study on the rat that removal of the submaxillary

glands is responsible for the increased sensitivity to the hemorrhagic effects of dicumarol. Grad and Leblond (224) reported that the testis and thyroid hormones exert a synergistic action on the serous tubules of the submaxillary gland in the rat. Fawcett and Kirkwood (225, 226) found a high concentration of thyroid iodinase in the submaxillary salivary gland. They postulate that the glands may play an important role in the metabolism of extrathyroidal 'organic' iodine and may function as "reverse thyroids". However, a number of workers have not been able to confirm the findings (227, 228, 229, 230). It has been reported (231) also that the salivary glands play an important part in regulating the activity of the thyroid glands by way of the utilization or production of a thyroid stimulating hormone. The thyroid gland from salivarectomized rat showed a four times greater uptake of TSH than the control animals. The relationship between the submaxillary salivary glands and other endocrine glands has been reviewed by Volker (232). However, the majority of the postulated relationships to the adrenal glands, estrogen metabolism have not been substantiated.

The elevated PBI values obtained in our study (page 90) would indicate a state of low grade hyperthyroidism in view of the histological appearance of the tissue (page 56) and the slightly increased weight of the glands (page 90).

Among the prominent changes in the salivarectomized animal were an increase in the mean systolic and diastolic blood pressure from 110 mm (control) to 160 mm/Hg, a thickening of the wall of the small vessels (page 54) and a small increase in the weight of the heart, with hypertrophy of the muscle

fibers (page 92). Salivarectomy alone does not give rise to spontaneous bleeding.

Dicumarol, ingested along with the food, apparently is readily absorbed presumably in close association with the lipid materials in the diet. The normal adult rat, represented by our sham-operated control, can detoxify and eliminate as well as store the drug in the adipose tissue. Ingestion by the salivarectomized animal, however, results in abrupt retardation of the growth rate and rapid development of the hemorrhagic state. The arteriographic evidence in our study (page 86) indicated that the small vessels of the intestinal wall are among the first to become unstable and to break down (page 76). Failure to absorb and possibly even to digest the food in the gastrointestinal tract reduced the animals virtually to a state starvation. This was further indicated by the observed rapid breakdown of glycogen in the liver (page 100).

It is of particular interest that the onset of bleeding occurred (page 65) before any fall in the prothrombin level of the blood was detectable (page 108). The initiation of bleeding was favoured furthermore by the thinning of the wall of the small vessels (page 53) and the increased blood pressure. Another feature of especial significance is that bleeding apparently begins around the arteriolar segment of the microcirculation and not at the venular segment as suggested by Zweifach.

Vascular breakdown is most probably initiated from the cellular elements of perivascular tissue and not from the blood. There was evidence of dissolution

of the ground substance of the supporting tissues (page 67). The presence of acid phosphatase-containing granular structures in the endothelial cells and in the perivascular mast cells (page 127) indicated the presence of 'lysosomes' and the release of their hydrolytic enzymes in vitro under the influence of the dicumarol (pages 230, 231).

It may appear paradoxical that vascular fragility is accompanied by an increased level of hydrolase inhibitor activity. This was shown also by others in our laboratory to occur in inflammation (233). As the bleeding in the animal becomes more severe and generalized, the inhibitor activity also tends to rise. Thus, there appears to be activation of a defence mechanism to counteract the connective tissue breakdown (or inactivate the enzymes responsible for the breakdown).

In the hemophiliac, in the bleeding phase, the mean serum level of antihyaluronidase was found to be 82 per cent compared to 60 per cent in the normal. The mean antitrypsin activity was found to be 90 per cent compared to 70 per cent in the normal. Hyaluronidase was shown to activate antithrombin which acts as a potent anticoagulant.

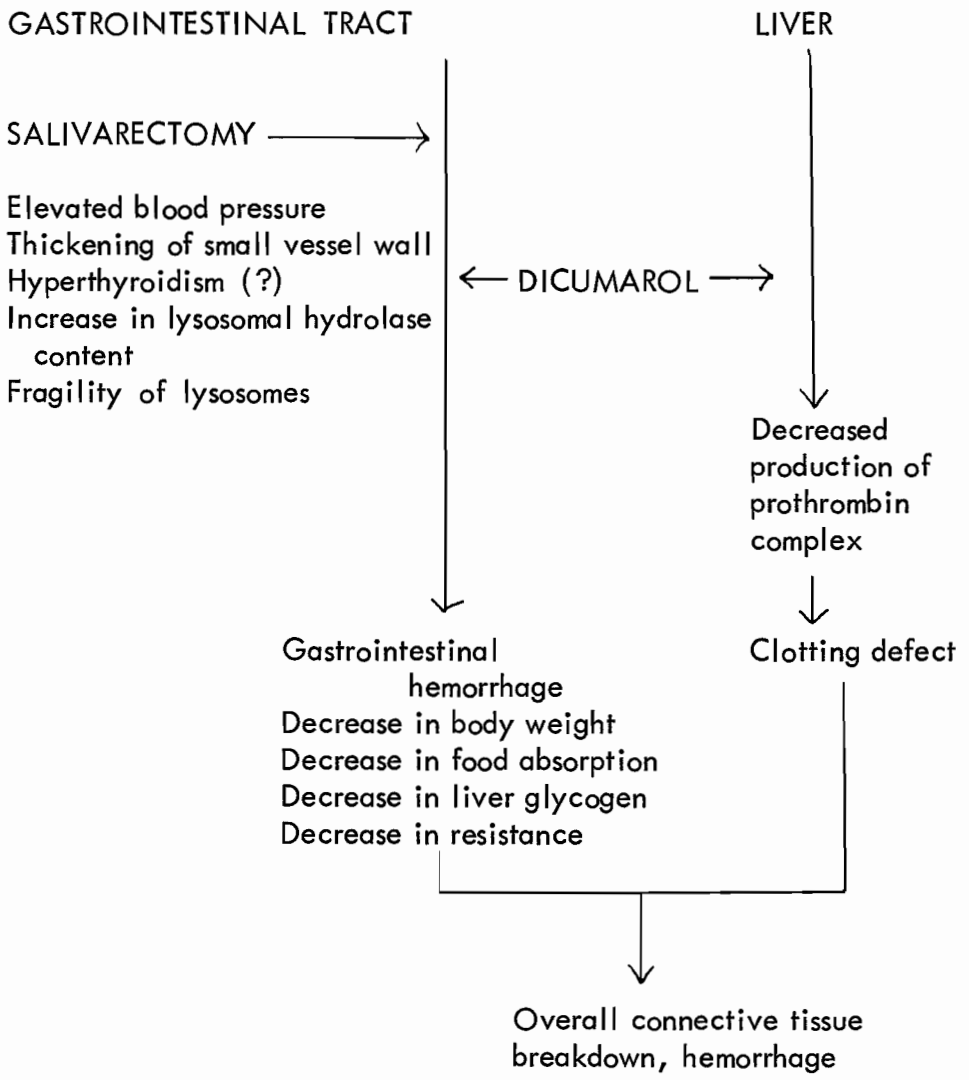
The observed parallels between the state of vascular fragility in the hemophiliac and that seen in the dicumarol treated rat lends validity to the use of the salivarectomized animal for the study of vascular fragility.

While the conditions which initiate the development of vascular fragility may differ in the various hemorrhagic diseases, dissolution or weakening of the perivascular sheath and the adjacent connective tissue appears to be a

common occurrence in all cases. Elevation of the inhibitor activity of the serum is among the most useful indices of the state of vascular fragility.

Among the difficulties in the biochemical approach to the study, and particularly the involvement of the blood coagulation mechanism in vascular fragility is that few of the coagulation factors have been prepared in pure form, and little is known about their physico-chemical properties such as ionization, active centres and others. The existing clinical methods used for diagnostic purposes measure the activity rather than the absolute quantity of the factors. While the procedures are very useful in clinical diagnosis, they are of limited value for basic scientific studies. For example, the platelet cofactor I activity may in certain circumstances be found to be lower than normal. This may arise from a deficiency in the concentration of the factor, from the presence of an increased amount of inhibitor or from some change in the physico-chemical characteristics of the molecule. Thus, the simple measurement of activity does not tell which of these conditions is responsible for a lowered activity.

The author's concept of the sequence of events in the development of the state of vascular fragility and internal bleeding in the salivarectomized rat receiving a dicumarol containing diet may be summarized as follows:




It is evident that a study of this magnitude calls for collaboration of workers from many disciplines - the biochemist, biophysicist, histologist, physiologist, immunologist, microcirculatory specialist and others. Before this elaborate type of teamwork is achieved a picture of the overall problem and its facets has to be appreciated. It is hoped that our effort may help to promote wider team work on the problem.

## SUMMARY

The following is an outline of the main findings in the study:

1. The salivarectomized rat, fed a dicumarol containing Purina ration (0.5%), has proved to be a satisfactory experimental subject for the production of uniformly distributed, reproducible and controllable hemorrhage, and for the study of vascular fragility.
2. Three to four weeks after removal of the submaxillary salivary glands from young rats (60 g), the following changes are apparent:
   (a) thickening of the wall of the small vessels (arterioles and small arteries),
   (b) elevation of the systolic and diastolic blood pressure,
   (c) increase in pulse pressure,
   (d) increase in the relative weight of the heart, kidneys and thyroid glands,
   (e) hypertrophy of muscle fibers in the heart,
   (f) elevation of the serum protein-bound iodine,
   (g) hyperplasia of the thyroid glands with a low cuboidal epithelium, small follicles and pale vacuolated colloid,
   (h) abrupt fall in the growth rate,
   (i) relative fragility of the lysosomal membranes.
3. Dicumarol causes the release of acid-phosphatase and beta-glucuronidase from rat liver lysosomes *in vitro*.
4. Acid phosphatase-containing granules are demonstrable in abundance in the cytoplasm of the endothelial cells of the small vessels and in the peri-

vascular mast cells.

5. Dicumarol administration to stabilized salivarectomized rats produces the following changes:

   (a) early breakdown (48 hours after administration) of the small vessels in the gastrointestinal tract as shown by

      (i) arteriographic demonstration of vascular fragility,

      (ii) accumulation of red blood cells in the cortical sinusoids of the mesenteric lymph nodes,

      (iii) submucosal hemorrhages in the stomach and small intestine,

      (iv) tarry stools,

   (b) impaired absorption of nutrients from the gastrointestinal tract as indicated by

      (i) loss in body weight,

      (ii) fall in liver glycogen,

      (iii) decrease in the urinary excretion of nitrogen,

      (iv) decrease in the absorption of dicumarol, and in the concentration of the drug in the liver, epididymis, blood, muscle and small intestine,

   (c) overall loose connective tissue breakdown and hemorrhage as demonstrated by histological evidence.

6. Vascular fragility and increased vascular permeability are distinct phenomena as evidenced by

   (a) the absence of edema fluid in the hemorrhagic areas,

   (b) the absence of red blood cells in the edematous areas.

7. Vascular fragility in the salivarectomized rats receiving a dicumarol containing diet is localized at the arteriolar or precapillary part of the

microcirculation as shown by

(a) arteriography (contrast medium does not enter the capillary network),

(b) the absence of abnormality of the capillaries by histological examination.

8. Presence of central lobular necrosis in the liver parenchyma in the salivarectomized rats receiving a dicumarol containing diet.
9. No increase in the tryptic, chymotryptic or hyaluronidase activity of the serum of salivarectomized rats in the course of bleeding.
10. Elevation of the antitrypsin, antichymotrypsin and antihyaluronidase activity in the serum of salivarectomized rats in the course of bleeding.
11. Hemophilic serum obtained at the height of a bleeding episode had a high antithrombin, antihyaluronidase, antitrypsin and antichymotrypsin activity.
12. Addition or administration of platelet cofactor I concentrate will decrease the thrombin inhibitor activity of hemophilic serum in vitro and of the plasma in vivo.
13. The inhibition of thrombin by antithrombin is the noncompetitive type.
14. Hyaluronidase apparently can 'activate' antithrombin in vitro.
15. Bleeding begins before any change is detectable in the state of the blood clotting system as indicated by measurement of the 'prothrombin time'.
16. Arteriography is a useful method for the demonstration of vascular fragility.

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## CONTRIBUTIONS TO KNOWLEDGE

The author considers that the following ideas and findings mentioned in the thesis represent new contributions to knowledge.

1. The application of radiography for revealing the state of vascular fragility.
2. The method of producing experimental hemorrhage in the rat by salivarectomy and dicumarol administration.
3. The inference and demonstration of low grade hypertension and thickening of the arteriolar and small arterial wall in the salivarectomized rat.
4. The close relation between, if not the identity of antithrombin and anti-platelet cofactor I.
5. Demonstration of fragility of the lysosomes as a result of salivarectomy in the rat.
6. The release of lysosomal hydrolases by dicumarol *in vitro*.
7. The 'activation' of serum antithrombin by hyaluronidase *in vitro*.