

[54] **METHOD OF SCREENING
SUBSTANCES FOR USE IN THE
TREATMENT OF CIRCULATORY
SYSTEM DISEASES**

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[52] U.S. Cl.**128/2.1 R, 23/230 B, 424/9**

[51] Int. Cl.**A61b 5/04**

[58] Field of Search.....128/2, 2.1; 23/230 B; 424/2,
424/3, 7, 9

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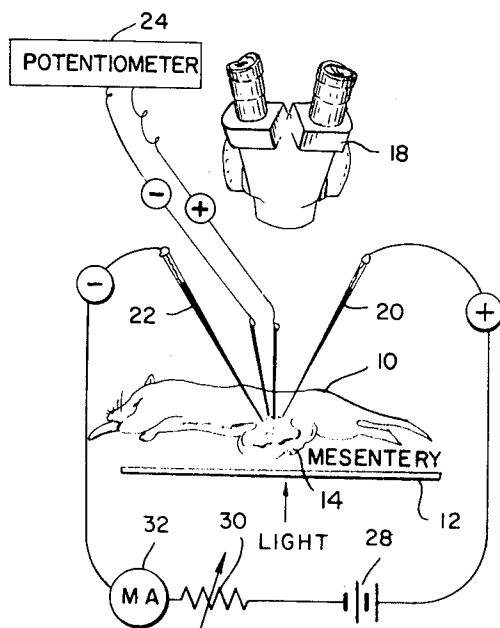
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Primary Examiner—Kyle L. Howell
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[57] **ABSTRACT**

A method as set forth for screening chemical agents and compounds to determine their usefulness in the treatment of circulatory system diseases and to enable distinguishing the anticoagulant and antithrombotic characteristics of the same. The method consists of a plurality of interrelated steps including the evaluation of the agent or compound on an exposed laboratory animal mesentery. Also included are tests on streaming potential in blood vessels, both in vivo and in vitro. In addition, the method includes checking the effect of the tested substances on electro-osmosis and evaluating the effect of the substance undergoing test on the charge of blood cells. Still further, the test includes checking the effect of the substance on the transport of ions across blood vessel walls and on the sorption and desorption of ions with respect to the circulatory system. In addition, the effect of the substance on destruction of various cells and proteins in blood is determined. The above characteristics are evaluated to determine the antithrombotic, antiatherogenic usefulness of the substance undergoing test.

16 Claims, 26 Drawing Figures



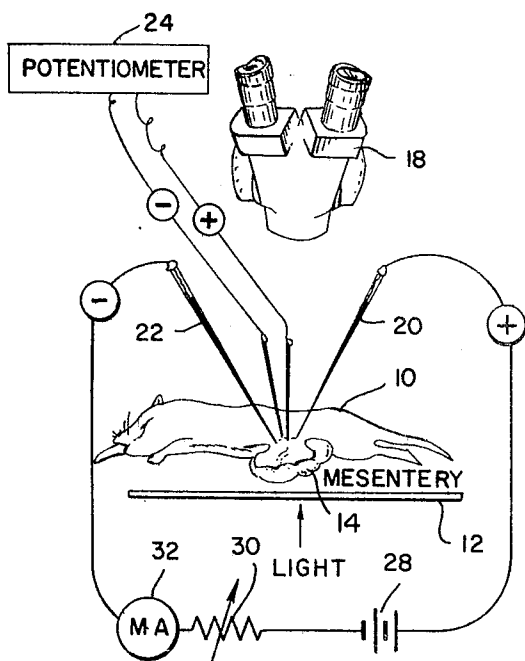


FIG. 1

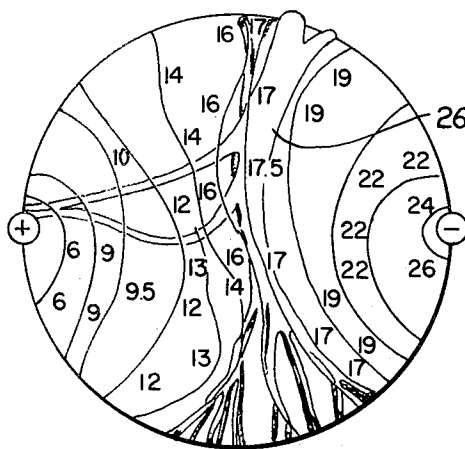


FIG. 2

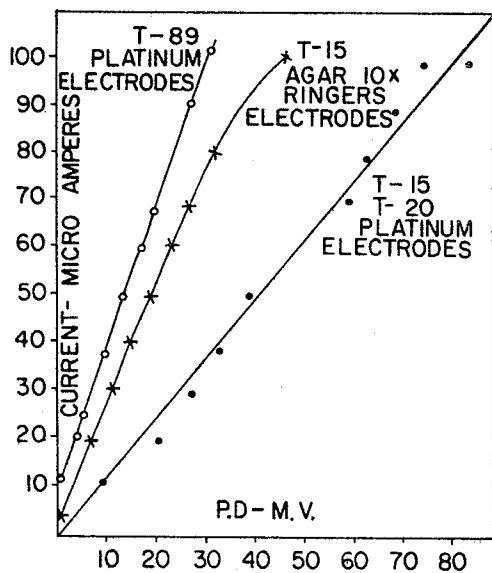


FIG. 3

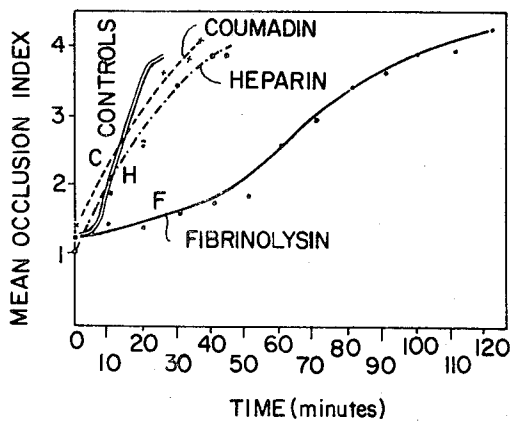


FIG. 4

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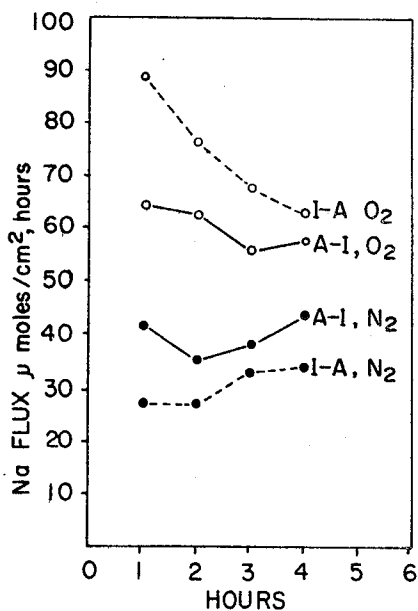


FIG. 5

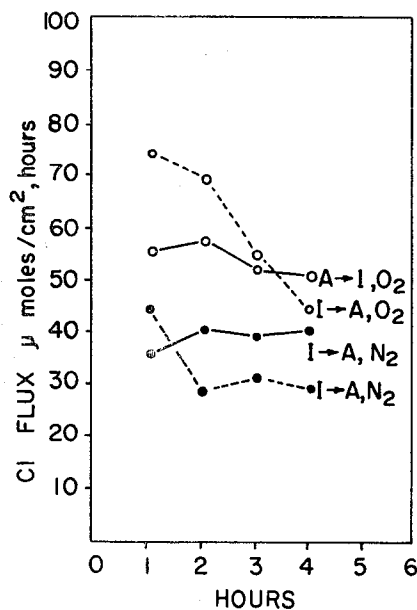


FIG. 6

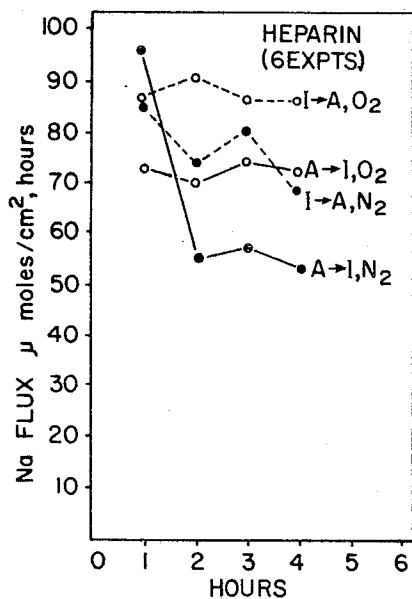


FIG. 7

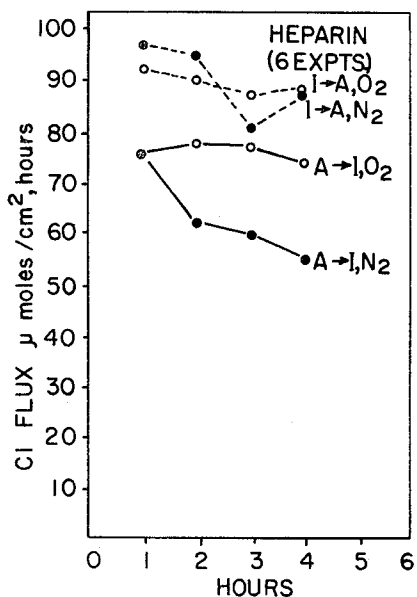


FIG. 8

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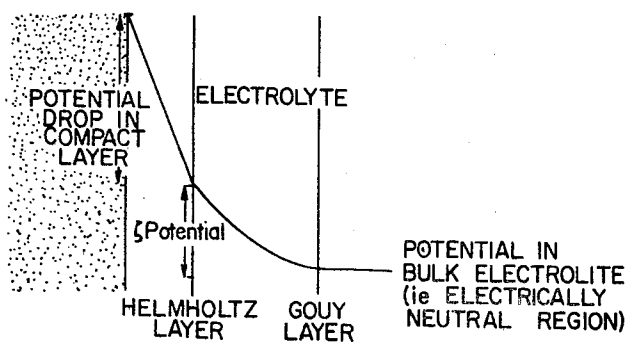


FIG. 9

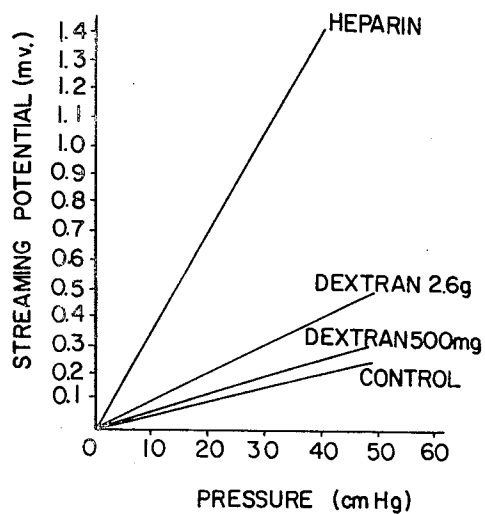


FIG. II

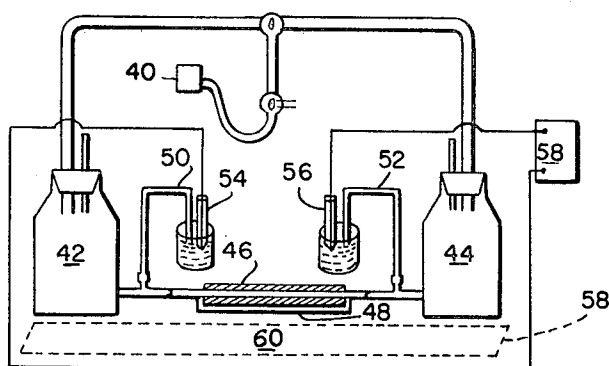


FIG. 10

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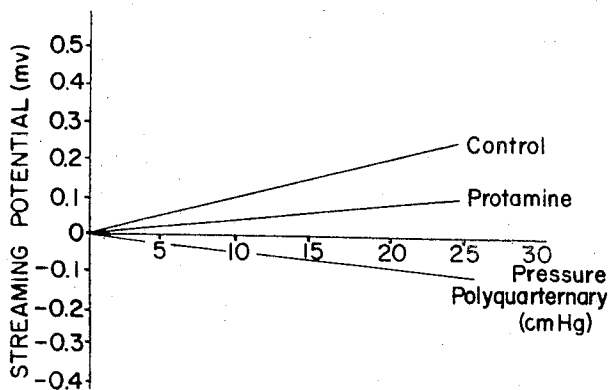


FIG. 12

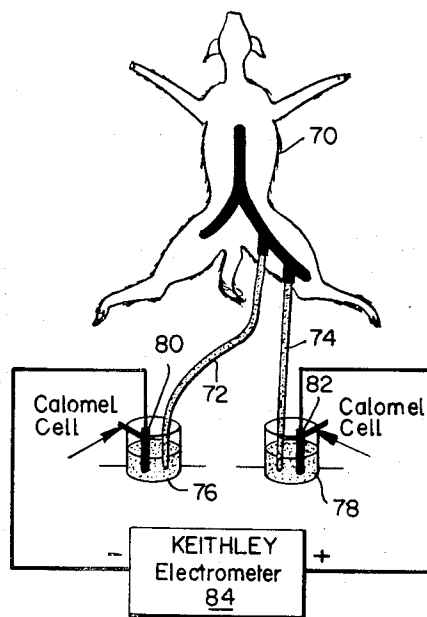


FIG. 13

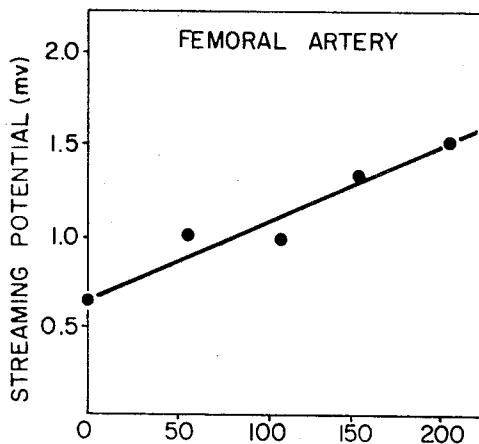


FIG. 14

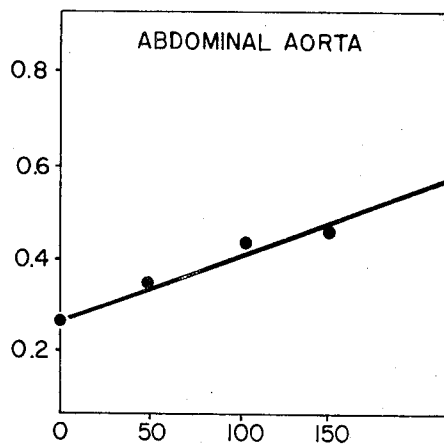


FIG. 15

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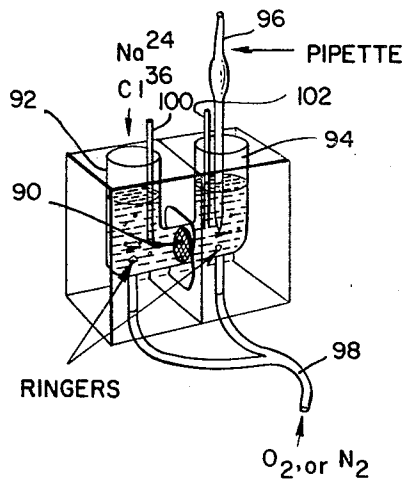


FIG. 16

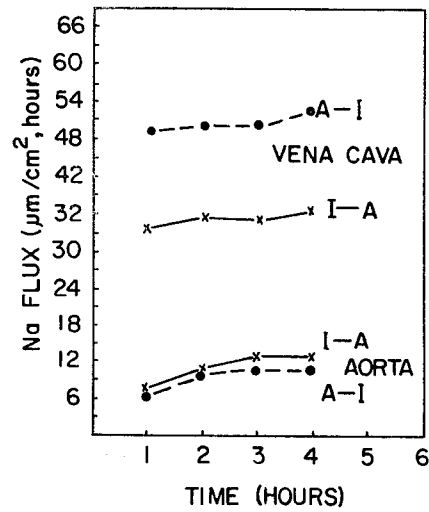


FIG. 18

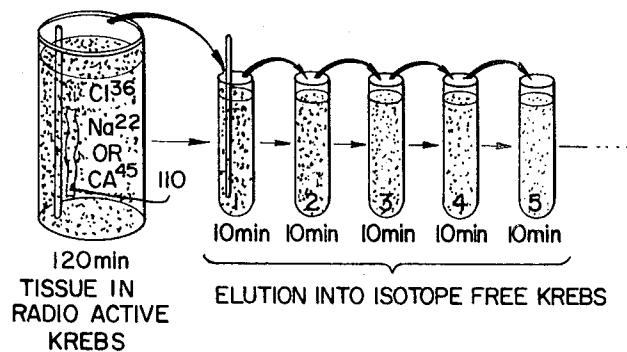


FIG. 17

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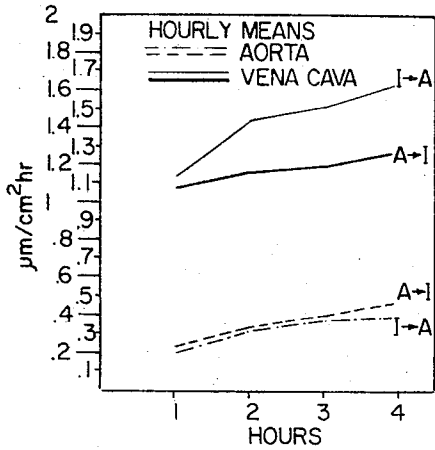


FIG. 19

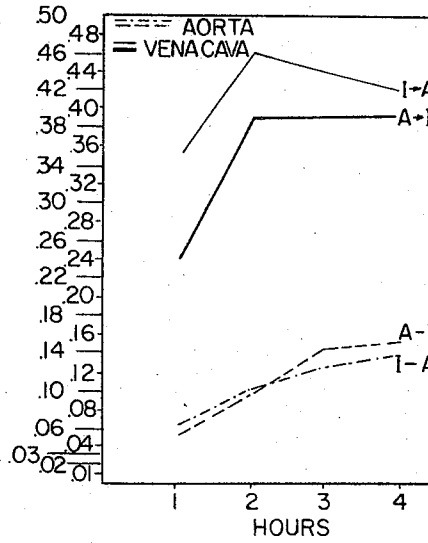


FIG. 20

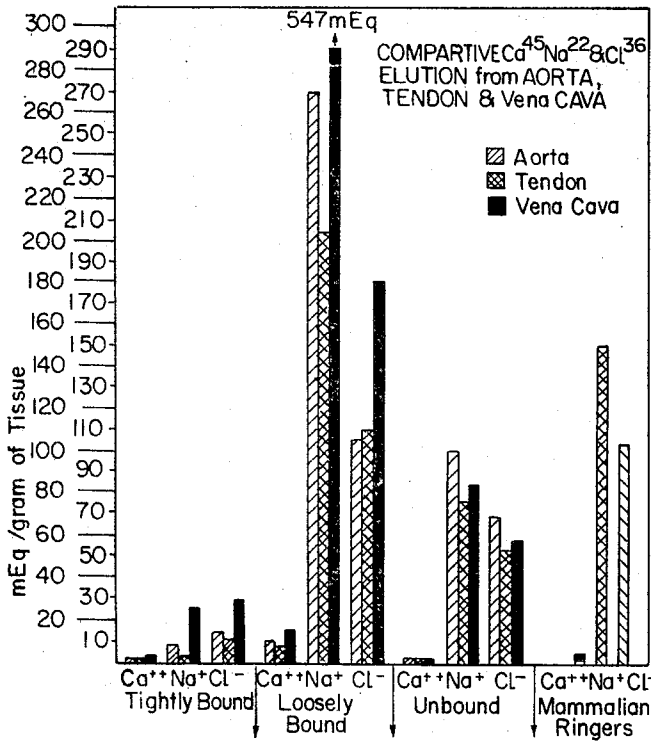


FIG. 22

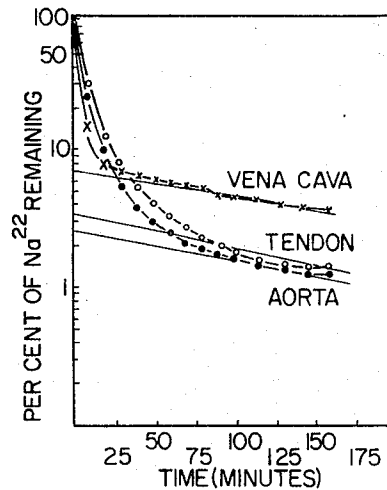


FIG. 21

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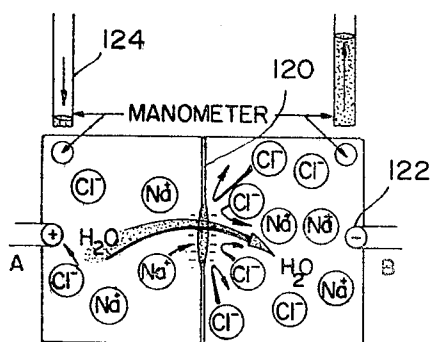


FIG. 23

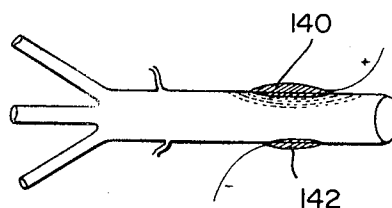


FIG. 24

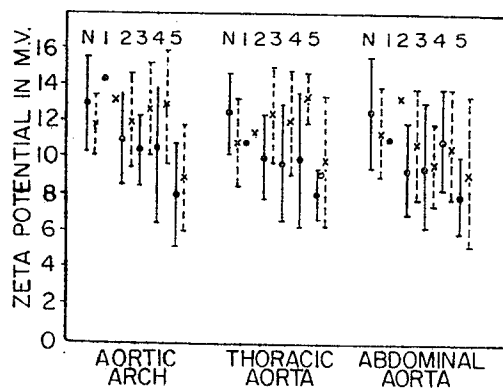


FIG. 25

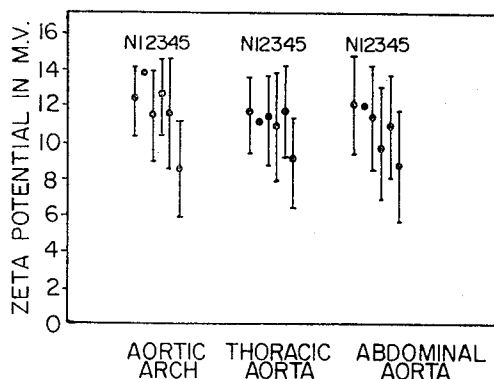


FIG. 26

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METHOD OF SCREENING SUBSTANCES FOR USE IN THE TREATMENT OF CIRCULATORY SYSTEM DISEASES

BACKGROUND

Cardiovascular disease is one of the leading causes of death in the world today. Cardiovascular, cerebrovascular and renovascular deaths accounted for better than 50 percent of the total deaths in the United States in 1967 according to a United States Public Health Service report concerning mortality. The vast majority of these deaths were related to myocardial infarction caused by thrombosis or terminal occlusion of coronary arteries.

For many years, attempts have been made to evaluate the mechanisms by which the normal vascular tree maintains homeostasis and prevents abnormal atheroma formation and abnormal thrombosis. Among other considerations, such evaluation places under investigation the mechanisms of thrombosis and coagulation and possibly atherogenesis.

It must immediately be noted that intravascular thrombosis and coagulation of blood *in vitro* are two separate though related phenomena. One of the major differences is that, in thrombosis, platelets are some of the first elements to deposit on an intravascular surface which is developing thrombosis. The platelets undergo changes, release ADP, coalesce, aggregate more platelets and ultimately produce catalysis of thrombosis in the local area. Coagulation occurs by a different mechanism probably due to the interaction between the foreign interface on which the blood is placed and the blood elements or enzymes which are productive of clotting. Activation goes through a regular enzymatic cycle until prothrombin, catalyzed to thrombin in the presence of calcium, produces fibrinogen conversion to fibrin with the establishment of a clot.

In addition to the above, the two mechanisms are different at least in that the initial triggering events may not always be the same. They are similar in that a gel of blood and its components is formed causing intravascular occlusion and distal ischemia (loss of oxygen supply to tissues normally supplied by the occluded blood vessel).

For over two centuries, attempts have been made to determine the mechanism by which thrombosis takes place. There has, along these lines, been some progress describing the thirteen enzymes involved in thrombosis. However, there has been no means or system by which the system of vascular homeostasis could be evaluated until approximately 1927 when Harold Abramson first described cataphoresis (that is, the movement of negatively charged blood particles to a positive electrode in an electrophoresis cell. In 1951, there was first described the reversal of charge of the blood vessel wall as an element in the induction of intravascular thrombosis. Since then, it has become increasingly obvious that at least the induction of thrombosis is an electrochemical phenomenon. It is not yet proven but it now seems reasonably certain that the mechanism by which intravascular occlusion takes place is also related to interfacial and/or electrochemical reactions that catalyze thrombosis at critical potentials by activating enzymes.

With the realization that thrombosis is probably an electrochemical phenomenon, it became possible to

conceive of various studies which would yield information concerning the nature of blood vessel wall function, normally serving to maintain homeostasis thus preventing abnormal thrombosis.

Initially, it was thought that the blood vessel wall must be metabolically active. The first studies carried out to test this hypothesis were related to studies of ion turnover rates and active transport across blood vessel wall and its various membranes. This revealed a considerable amount of information relating to sorption and desorption of ions, specifically with respect to potassium and calcium, while active transport and very rapid movement of sodium and chloride across blood vessel walls were found. Transport was observed at very low net potentials so that some sort of exchange diffusion undoubtedly goes on in this system.

After the initial studies of the active transport of various ions, an investigation was made concerning the metabolic relationships involved, and study of turnover rates of various ions by the tissues of the blood vessel wall. It was found that potassium and calcium ions were rapidly turned over and sorbed out of proportion to their concentration in blood and plasma whereas sodium and chloride were not quite so intensely absorbed. It was also found that the negative charge of the blood vessel wall was closely related to the presence of the sorbed positively charged K^+ and Ca^{++} ions. It appeared that they were being absorbed into the cell plasma membrane in polarized fashion producing an electric double layer related to the negative counter charges on the surface of the cell walls.

These phenomena and reflections concerning them led to the study of the pores through which the various ions moved. Thus, electro-osmosis, study of the charge and transport rate of various ions across the charged wall pores, commenced. Electro-osmosis is an area-independent phenomenon. As long as there is one normal pore available per given unit surface area, the system will work relatively well. It was rapidly determined that the pores of the blood vessel wall at normal human and other mammalian pH's were negatively charged with a fairly high negative surface charge concentration. The pore charges acted in a classical fashion becoming more positive as the ambient environment became more acidic with an isoelectric point at a pH of approximately 4.8. Below this pH, the pores became positive. Ion movement through the pores was quite interesting in that it occurred with bulk water transport. Thus the blood vessel wall was observed to act as a classic electro-osmotic membrane.

Simultaneously with the electro-osmosis determinations, it was found that the surfaces of the blood vessel wall presented to the blood must also be participating in these electrochemical phenomena, and reactions. If the wall surfaces were charged, they should be producing streaming potentials. Attempts to measure streaming potentials were commenced and it took much hard work to develop the techniques which permitted measuring streaming potentials of biological membranes *in vivo* and *in vitro*. These measurements had never successfully been made in living systems prior to the present experiments. The findings reveal that not only the vessel wall pores but the surfaces of blood cell walls are negatively charged and that this negative charge could be effectively changed such as, for example, by

changing pH, loss of normal ion concentrations in blood, and by injury. In fact, it was shown that the application of injurious materials to the walls, crushing or other injury would seriously decrease the net charge of the blood vessel wall as measured in the streaming potential experiments.

However, blood flows through the blood vessel lumen. It also must be taken into account that blood per se is electrochemically sensitive (that is, the cells and proteins of blood have their own charges). Moreover, blood acts as an unstable colloid and is subject to colloidal chemical studies and manipulations. The early studies of Abramson and the subsequent ones noted herein concerning the electrophoresis of cells and proteins of blood indicate that they have a homogeneous negative charge and are susceptible to various types of noxious agents. The latter studies also ultimately revealed that heparin, the universal anticoagulant, made these already negative cells even more negative. Specifically, electrophoresis permitted studying those components of blood which flowed through the blood vessels whose physical chemistry was finally being understood.

All of these studies began to give an increasingly total picture of the entire environmental situation of the vascular tree and of the blood flowing in it, and permitted an understanding of the mechanisms by which the tree normally functioned and what happened to these mechanisms when the system broke down. Each of the studies which were carried out revealed a different part of the picture and a different important physical chemical aspect of the physiologic which participated in the establishment of the entire homeostatic picture.

Additional studies which took place involved measurement of the effect of a chemical or material on blood in vitro. These studies determined the anticoagulant effects of any chemical or material to keep blood, in vitro or in a foreign vessel, from clotting such as is found with heparin. It was found that some materials, for instance, will effect blood coagulation in vitro per se while others will in the usual concentration only act to prevent thrombosis in vivo.

It is frequently important in vascular surgery and is increasingly important in preventing abnormal atherosclerosis and abnormal thrombosis to be able to study the effect of a chemical or to have a technique by which one might study the effects of a chemical or pharmacological agent on the various components of the vascular tree already described. In vascular surgery, for instance, if one wishes to use both an antithrombotic and anticoagulant material, one would use heparin.

There are times such as, for example, following the implantation of a porous prosthetic device where one wishes to prevent abnormal thrombosis but cannot use an anticoagulant material which might cause the patient to bleed to death since the anticoagulation would permit blood to ooze through the pores of the prosthetic graft. In this instance, one must use an antithrombotic but not anticoagulant material, the terms being specific. One might, for example, use limited amounts of dextran which has relatively little anticoagulant effect in the amounts used for this purpose, but might have very dramatic antithrombotic effects in

small vessels distal to a vascular prosthetic runoff or in the wire graft surfaces itself.

In addition, it is desirable sometimes to have or produce, over a long-term, the subtle effects of sophisticated antithrombotic chemicals rather than the brute force effect such as the anticoagulation produced by heparin or the anticoagulation produced, by subtoxic amounts of dicoumarol derivatives, which in toxic amounts are used as rat poisons to cause the rats to bleed to death into their capillary beds.

Frequently, it is not possible to give a patient heparin over a long-term because of his previous experiences with bleeding peptic ulcer or a known tendency to bleed from any one of a number of areas, or it is not advisable to treat patients with heparin who are hypertensive so that there would be a fear of bleeding into the brain tissues. In such cases, it might be possible to use a sophisticated antithrombotic material while not effectively manipulating or changing the normal coagulation characteristics of the blood per se. In other instances, it might be desirable to use a fibrinolytic material which would dissolve thrombi already present and perhaps prevent the further deposition of fibrin in an area which is incipiently coagulating.

SUMMARY OF THE INVENTION

In accordance with the invention, there has been evolved a process for evaluating the total characteristic effects of a chemical on the various components of the vascular tree. This system, which includes the aforementioned tests, will permit determining the effect of any material.

In accordance with the invention, the effects of a given chemical agent on controlled electrical thrombosis in the rat mesentery in vivo are measured. This test is a controlled passage of a small electric current (e.g., 10 milliamperes) across the surface of an exposed rat mesentery bathed in gelatin Ringers solution to maintain vascular oncotic pressure while it is observed through a microscope. Occlusion of the small blood vessels in the mesentery is observed while a current is passed across them. These vessels coagulate in highly controlled experiments in approximately 30 minutes. The coagulation time in this controlled environment is markedly elongated by heparin and other overtly anticoagulant materials such as fibrinolysis which prevents fibrin formation. Other materials such as, for example, coumadin acutely (a bishydroxycoumarin derivative) have little effect on thrombosis of vessels in the rat mesentery since they do not specifically effect blood coagulation per se or do not participate in the coagulation or thrombosis schema in vitro or in vivo.

Finally, the toxic effects of an injection of a material into a rat in which rat mesentery thrombosis is produced gives an indication of the toxicity of the material in a living biological system (namely, the rat).

Thus, this last-mentioned test has two specific components: (1) the lethal characteristics of a chemical in the biological system of a rat and (2) its effect to prevent thrombosis in a controlled rat mesentery coagulation experiment.

All of the above steps give information about different components of the tree as follows:

1. Streaming potential tells about the surface of a blood vessel wall that the blood sees. This surface is

played upon and contributed to by electro-osmosis across the wall, blood pressure forcing positive ions out through negatively charged pores in the wall (transverse streaming potential or pressure electro-osmosis), bulk water transport across the pores, active secretion of anticoagulant materials by the wall (particularly veins), active sorption and desorption by the wall cells maintaining the homeostatic nature of the evenly spaced negative charges in the electric double layer which the blood sees.

2. Electro-osmosis gives information concerning the ion movements, pore size and type of sorption and desorption on the surfaces of the pores that normally take place in the wall. It tells one how various pharmacologic agents can effect the pores and the pore charge and transport across the wall pores. For example, heparin tends to effect these pores relatively little, actually, because it is a big molecule and has difficulty getting into the rather small pores. The smaller chemical agents have a more significant effect in this area.

The effect of heparin and other agents on active transport is easily determined by adding it to a hot (ion isotope) bathing solution used to measure ion movement across the wall, in an ion flux experiment. Sorption and desorption is measured by actually putting the material in a hot (radio isotope-containing) solution; permitting the wall to soak up the radio active ion, and then watching the rate at which it comes off in a non-isotopic equivalent solution. The effect of various pharmacological agents on the ion transport and ion elution phenomena are thus easily determined.

3. Coagulation, per se, can be easily determined by taking blood from an experimental animal and putting it into a test tube containing the chemical or pharmacologic agent that one wishes to determine the anticoagulant properties of.

4. The rat mesentery preparation requires taking a pharmacological or chemical agent, injecting it into a rat at the desired concentration per gram of body weight and determining its effect to prolong electrical coagulation time in the rat mesentery.

5. The platelet release reaction is a measure of ADP, ATP, AMP, and glucose phosphate release from platelets. The reaction is activated by a number of foreign surfaces or by chemical or similar reactions between a large concentration of washed rat or human platelets and the chemical surface or material to which the platelets are exposed. ADP release from platelets is known to be catalytic in the production of intravascular thrombosis. Release of ADP from concentrated platelet preparations by any material can be presumed responsible for intervascular thrombus activation, acceleration or both. The test is a very sophisticated one and has considerable meaning in the context at hand.

6. Blood destruction is determined by taking aliquots of whole oxilated blood and putting them in contact with powders of a given material. Erythrocyte, leukocyte and platelet counts are taken before, at fixed periods during and at the end of the test. This is an accurate technique for measuring overt destructive characteristics of a material on blood. It is both inexpensive, accurate and when positive quite indicative of the blood destructive characteristics of a given material.

BRIEF DESCRIPTION OF DRAWING

FIG. 1 is a partially pictorial, partially diagrammatic illustration of an apparatus for checking the effect of a substance on the occlusion of blood vessels in a test animal mesentery;

FIG. 2 is a diagrammatic illustration of equipotential lines created by current passing through the mesentery as illustrated in FIG. 1;

FIG. 3 is a graph showing the relationship between applied current and measured potential difference in the apparatus in FIG. 1;

FIG. 4 is a graph illustrating the measurements of occlusion with the passage of time in the apparatus of FIG. 1;

FIG. 5 is a graph relating to a second test of the invention with respect to the transport of ions through the wall of a circulatory system in vitro;

FIG. 6 is a graph similar to that of FIG. 5 under different conditions;

FIGS. 7 and 8 are graphs similar to those of FIGS. 5 and 6;

FIG. 9 is a potential distribution chart with respect to a solid-solution interface with respect to the movement of ions;

FIG. 10 is a diagrammatic illustration of an apparatus for the determination of streaming potentials in vitro as employed in another test of the invention;

FIG. 11 is a chart illustrating the effect of certain compounds on in vitro streaming potential;

FIG. 12 is another chart showing the effect of certain substances on in vitro streaming potential;

FIG. 13 is a schematic representation of an experimental arrangement for the measurement of in vivo streaming potentials as employed in another test of the invention;

FIGS. 14 and 15 are charts illustrating the dependence of streaming potential on the amount of substances infused in a test animal;

FIG. 16 is a diagrammatic illustration of an apparatus for studying the passage of ions through a membrane by means of ion diffusion;

FIG. 17 diagrammatically illustrates a test for the study of ion elution as employed in accordance with the invention;

FIG. 18 is a chart illustrating the movement of certain ions through aorta and vena cava walls;

FIGS. 19 and 20 are charts also relating to ion flux;

FIG. 21 is a chart illustrating ion elution;

FIG. 22 is a chart indicating the concentrations of ions sorbed in the pores and surfaces of test animal aortas and vena cava;

FIG. 23 diagrammatically illustrates an apparatus for examining phenomena taking place during electro-osmosis;

FIG. 24 diagrammatically illustrates the application of electrodes to opposite sides of a blood vessel for the study of vascular factors in blood vessel occlusions; and

FIGS. 25 and 26 are charts showing the values of zeta potentials of human aortas as a function of the degree of arteriosclerosis.

DETAILED DESCRIPTION

Before going into the details of the various steps of the process of the invention, some of the general results

of investigating different materials will next be pointed out below:

Heparin. This substance has dramatic negative charge effects on both the blood vessel wall surfaces (surface potential, electro-osmosis) and the blood cells and proteins (electrophoresis). It has relatively little effect on the blood vessel wall pores because of its large size although it has a limited negative charge effect here as well. Its effect on clotting time in vitro is infinite since it is anticoagulant in vitro. Its effect on occlusion times in the rat mesentery is up to four times that of controls. It has essentially no blood destructive characteristics, producing no destruction of red cells, white cells or platelets. It has antithrombotic as well as anticoagulant effects, makes all of the components of the vascular tree more negatively charged, has in vitro anticoagulant effects, and has also been shown to be antiatherogenic in that it reduces hyperlipemia by reducing the size of chylomicrons and producing a more rapid absorption of the fat in blood. This substance is therefore a test standard against which most other materials can be evaluated in this system.

Protamine. This substance, which is the antagonist of heparin, has exactly the opposite effect. It makes the surface charges of blood cells and proteins more positive, makes the blood vessel wall surfaces more positive, makes the pore charges on the walls more positive, and instigates more rapid coagulation time. It reverses the negative charge, anticoagulant and antithrombotic effects of heparin, stoichiometrically, 2 milligrams of protamine for approximately 1 milligram of heparin and has very definite positive, thrombotic charge effects. It reduces the clotting time of blood in glass to its minimum level approximating 2 to 3 minutes.

SP54. This substance is a sulfonated polyglucoside made by BeneChemie in Germany which has some of the charge effects of heparin, matching its effects on blood cells and proteins, and blood vessel wall and having an increased effect on blood vessel wall pore apparently because of its small size. However, it does have discernable blood destructive effects producing a plus 2 hemoglobinnemia due to a release of hemoglobin from damaged red cells in vitro.

Chondroitin Sulfate. A heparin-like derivative extracted from blood vessel walls. It has only a grade 2 negative charge effect on the blood vessel wall, is considerably less negatively charged than heparin, increases the rat mesentery occlusion time to approximately 3 to 4 times that of control, has a moderate anticoagulant effect in vitro, and a rather sophisticated antithrombotic effect in vivo. Its blood destructive characteristics are nil.

Ascorbic Acid. Long ascribed a homeostatic effect on capillary beds, this substance is noted to have an extraordinarily small charge effect in streaming potential and electro-osmosis experiments producing very little effect on either transverse pores or surfaces or surface charge of the blood vessel wall. It slightly increases rat mesentery occlusion time in the rat mesentery electrical occlusion experiment, has a minimum effect to increase clotting time in vitro and produces relatively little blood destruction.

Cholesterol. This substance has almost no effect on any of the studied parameters. It thus cannot be considered an anticoagulant or overtly coagulant material.

Dimethyl amino ethanol. This substance is a detergentlike material which has been shown to have a rather sophisticated effect on the blood vessel wall and pore charge. It is a small molecule. It doubles the rat mesentery occlusion time. It has very small effects, except in very high concentrations, on clotting time and produces relatively little blood destruction. While it has relatively little effect on clotting, it is a rather potent antithrombotic agent apparently making both the blood vessel wall and blood cells more negative.

Ethyl maleimide. This reverses the charge effects on blood vessel wall because of its chemical surface activities. It has effects opposite to those of heparin and the other negatively charged chemicals and drugs. It shortens the rat mesentery occlusion time, makes the blood vessel wall pores more positive, shortens clotting time and causes approximately plus one blood destruction of both red cells and white cells with moderate hemoglobinnemia in vitro.

Potassium SPG. This is a maximally sulfonated polyglucose which was found to have rather dramatic negative charge effects on blood vessel walls with respect to streaming potential and on the blood vessel wall pores in vitro in the electro-osmosis experiments. It produced 4 plus blood destruction in test measurements. On injection into a rat during the rat mesentery occlusion time studies, the sample was found to produce death rapidly by a series of convulsions except at the lowest concentrations used. This heparin-like material therefore is extraordinarily toxic in rats. Even at low concentrations approximating 0.1 milligrams per hundred grams, it is found to be lethal.

Polyquaternary. This is a positively charged ammonium salt which also has very dramatic blood coagulation effects, making the vessel wall more positive. It decreases rat mesentery occlusion times. Clotting times were dramatically shortened by this material when added to blood in a glass or test tube. Its blood destructive characteristics are plus 2.

Dextran. This produces a dramatic charge effect on blood vessel walls making them progressively more negative as stoichiometric amounts are added to the blood stream. For each unit of additional dextran in vivo, the streaming potential in vivo doubles. It has reduce effect on the rat mesentery occlusion time. It effects the clotting time minimally in low concentration even though it produces no blood destruction. Thus dextran will effect the blood vessel wall per se, while it has relatively little effect on anticoagulation in physiologic concentrations and in fact may occasionally shorten normal coagulation times in vitro.

Thus it can be seen that each of the pharmacological agents tested will produce a different schema of results in the battery of tests to which the material is subjected and that the tests will yield information concerning which agent or chemical will produce the desired effects in a given electrochemical area of the vascular tree, at what concentrations the effect is obtained, the upper limits of the effect in terms of the maximum dosages necessary to produce the effect, toxicity, and so forth. This system therefore provides a most useful screen for testing the effect of drugs, chemicals and pharmacological agents on the various homeostatic mechanisms which normally serve to maintain the integrity of the vascular tree. The tests study interdepen-

voltage intercepted by any single small blood vessel was in the range of 1–3 mv maximum, wall to wall, for currents of 20 μ a, and somewhat larger for higher currents. This constituted the field gradient across any given vessel intercepted by the lines of current flow for each test.

It is not possible to measure the voltage drop between inside and outside the vessel wall in this experiment because this would necessitate placing an electrode within the blood vessel under study, an act which would itself compromise blood flow through the vessel and thus would compromise the results of the test. c) The potential difference pole to pole at 20 μ a of current flow was in the range of 20 mv and increased with increasing currents (FIG. 3).

Little of the current which actually traverses the mesentery is effective transvascular current, since in large proportion, it flows over the surface of the thin mesentery through the bathing solution. It is believed that a probable maximum of one-quarter of the total current at 20 μ a total flow actually traverses the blood vessels in the rat mesoappendix in these tests.

Microscopic observation indicates that precipitation of cells, ostensibly beginning thrombus formation, first occurs on the vessel wall nearer the positive electrodes. Many small free emboli can be noted during the procedure. Some of these can be observed to form within the observed vessel and then to break loose and course down the vessels. If the current is cut off after the observed coalescence of cells and rouleau formation commences, additional clumps of cells are frequently observed to break off from vessel walls and float away. Thus the process appears partially reversible as long as the vessels are not mechanically injured. The position of the formation of the thrombus usually corresponds to the points of maximal current concentration as determined by field measurements.

If a vessel is injured so that blood extravasated during the experiment, the free red cells are seen to swirl off toward the positive electrode where they quickly coalesce. The few cells which remain about the negative current electrodes are seen to maintain their free condition for a considerably longer period.

A current flow of a very few microamperes requiring an electrode-to-electrode voltage of 20–30 mv and a maximal transvascular potential difference of 1–3 mv can result in occlusion of small vessels by clumped red cells, white cells and other elements.

The above test is employed, according to the invention, as one of a series of tests and is employed in such manner that the test animal is treated with the chemical agent or compound being examined. The occlusion time is compared with that of a control and is graded with respect thereto.

For further details on the above test, reference can be made to "Effect of Small Electric Currents on Intravascular Thrombosis in the Visualized Rat Mesentery," *The American Journal of Physiology*, Vol. 198, No. 5, May, 1960.

EXAMPLES

Forty rats of the Wistar strain, of both sexes and weighing between 100 and 200 grams, were used as experimental subjects. They were anesthetized by intraperitoneal injection of pentobarbital sodium (6 mg. per 100 Gm. body weight), a midline incision was made in the lower abdomen, and the cecum and mesoappen-

dix were extracted. The rat was strapped to a rat board, through the center of which projected a plastic, transilluminated, highly polished cylinder. The rat board itself was attached to a micromanipulator so that the entire preparation was movable. The mesoappendix was mounted on the surface of the transilluminated cylinder under the objective of a binocular stereoscopic dissecting microscope. The tissue was continuously bathed with fresh 1 percent gelatin-Ringer's solution at a pH of 7.4 and a temperature of 35° to 37° C. The rat cecum was completely surrounded during the experiment with cotton saturated with Ringer's solution. The undersurface of the mesentery was moistened by the capillary action of the underlying cotton surrounding the transilluminated cylinder. In all experiments, a 30 μ amp. current was passed across the membrane via positive and negative platinum electrodes. Current source consisting of a 6 volt dry cell battery 28 (FIG. 1) connected in series with a variable resistance 30 and microammeter 32 (FIG. 1).

In order to obtain quantitative determination of the effect of the current on the vessels of the mesoappendix, a numbering system was used to grade the state of vascular occlusion. The patency of each vessel was given a number from 1 to 4, the larger numbers indicating an increasing level of occlusion:

1. Completely patent vessel.
2. Some disturbance of flow. Blood cells noted to stick to the vessel wall.
3. Overt evidence of partial occlusion.
4. Complete occlusion of blood vessel with blood elements, presumably thrombosis.

The visualized vessels were examined immediately after the experiment started and every 5 to 15 minutes thereafter. The size of the vessels counted ranged in diameter from 20 to 50 μ .

Anticoagulant agents used were administered in a concentration known to produce maximum effect.

Control group. Ten rats not treated by any therapeutic agent acted as controls.

Coumadin group. In 10 experiments, coumadin was administered orally (0.5 mg. per 125 Gm. body weight) over a period of 12 hours, beginning 36 hours prior to the application of current. This overdose of coumadin prolonged the one-stage prothrombin time to over 8 minutes, compared to an average control prothrombin time of 18 seconds.

Heparin. In 10 experiments, heparin was administered intraperitoneally (0.003 mg. per gram body weight) one hour prior to anesthesia, and 1½ hours prior to the application of current. The overdose of heparin given prolonged clotting to such an extent that it was in excess of 24 hours.

Thrombolysin. In 10 experiments, fibrinolysin plus activator was administered intraperitoneally (25,000 units per animal) 1½ hours prior to the application of current. The index of fibrinolysin activity utilized was the 24 hour clot lysis method. A hematocrit of over 15 percent (of the serum of the incubated whole blood) was considered significant. The measured hematocrit values ranged from 20 to 30 percent.

The results are detailed in Table III and in FIG. 4. The results were subjected to the critical ratio test for significance. This consisted of the determination of the number of standard deviations from the mean for the summed mean 50 percent occlusion times.

Controls. Results from 10 experiments are to be found in Table III. Mean 50 percent occlusion times for this group of experiments was 18.2 minutes.

Coumadin group. In the next group of experiments, no significant alteration of the rate of vascular occlusion was produced by coumadin when compared to control experiments (FIG. 4).

Heparin group. The results of 10 experiments are shown in Table III. It is seen that heparin resulted in a statistically significant prolongation of the rate of vascular occlusion when compared with controls and the results in coumadinized animals.

Fibrinolysin group. In this group, a pronounced prolongation in the occlusion time in comparison with control, coumadin, or heparin groups was noted. The difference is highly significant statistically (Table III, FIG. 4).

TABLE III

Type of experiment	Number of experiments	Totals for blood vessels in groups (average)*								Occlusion time (average minutes)	Critical ratio (ΔT)†
		Beginning				End					
		1	2	3	4	1	2	3	4		
Controls.....	10	10	0	0	0	0	0.1	2.2	7.7	18.2	-----
Coumadin.....	10	9.5	0.3	0.2	0	0	0	0	10	25.4	1.67
Heparin.....	10	9.9	0.1	0	0	0	0.4	0.4	9.2	30.6	5.10
Fibrinolysin.....	10	8.9	0.9	0.2	0	3	0	0	7.0	† 69.5	>23

*All experiments at 30 ma.

†Ratio less than 2.5 indicates insignificant effect of pharmacologic agent, i.e., no significant difference between experimental group and control.

The highly significant results with fibrinolysin suggest that fibrinogen-fibrin conversion is critically involved in direct-current thrombosis. This finding has previously been suggested by in vitro determinations with fibrinogen-saline solutions subjected to positive-pole direct current and by in vivo clinical experience.

Heparin, as would be expected in the dosages used, has a moderate and statistically significant inhibitory effect on direct-current thrombosis, but does not ultimately prevent intravascular thrombosis by the applied direct current. Heparin appears to effect intravascular occlusion at a biochemical level proximal to fibrinogen-fibrin conversion, possibly at the prothrombin-thrombin conversion stage in blood clotting. The results suggest that the primary effect of heparin on intravascular thrombosis may be related to changes in vessel wall metabolism rather than in prolongation of the clotting time.

Coumadin had a minimal and statistically insignificant effect on the inhibition of direct-current coagulation in this experiment.

Finally, the experiments demonstrate that the mean 50 percent occlusion time for animals receiving fibrinolysin is thrice that for animals receiving coumadin and the control group, and almost twice that for animals receiving heparin.

The investigation thus gives considerable insight concerning bleeding states in which one might expect to use direct-current coagulation to produce hemostasis. It should work most effectively in states in which coumadin has been used as an anticoagulant agent. Heparin would present a somewhat increased problem in the effective utilization of direct-current coagulation, but not nearly as great a problem as does hypofibrinogenemic states. It would seem that one should not expect direct-current coagulation to work effectively in the presence of an increased fibrinolytic process in the bloodstream.

ION TRANSPORT

A discussion of ion transport mechanisms appears in "Heparin-Anticoagulant Plus," *Extrait du Bulletin de la Societe Internationale de Chirurgie*, Vol. XXI, 1962, No. 4, Pgs. 325-334.

In a test for the presence of active transport mechanisms for specific ions across the blood vessel wall membrane, it was established that active ionic transport of sodium and chloride ions does exist. As a byproduct, there were discovered hitherto undescribed effects, beneficial and otherwise, produced by widely used pharmacological agents.

Using radioisotope tracer techniques, the in vitro rates of transport of sodium and chloride ions across aorta and vena caval wall membranes were measured. The test membranes were gathered by the removal of

the descending thoracic aorta and thoracic cavae from dogs immediately following sacrifice by intravenous air embolism. Segments of these vessels were incised longitudinally to produce flat membranes which were used to separate specially designed paired chambers; the only method of entry from one chamber to the other was through the vascular membrane. Each chamber was filled with a bathing solution consisting of Krebs' saline serum substitute at 37° C. To test the membrane for active transport mechanisms a radioactive isotope of one of several ions was added to one side of the paired chambers. The rate of appearance of the isotope in the uncharged chamber thus became a measure of the transport of this ion across the vascular membrane. The results of experiments using sodium 24 and chloride 36 will be described. Each test involved the use of four membranes; two membranes were used for duplicate determinations of sodium and chloride transport from intimal to adventitial surface of the membrane, while the two remaining membranes were used for duplicate determinations of Na and Cl transport from the adventitial to the intimal surface of the test membranes. At the end of each hour, one cc samples of recipient chamber contents were assayed for radioactive isotope concentration using a standard gas flow Geiger Muller Counter. The determinations were converted to the bi-directional rate of ionic transport across the vascular membrane expressed as micromoles per centimeter square, hour.

Identical determinations were conducted under four different membrane ambient conditions; under aerobic conditions produced by bubbling 95 percent oxygen — 5% CO₂ gaseous mixture through each chamber; under anaerobic conditions produced by 95% N₂ — 5% CO₂; under aerobic conditions in the presence of heparin; and under anaerobic conditions in the presence of heparin.

The results will be presented under the following headings:

Ionic transport across both the aortic and vena caval membrane preparations under:

1. Aerobic conditions.
2. Anaerobic conditions.

TABLE IV

Na Flux. Non-Heparinized Aorta. Matched Aerobic-Anaerobic Pairs

Expt.	Gas	Direction of dominant flux		Mean hourly flux, $\mu\text{M./cm.}^2$, hr.		Net flux (A-I) - (I-A)	Expt.* (N ₂) - (O ₂)
		A-I	I-A	fi (A-I)	fo (I-A)		
64.....	N ₂	x	-----	14.78	10.20	+4.58	+4.27
65.....	O ₂	-----	x	12.66	12.34	+0.31	
66.....	N ₂	x	-----	12.62	11.22	+1.30	+4.75
67.....	O ₂	-----	x	11.46	14.81	-3.45	
68.....	N ₂	x	-----	10.70	8.10	+2.60	+3.02
69.....	O ₂	-----	x	11.23	11.66	-0.42	
70.....	N ₂	x	-----	13.37	14.72	-1.45	+1.20
71.....	O ₂	-----	x	8.86	11.60	-2.65	
78.....	N ₂	x	-----	14.70	11.60	+3.10	+4.70
79.....	O ₂	-----	x	13.10	14.70	-1.60	
345.....	N ₂	x	-----	10.46	8.06	+2.40	+0.51
344.....	O ₂	-----	x	8.44	6.55	+1.89	
347.....	N ₂	x	-----	12.42	6.83	+5.59	+6.71
346.....	O ₂	-----	x	7.77	8.89	-1.12	
349.....	N ₂	x	-----	16.89	11.90	+5.99	+8.45
348.....	O ₂	-----	x	9.22	11.68	-2.46	
601.....	N ₂	None	-----	12.26	12.17	+0.09	+1.50
600.....	O ₂	x	-----	13.16	11.51	+1.59	
603.....	N ₂	-----	x	6.94	7.46	-0.52	-1.56
602.....	O ₂	x	-----	11.09	10.06	+1.04	
605.....	N ₂	x	-----	15.29	8.85	+6.39	+6.51
604.....	O ₂	-----	x	13.20	13.32	-0.12	

* Possibility that results are due to chance. T.=2.4753; P.=1.7%.

- (3) Aerobic conditions with heparin.
- (4) Anaerobic conditions with heparin.

TABLE V

Na Flux. Heparinized Aorta. Matched Aerobic-Anaerobic Pairs

Expt.	Gas	Direction of dominant flux		Mean hourly flux, $\mu\text{M./cm.}^2$, hr.		Net flux (A-I) - (I-A)	Expt.* (N ₂) - (O ₂)
		A-I	I-A	fi (A-I)	fo (I-A)		
72.....	N ₂	-----	x	14.38	15.02	-0.64	+4.27
73.....	O ₂	-----	x	9.94	13.85	-4.91	
75.....	N ₂	-----	x	9.75	9.92	-0.17	-3.44
74.....	O ₂	x	-----	9.13	6.86	+2.27	
77.....	N ₂	-----	x	14.20	15.50	-1.30	-3.75
76.....	O ₂	x	-----	11.80	9.35	+2.45	
83.....	N ₂	x	-----	10.10	9.80	+0.30	-1.67
82.....	O ₂	x	-----	12.70	10.80	+1.97	
565.....	N ₂	-----	x	10.43	11.41	-0.98	-0.24
564.....	O ₂	-----	x	9.25	9.90	-0.74	
567.....	N ₂	-----	x	12.56	15.84	-3.28	-4.00
566.....	O ₂	x	-----	15.74	15.02	+0.72	
569.....	N ₂	x	-----	17.56	16.42	+1.15	-1.73
568.....	O ₂	x	-----	16.29	13.41	+2.80	
571.....	N ₂	x	-----	17.09	16.25	+0.84	+0.96
570.....	O ₂	x	-----	13.90	14.02	-0.12	
573.....	N ₂	x	-----	27.75	19.60	+8.15	-3.37
572.....	O ₂	x	-----	22.75	11.23	+11.52	
563.....	N ₂	x	-----	14.25	14.56	-0.31	-4.86
562.....	O ₂	x	-----	13.76	9.21	+4.55	

* Possibility that results are due to chance. T.=1.8042; P.=5.0%.

RESULTS

As can be seen from Table IV, net transport of both sodium and chloride ions across the aortic wall under aerobic conditions is most frequently in the direction from intima to adventitia. Anaerobic conditions usually lead to abolition or reversal of this net transport. The net comparative effects of aerobic-anaerobic conditions on ion transport across matched pairs of aortic membranes are best viewed in the last columns of Tables IV (non-heparinized) and V (heparinized). It can be seen that the remainder of the anaerobic net flux less the aerobic net flux usually is positive. The reverse is true when aerobic net transport is subtracted from anaerobic net transport in experiments using the heparinized aorta (Table V).

It can further be seen from Table V that heparin decreases or prevents the anaerobic effect on ion transport in an apparent attempt to normalize ionic transport, across the aortic wall under anaerobic conditions such as might be produced distal to an arterial obstruction.

FIGS. 5-8 demonstrate dramatically in graphic form the same protective effect of heparin for the vena caval wall. Heparin not only protects the vena caval wall against anaerobic reversal of transport, but helps maintain measured net transport in the anaerobic experiments so that the magnitude of Na and Cl transport across the anaerobic vena caval membrane is equal to aerobic transport.

A good deal of clinical evidence is available which indicates that the pain and discomfort of thrombophlebitis is rapidly alleviated by the injection of intravenous heparin. It is difficult to explain this relief of pain by the simple doubling of the blood coagulation time. Moreover, the anticoagulant effect is not synchronous with the relief of pain, which indeed persists through the periods of normal coagulation time in the cyclical regimen of heparin therapy. These observations point out the presence of other mechanisms to explain the relief of pain produced by heparin.

The question arises as to what these mechanisms are. Inasmuch as the sensory arch of the pain reflex originates from the vessel wall or perivascular tissues it would appear reasonable that the pain relieving effects of heparin come from the effects of heparin on the blood vessel wall.

Heparin has been shown to block or delay the thrombotic effects of electric currents produced by electrodes attached to the outer surfaces of canine femoral and carotid arteries but does not prevent precipitation of a thrombus on the positive electrode inserted into heparinized blood in vitro.

These observations raise the question of whether or not the antithrombotic effects of heparin are caused by its antithrombin effect per se, or is due to its direct effect on the blood vessel wall. The fact that heparin will usually not prevent thrombosis in distally occluded atherosclerotic blood vessels supports this latter possibility.

At operation when a phlebotic vein is opened, it can be grossly determined that the occluded vessel universally contains very dark or black, apparently completely deoxygenated blood. It would appear therefore that these occluded segments are exposed to far advanced anaerobic conditions. The present investigation would indicate that the effect of heparin in reversing the abnormal ionic transport mechanisms, engendered by anaerobic conditions, is in the direction of returning the vessel wall metabolism to a normal state, which is pain free and antithrombotic. It is also possible that the direct effect of heparin upon the blood vessel wall is related to the lipid clearing effect.

STREAMING POTENTIALS AND IN VIVO AND IN VITRO TESTS FOR ANTITHROMBOTIC DRUGS

In Surgery, St. Louis, Vol. 64, No. 4, Pgs. 827-833, Oct. 1968, a report is made on the effect of thrombotic and antithrombotic drugs on the surface charge characteristics of canine blood vessels.

A knowledge of the surface charge characteristics of a blood vessel wall is useful from the point of view of elucidating: (1) the mechanism of intravascular thrombosis; (2) the mechanism of drug action in the prevention of thrombosis; and (3) the essential criteria in the selection of prosthetic materials. An electric double layer exists at all solid-solution interfaces. The potential across the interface depends mainly on the charge density of the surface and on the ionic concentration of

the solution. Measurements of the electrokinetic characteristics (streaming potentials, electro-osmosis, etc.) provide information on the potential difference across a solid-solution interface. The streaming potential (E) is the potential developed in a narrow tube (e.g., glass tube, blood vessels) between two identical reference electrodes when there is a pressure difference (P) across them because of the flow of solution. It is linearly related to the zeta potential (ζ), which represents a part of the potential drop across a solid solution interface (FIG. 9) by the equation:

$$\zeta = (4\pi\eta KE)/PD \quad (1)$$

where, K , and D are the viscosity, specific conductivity, and dielectric constant of the solution, respectively. The relation between the charge density in the diffuse layer (q_D) and the zeta potential for a z - z valent electrolyte is given by:

$$q_D = (2kTcD)/\pi \sinh(ze/2kT) \zeta \quad (2)$$

where c is the electrolyte concentration; e is the electronic charge; and k is Boltzmann's constant. At very low electrolyte concentrations, q_D corresponds to the surface charge density of the solid phase. It is not possible to obtain the surface charge density from the zeta potentials at electrolyte concentrations above $10^{-3}M$. However, for a constant electrolyte concentration, a higher zeta potential, which corresponds to a higher streaming potential according to equation 1, signifies a higher surface charge density of the solid. The surface charge characteristics of the vascular wall are obtained from streaming potential and electro-osmosis measurements. The influence of pH, aging, and variation of concentration of the bathing solution on the electrokinetic characteristics of the blood vessel wall have also been determined.

The tests discussed below were conducted to determine the influence of thrombotic and antithrombotic drugs on the streaming potentials; the changes in this experimentally determined parameter were used as a measure of the surface charge effects of the drugs across canine aorta, carotid, and femoral arteries. The experiments with carotid arteries were carried out in vitro as a function of flow rate of electrolyte solution through the vessel. The streaming potential measurements across the aorta and femoral artery were carried out in vivo at the normal flow rate of blood. Interpretation of the effects of these drugs on streaming potentials, in conjunction with their molecular chemistry, provides further insight into the nature of the vascular surface charges as well as the common mechanisms of drug antithrombotic activity.

In vitro streaming potential measurements across carotid arteries: Carotid arteries were obtained from mongrel dogs killed by sodium pentobarbital or air embolism. The arteries were placed in the apparatus shown in FIG. 10.

This apparatus for the determination of streaming potentials, in vitro, across carotid arteries comprises: a nitrogen cylinder 40; two graduated, aluminum foil-covered bottles 42 and 44; a sponge 46 soaked with electrolyte; insulation 48; agar bridges 50 and 52; calomel electrodes 54 and 56; Keithley electrometer 58; and grounded aluminum plate 60.

Modified Krebs solution, which has the same ionic composition as blood, was used as the bathing solution.

Nitrogen gas was employed at varying pressures to produce uniform flow rates. Potentials between the calomel electrodes 7 were measured with the Keithley electrometer 58. The downstream electrode was always connected to the positive terminal of the electrometer.

Streaming potential measurements were made across the ends of the carotid arteries at pressures of 10, 20, 30, 40 and 50 cm. Hg during flow of Krebs solution per liter in the absence and in the presence of one of the drugs, heparin, protamine, polyquaternary, or low molecular weight dextran. The concentration of the first three compounds was 100 mg. per 1,000 ml. of Krebs solution. Dextran was used at concentration of 500 mg. and at 2.6 Gm. per 1,000 ml. Krebs solution.

As shown in FIGS. 11 and 12, all the streaming potential-pressure relations are linear. Heparin increases the slope of the curve above that of the control to the greatest extent. Low molecular weight dextran, at a concentration of 2.6 Gm. per 100 ml. Krebs solution, raised the streaming potential to a lesser degree. Increases in streaming potentials in the presence of dextran at a lower concentration are small at all flow rates. Protamine lowers the streaming potential-flow rate relation to below that of control value. Polyquaternary produced a reversal of sign of the streaming potential.

In vivo streaming potential measurements across femoral arteries: Healthy mongrel dogs (e.g., dog 70, FIG. 13), with an average weight of 20 kilograms, were used. General anesthesia was induced by injecting sodium pentobarbital, 30 mg. per kilogram, intravenously. Polyethylene tubes 72 and 74 were inserted through side branches in the aorta and femoral arteries at known distances apart and connected to beakers 76 and 78 containing calomel electrodes 80 and 82. The back flow of blood through the polyethylene tubes, which then clotted, served as electrolyte bridges. The calomel electrodes were connected to a battery operated Keithley electrometer 84—thus both measuring electrodes were effectively off ground. The experimental arrangement is schematically represented in FIG. 13. Measurements are made at the normal flow rate of blood.

Streaming potentials under normal conditions averaged 0.2 mv. across the aorta and 0.5 mv. across the femoral artery. Intravenous low molecular weight dextran (average mol. wt. 40,000; 10 percent w/v) markedly increased the streaming potentials, and thus demonstrated a marked increase in the negative surface charge on the intima. The increase in each case is linearly related to the amount of dextran infused (FIGS. 14 and 15). After infusion of 200 ml. of low molecular weight dextran, measured streaming potentials increased to an average of 0.5 mv. across the aorta and 1.6 mv. across the femoral artery.

Similar effects were produced by injecting heparin into the experimental animal. Intravenous heparin (3 mg. per kilogram) increased the streaming potential to 0.7 mv. across the aorta and 0.8 mv. across the femoral artery. Subsequent injection of 50 mg. intravenous protamine decreased the streaming potential to their original values (Table VI).

TABLE VI

Effect of heparin and of protamine on in vivo streaming potential across canine femoral artery.

Streaming potential (mv.)					
With no drug		With 50 mg. I. V. heparin		With 50 mg. I.V. heparin followed by 50 mg. I.V. protamine	
Aorta	Femoral artery	Aorta	Femoral artery	Aorta	Femoral artery
0.3	0.5	0.75	0.8	0.3	0.5

Two drugs, heparin and dextran, elevate the streaming potential above the control values. These two drugs are believed to be hydrolyzed to acidic products at physiological pH. Heparin is an acid mucopolysaccharide with a molecular weight of about 17,000. Its hydrolysis yields repeating units of D-glucuronic acid with an O-sulfate group at C-2 and D-glucosamine N-sulfate with an additional O-sulfate at C-6. Heparin differs from other mucopolysaccharides in that there are sulfate groups bound to amino groups to form sulfamic linkages. This type of linkage is unique in nature. The content of esterified sulfuric acid is approximately 40 percent. This structure appears to make heparin the strongest organic acid occurring in mammalia. Antithrombotic activity is related to sulfonic acid content, and hydrolysis of the ester linkage results in a loss of activity. In addition, a minimum molecular weight is required since synthetic polymers of a monomer identical with that of heparin are not biologically effective. The low molecular weight dextran used had an approximate molecular weight of 40,000. It is a branched polysaccharide composed of glucose units linked primarily by 1:6 glucosidic linkages. Low molecular weight dextran has been shown by electrophoresis studies to maintain erythrocyte negativity. Biologic activity of dextran as an antithrombotic agent may possibly be due to hydrolysis producing free acidic groups.

Conversely, protamine and polyquaternary, both strongly basic molecules, decrease the streaming potential. Protamine has a low molecular weight containing a large amount of arginine and therefore is strongly basic. Protamine combines with the acidic heparin molecule forming a stable salt with loss of anticoagulant activity. Polyquaternary is a long chain detergent containing many basic ammonium groups. This compound is therefore strongly basic. It has been shown to reverse the signs of the zeta potential of erythrocyte and intimal membranes as well as the sign of the streaming potential.

The first group of drugs, which increases the streaming potential, is associated with antithrombotic activity, and the second group, which diminishes the streaming potential, possesses thrombogenic or heparin-antagonistic effects. Increases in the slopes of the streaming potential-pressure relations in the presence of antithrombogenic drugs (FIG. 11) are associated with increases in zeta potentials. As was pointed out above, a higher magnitude for the zeta potential corresponds to a higher mean negative surface charge density on blood vessel wall. Conversely, thrombogenic drugs decrease the magnitude of the surface charge density or even change its sign (FIG. 12). A change in the surface charge density in the presence of these drugs can only be caused by their absorption on the blood vessel wall. In this way, acidic compounds increase membrane negativity and basic compounds decrease or even reverse the sign of the membrane charge.

Acidic molecules with known antithrombotic properties increase the streaming potential while throm-

bogenic and heparinantagonistic molecules decrease or reverse the sign of the streaming potential. It is very probable that the mechanism of their action is by the adsorption on intimal surface, with a consequent increase of its surface charge density in the presence of antithrombogenic drugs (and the reverse situation with thrombogenic drugs), as manifested by an increase in the streaming potential. Desirable properties for an antithrombotic agent include a strongly acidic charge, ease of adsorption onto the blood vessel wall, and a high molecular weight.

RELATIONSHIP OF IONIC STRUCTURE OF BLOOD-INTIMAL INTERFACE TO INTRAVASCULAR THROMBOSIS

Attempts have been made to study the rate of ingrowth of living cells into vascular grafts. The technique used was that of implanting electrode pairs across the graft and recipient artery in dogs. Electrodes implanted within the lumen of the blood vessel or graft were usually negative relative to the external electrode. Reversal of polarity of the luminal electrode usually indicated intravascular thrombosis at some point near the intimal electrode. Because of these observations, the investigators studied the effects of applied charge, "pseudoinjury current," in producing thrombosis within blood vessels.

Application of a pair of electrodes, one positive and one negative, to the blood vessel wall resulted in the production of a thrombus beneath the positive electrode only. The negative electrode could be used to prevent thrombosis in both injured and very small anastomosed blood vessels.

These findings impelled additional studies of blood-intimal interface in an attempt to elucidate phenomena occurring normally between the intima and the circulating blood which served to maintain blood in the liquid state and were disturbed by these applied charges. Early experiments seemed to indicate that a spontaneous potential difference existed across the normal blood vessel wall. However, it was soon shown that little or no potential difference was measurable across the isolated blood vessel wall bathed with identical solutions on either side. Simultaneously, it appeared increasingly probable that the potential difference being looked for existed at the microvolt level and was exerted over short distances of a micron or less.

Because of technical difficulties implicit in carrying out in the intact animal the experiments to be described below, most of the subsequent experiments have been carried out in vitro. It is assumed that the observations reported here are in some degree applicable to phenomena naturally occurring in vivo at the blood-intimal interface. Studies of canine and rabbit blood vessel walls have been directed along three lines: (A) ion diffusion (flux) studies; (B) ion elution studies; and (C) electro-osmosis experiments.

Ion flux studies have measured the rate at which radioactive tracers of several ions (Na^{22} , Na^{24} , Cl^{36} , and Ca^{45}) move across the blood vessel wall into the fluid compartments on either side. They are to some degree a measure of the pore size of the vessel wall and its permeability to a given ion. They are also a measure of the rate at which the blood vessel wall picks up the ions and transports them, in either direction, across the wall.

Ion elution studies measure the rate at which the cells of the intact blood vessel wall actually absorb and release the same ions. These studies of the "third space," the vessel wall, complete the picture partially elucidated by ion movements across the wall. In this experiment the release of radioactive ions from tissues previously loaded with radioisotope is measured.

Electro-osmosis experiments are the converse of electrophoresis experiments. Instead of having the charged particles move from one pole to the other pole in a fluid medium, fluid is forced across the charged membrane which is fixed and cannot move. An electromotive force is applied across the vascular membrane. The water molecules of the solution bathing the wall move from one side of the membrane to the other, depressing the manometer fluid level on one side while, raising it on the other. The direction and rate of solution flow across the membrane indicates its charge sign and magnitude, respectively. It is possible, by means of the data from these three sets of experiments, to analyze, at least in part, the structural and metabolic characteristics of the membranes.

Ion diffusion studies: Ion diffusion studies are made by using a segment 90 of a blood vessel wall to divide the two halves 92 and 94 of an ion diffusion chamber (FIG. 16). An excised blood vessel, canine or human, is split longitudinally and divided into segments. Multiple matching vessel wall segments are placed over the openings between cell halves. The cells are filled with identical, oxygenated, mammalian Ringer's solution at a pH of 7.4 and 37° C. One microcurie of Na²², Na²⁴, Cl³⁶, Ca⁴⁵, or K⁴² is placed in the donor solution. The rate at which the ions diffuse across the membrane from intima to adventitia in one cell and from adventitia to intima in a second cell in each cell pair is measured by pipetting off samples at 96 of the recipient solutions at periodic intervals. The samples are dried and the radioisotope tracer that has diffused across the wall is counted in a Geiger counter. The transmural isotope fluxes across the wall in each direction are then compared.

In FIG. 16, perfusing gases are usually bubbled via tube 98 in through the bottom of the cell. Agar bridges 100 and 102 attached through calomel cells to a potentiometer serve to measure the potential difference developed by the wall. Radioisotopes of the ion being studied are added to the bathing solution on the left. The rate of ion movement across the membrane is measured by pipetting samples of recipient solution on the right-hand side of the cell at regular intervals, drying the samples, and counting them in a Geiger counter.

Ion Elution studies: Ion elution studies are determined by actually placing blood vessels and other tissue membranes for comparison into physiologic Ringer's solution, containing an isotope of the ion to be studied, usually either Na²², Cl³⁶, or Ca⁴⁵. The membranes are thus loaded with the isotope. The membranes are next removed from the isotope-containing solution and placed, for periodic intervals, into a series of isotope-free aliquots of solution (FIG. 17). The rate at which the isotope escapes from the tissues represents a measure of the turnover rate of the ion studied.

Electro-osmosis determinations: Electro-osmosis studies are carried out by placing the vessel-wall membrane over the openings between two half cells. A current (usually 10 ma) at high voltage (150 volts) is

passed across the membrane through electrodes at either end of the cell. Manometers inserted in the tops of the cells measure the direction and rate at which water moves across the membrane from one half-cell into the other half-cell. The direction at which the fluid moves is an indication of the charge of the membrane. The rate at which it moves indicates the charge density per unit area. If experiment duration, specific conductivity of the cell, dielectric constant of the solution, and fluid viscosity are known, the zeta potential of the membrane surfaces can be determined from the equation

$$\zeta = (4 \pi nKV/Di)$$

where ζ = zeta potential in millivolts

n = fluid viscosity in poise

K = specific conductivity of the cell

D = dielectric constant

i = current in milliamperes.

Several hundred studies of net diffusion of Na²², Na²⁴, Cl³⁶, K⁴², and Ca⁴⁵, across canine and rabbit aorta and vena cava walls have been completed. These studies show that there is net transport of ions under various conditions across these blood vessels.

As shown in FIG. 18, there is small net diffusion of both Na⁺ and Cl⁻ of the order of magnitude of 3 μ M. per square centimeter per hour from intima to adventitia across canine aortic walls. Net movement of Na⁺ and Cl⁻ ion across canine vena cava wall is in the opposite direction and occurs at about 10 times the rate of movement of these ions across aortic wall.

Contrariwise, there is very little movement of either K⁺ or Ca⁺⁺ across either aortic or vena cava wall. The net flux which does occur is in the direction opposite to Na⁺ and Cl⁻ flux (FIGS. 19 and 20).

The ion elution studies of Ca⁴⁵, Na²², and Cl³⁶ from blood vessel walls show that each of the tissues studied (aorta, vena cava, and tendon) have different turnover rates for the three ions.

In addition, there is good evidence that the blood vessel walls tend to absorb large quantities of these ions, concentrating them between 3 and 20 times their concentration in the bathing solutions. Additional evidence from curves similar to those shown in FIG. 21 indicate that the escape of the isotope from the tissues studied can be divided into two components: loosely bound and tightly bound ions. The escape rate of the loosely bound ion is indicated by that part of the curve with a rapid slope in FIG. 21. The tightly bound isotope is indicated by that segment of the curve with the lesser slope when extrapolated to zero time.

FIG. 22 indicates the relative concentration of the various ions absorbed onto the tissues and cell surfaces of aorta, vena cava, and Achilles tendon. The Ca⁴⁵⁺⁺ is most entirely absorbed in the studies which have been completed. It is concentrated to a level at least 8 times that present in the fluid environment about the tissue fibers.

Other experiments from this group of studies have demonstrated that the presence of Na⁺ in the bathing solution is essential to the complete uptake of Ca⁺⁺, implying an ion matrix formation at the tissue interfaces between Ca⁺⁺ and Na⁺. The third experiment has shown that Ca⁺⁺ must be present in the bathing solution in order to obtain complete elution of Ca⁴⁵⁺⁺ bound to the aortic and vena cava wall segments. Thus, a portion of the bound Ca is only self-exchangeable

with another Ca^{++} ion. Finally, ion movement across the blood vessel walls takes place in an inverse relationship to absorption on the tissues of the blood vessel wall. The possible relationship between the measured membrane tissue charge, ion movement across membranes, and ion absorption on the studied membranes will be discussed later.

Electro-osmosis studies. As shown in Table VII, fiber and cell surfaces of the canine aorta and vena cava appear negatively charged during electro-osmosis experiments.

TABLE VII

Electro-Osmosis Data: Canine Aorta and Vena Cava

Tissue	Gas		Solution	Electro-osmosis direction		Mean potential (mv. at 10 mamp.)	Area of membrane (cm. ²)	Statecoulombs per cm. ² at 10 mamp.	Net negative ions per cm. ² $\times 10^{10}$
	85%	15%		+	-				
Canine aorta.....	O ₂	CO ₂	Sugar-free Krebs.....	I →	A	8.33 ± 5.19	1	.121	2.53
Do.....	O ₂	CO ₂	do.....	A →	I	17.74 ± 9.10	1	.255	5.61
Mean.....						10.68 ± 7.27		.156 ± .0001	3.25 ± .0003
Canine vena cava....	O ₂	CO ₂	Sugar-free Krebs.....	I →	A	5.54 ± 1.17	1	.080	1.67

The current can drive Na ions across the vessel wall 120 (FIG. 23) through the pores from left to right because the Na ion and pores are apparently oppositely charged. However, the negatively charged Cl ions, which must carry negative charge from the negative electrode on the right side to the positive electrode on the left, cannot easily go through the negatively charged pores of the wall. Therefore, a layer of negatively charged Cl- ions in addition to an excess of Na⁺ ions builds up on the right side of the negatively charged wall. With the positive Na⁺, Ca⁺⁺, K⁺ ions, etc., coming across from the opposite side, a total net increase in both positive and negative ions occurs in the chamber containing the negative current electrode 122. Water obligatorily osmotes from the point of lower ion concentration to the point of higher ion concentration, lowering the manometer 124 on the positive side, raising it on the negative side. The rate at which the water moves across the membrane at a given current is a measure of the charge per unit area of the membrane.

In the above three experiments a study was made of three aspects of the same phenomenon: the surface characteristics of the cells and tissue fibers of blood vessel wall. Integrating the information obtained from various aspects of the three experiments described, it is possible to synthesize a partial picture of the charge characteristics of the membrane at the blood-intimal interface capable of producing these phenomena. This theoretical picture suggests that the surface of the intimal interface, as well as other pore surfaces, are negatively charged. Next to these surfaces there is a layer of positively charged ions, presumably Na⁺, Ca⁺⁺, and probably K⁺. In the next layer, which is much thicker, there is an excess of chloride ions and also the three cations previously mentioned. As one recedes from 10 to 100 from the tissue surface, the net ion charge tends to decrease progressively until equal numbers of ions of opposite charge are present in the solution.

It is theoretically conceivable that the net negative charge of the vessel wall tends to repel similarly charged proteins and cells flowing down the blood vessel. It is now necessary to remember, as Abramson demonstrated in 1927, that not only the red cells, white cells, and platelets are negatively charged, but also that

red cell "ghosts" are negatively charged. Thus, the red cell wall appears to have a fiber charge sign similar to that on vessel wall surfaces. It is conceivable that they have an electric double layer similar to that on the surfaces of the blood vessel walls themselves. Since these charged surfaces should naturally repel each other, this phenomenon is one of the mechanisms responsible for the prevention of intravascular thrombosis under normal conditions.

It is quite obvious that the blood vessel walls are non-homogenous membranes and a number of the

phenomena investigated occur in other than the blood-intimal interfaces. For instance, it is known that K⁺ and Ca⁺⁺ are absorbed by the muscle cells in the blood vessel walls. However, one experimental fact supporting the theory reported above is the fact that vena cava wall tissue, which has a much higher percentage of endothelium per unit weight, also has greater absorption of Na, Ca, and Cl ions under the conditions of this study. This would imply that the anatomically intact inner membrane of vessel absorbs, both intra- and extracellularly, a greater than average portion of the total ions absorbed, and may be especially involved in the ion transport process.

Three experiments are described above: (A) ion diffusion across blood vessel wall; (B) ion turnover rate by blood vessel wall (with Achilles tendon as control tissue); and (C) electro-osmosis studies of blood vessel wall. The information gleaned from the three experiments permits an appraisal of certain biophysical characteristics of the pores and surfaces of the tissues studied.

Mutual repulsion produced by the negative charge of both the blood vessel wall and the contained blood cells and proteins flowing in blood vessels is a factor in the prevention of intravascular thrombosis.

ELECTROCHEMICAL METABOLISM OF BLOOD VESSEL WALLS

It may be presupposed that most myocardial infarctions occur because of a defect in the oxygen-transport mechanisms supplying the myocardium. Such a defect can occur at any one of several levels: poor oxyhemoglobin dissociation, occlusion of capillary beds by "blood sludging," or occlusion of a major coronary artery. The latter would seem to be temporally related to the majority of myocardial infarctions.

If blood vessels and the blood itself are considered as a single system, the question arises as to what hemostatic mechanisms, both in the blood and in the blood vessel wall, prevent mural and intramural thrombosis. Knowledge of these mechanisms may eventually lead to prevention of the prime lethal arterial thrombosis—coronary artery thrombosis.

Study of the relationship of the blood to the blood vessel has been intensified with the advent of vascular

grafts and the problems of graft thrombosis. Our interest in vascular thrombosis began with correlated studies of the utility of freeze-dried homograft segments as vascular replacements and has continued during development of porous-plastic vascular prostheses.

Early in the studies concerned with graft healing, potentiometric electrodes were placed across grafts and recipient arteries of dogs in an attempt to measure the rate at which living tissue invaded the implanted grafts. These studies yielded some very unexpected measurements. A potential difference is measurable across the blood vessel wall *in vivo* following dissection and placement of electrodes across the wall.

The potential difference was greater immediately after grafting, decreasing over a period of 10 to 20 days. Because of this finding, the postoperative potential difference has in the past been considered a healing potential. It is now believed that much of the potential difference measured in this system is probably due to changing salt concentrations in the catheter electrode tips at the site of healing.

The intimal electrode is ordinarily negatively charged with respect to the adventitial electrode. On several occasions the transmural potential-difference polarity reversed. Concomitant with the reversal of polarity (the intima positively charged in relation to the adventitial), thrombosis of the vessel was frequently noted. The question was then raised as to whether the reversal of potential difference was the cause or effect of the vascular occlusion?

Experiments measuring the electrophoretic mobilities of various blood cells have indicated that all blood cells are negatively charged and move toward the positive pole in an electrophoretic cell with a mobility that is relatively constant for any given type of cell. It has also been indicated that the vascular wall must do something to prevent blood cells from being electrically attracted against the wall and sticking to the endothelium. As noted above, the cell charge is a factor in migration of inflammatory cells to areas of inflammation. It appeared possible that the presence of a positive charge on the intimal surface of the blood vessel wall attracted negatively charged platelets and other cells to the wall, producing a mural thrombus.

In an attempt to evaluate the possible relationship between cell charge, vessel-wall-intimal charge and intravascular thrombosis, a cylinder of exposed aorta or vena cava was placed in an electric field. The positively charged electrode 140 was placed on one side of the vessel wall, the negatively charged electrode 142 on the other side (FIG. 24). Electrical current passage between the electrodes produced deposition of a thrombus beneath the positively, but not beneath the negatively, charged electrode. The currents which were used to produce vascular thrombosis during these experiments were for the most part larger than currents which can be spontaneously produced by injured mammalian cells. For this reason, the occlusive effect of increasingly small electrical currents on visualized blood vessels in the rat mesoappendix, as discussed above, was measured. It was found that applied electrical currents of the order of 5 to 10 microamperes along a 20 millivolt potential difference resulted in occlusion of the small vessels of the rat mesoappendix. The effective transmural current flow and potential difference as determined by analysis of the experimental data and the determination of field, shape and size are of the order of one-tenth of those cited above. It appears

possible, therefore, for oriented currents of the order of magnitude of biological injury currents to produce occlusion of microscopic-sized blood vessels.

These findings suggested that thrombosis may be delayed or prevented by a properly oriented electrical field. A negatively charged electrode wrapped about the crushed femoral artery and vein in over 200 laboratory animals demonstrated a delay in thrombosis of the injured, negatively charged vessels when compared to the paired, untreated control vessels. Several additional experiments have now been carried out by other investigators demonstrating prevention of occlusion of large and small blood vessels by means of an oriented electrical field.

Clinical evaluation of the use of the hemostatic effect of an electrical current about a positively charged electrode to produce coagulation of blood from both normal persons and patients with hemophilia *in vitro*, and to stop bleeding in the tissues of laboratory animals and man, is now going on. These studies indicate that a properly oriented direct current is an effective hemostatic, coagulating agent. Even more important, *in vitro* experiments give evidence that small currents (0.1 to 1.0 microampere) will produce precipitation of a blood clot in both hemophilic and normal blood on the positively charged electrode of an electrode pair. In addition, small currents will convert fibrinogen to fibrin *in vitro*, producing what appears to be a fibrin gel within a test tube.

Accumulated data has given rise to the thought that the blood vessel wall expends energy in the transport of ions to maintain a stable blood-intimal ionic interface. It appears that this occurs in order to maintain the internal milieu and prevent intravascular thrombosis which might result due to adventitious and abnormal currents.

Several additional lines of clinical and experimental evidence gave substance to these speculations: first, it was found that when chronic intravascular potential-difference measurements were analyzed mathematically, the potential difference was shown to be produced by the perivascular tissues, not by the vessel wall itself; second, transvascular potential-difference measurements *in vitro* indicated that the normal potential difference across the isolated vessel wall was in the range of 1 millivolt (spontaneous or induced vascular transmural potential differences of greater magnitude are probably abnormal, and appear to act as a factor in intravascular thrombosis); and third, it was observed clinically that clots formed in solid-walled plastic arterial graft prostheses, but not in prosthetic arterial grafts woven of yarn manufactured from identical materials.

Several hundred studies have been completed measuring the *in vitro* transport of sodium, chloride and calcium ions across aorta and vena cava wall membranes of dogs. The studies have been carried out using radioactive ion tracer techniques. The results of these experiments indicate that transport of sodium and possibly chloride ions does take place. This ionic transport, which takes place at potential differences close to zero, is modified by anoxia, temperature, pH and trauma to the blood vessel wall. By definition, therefore, these ions move across the blood vessel wall, at least in part, by a process of active transport. All of these experiments appear to have one common denominator: they indicate that disturbances of normal transvascular ion concentrations, whether due to tissue injury, ap-

plied, oriented electrical currents, or to abnormal, oriented ion concentration per se, appear important to the process of intravascular thrombosis.

To test this hypothesis, a series of experiments was carried out to measure the occlusive effect of a current of injury in dying muscle in vivo on visualized blood vessels in a superimposed but separate rat mesentery (Table VIII).

TABLE VIII

Experiments on the Vascular Occluding Effect of a Current of Injury in Dying Muscle

Type of experiment	Number of experiments	Time of observation								Mean occlusion index	T	Significance *					
		Long-est (min.)	Short-est (min.)	Mean (min.)	Beginning†								End†				
					I	II	III	IV	Total				I	II	III	IV	Total
Controls.....	12	185	20	60	231	84	32	40	388	243	89	39	28	399	-0.894	1.24	0.23
Long controls.....	8	435	120	221	228	67	24	5	324	197	90	79	36	402	-0.42	3.68	0.003
Muscle mesentery.....	10	210	105	143	121	45	22	33	221	20	43	51	145	258	-13.62	9.42	10 ⁻¹³
Muscle dialysis-membrane-mesentery.....	10	200	30	121	119	79	63	51	312	24	45	82	181	332	-12.57	5.0	5×10 ⁻⁷
Muscle macerate.....	14	240	20	76	211	95	24	16	342	169	88	82	58	397	-0.61	4.35	0.0041
Thromboplastin.....	13	85	10	34	173	82	53	56	364	157	106	60	52	375	-2.208	1.95	0.05
Boiled muscle.....	10	195	50	91	101	19	8	16	144	104	33	22	25	184	-2.322	1.65	0.10
Lactate.....	14	220	26	81	84	125	135	133	477	86	146	105	124	461	+2.335	1.31	0.19
Dialysis membrane.....	4	75	20	63	56	19	1	1	77	45	16	6	1	68	-0.638	1.625	0.10

* Possibility that result is due to chance.

† Roman numerals refer to degree of occlusion of visualized vessels in the rat mesentery.

The injury potential difference produced by the muscle was measured by means of a pair of microelectrodes attached to a potentiometer. Each vessel visible in the mesoappendix was scanned regularly and assigned a number according to the degree of occlusion produced by the anoxic underlying muscle. The scanning process was repeated every 10 to 15 minutes during the course of the experiments to determine the progress of the occlusion. The effect of the anoxic dying muscle on blood flow through vessels in the mesoappendix has also been photographed. The rate of vascular occlusion appears proportional to the injury potential difference measured at the start of the experiment at the cut edge of muscle. During control experiments the vessels did not display these occlusive changes (Table VIII). Moreover, the resected bit of muscle lost most of its effect when macerated (Table VIII). The more the cellular structure was destroyed during maceration in mortar and pestle, the less the occlusive effect.

Boiled, the muscle lost entirely its thrombogenic effect (Table VIII). The effect persisted even though the muscle was separated from the mesentery by a 5-millicron pore dialysis membrane. Lactate decreased, rather than increased, the thrombotic effect of the test muscle, with resultant increased blood flow in that part of the vascular bed not previously completely occluded.

The results of similar experiments in which dying muscle was applied to the normal heart have recently been reported. Necrotic muscle applied to normal myocardium produced the electrocardiographic changes of myocardial infarction, caused conductance disturbances when applied to the conduction bundle, and prevented normal myocardial contraction in the areas directly beneath and close to the dying muscle. The effects were slowly reversible following removal of the injured muscle. Reversion to normal electrocardiographic activity took increasingly long periods of time if the dying muscle had been in contact with normal myocardium for periods greater than one half hour. Likewise, myocardium previously in contact with dying muscle only slowly regained its normal contractility fol-

lowing the removal of the dying muscle. It has been reported that the "necrotic" muscle causes vascular occlusion, but only disturbs myocardial conduction due to the release of potassium. It would appear that this evaluation is not entirely justified from the muscle-mesentery studies which have just been described. Whether the occlusive effects, if any, of the dying muscle on underlying myocardial capillaries are tempo-

rary or permanent also remains to be determined. It would be difficult to carry out the equivalent of the muscle-mesentery experiment in vivo on the heart, for it is impossible to transilluminate vessels within myocardium in the same fashion as one is able to transilluminate the mesentery.

These experiments may explain the phenomenon of marginal myocardial infarction. As an infarction occurs in muscle deprived of its blood supply, currents of injury occur in the nearest adjoining musculature. These abnormal currents establish a vicious cycle, producing occlusion in the capillary beds in the adjoining muscle and thus causing creeping necrosis. This in turn leads to death of the adjoining surrounding normal muscle, with further extension of the myocardial infarct into areas of marginal blood vessels of sufficient size to resist the thrombogenic effect.

Experiments outlining the relationship of applied fields and electrical currents to production and prevention of intravascular thrombosis have been described above. The hypothesis that the blood vessel wall ordinarily expends energy in the transport of ions in order to neutralize the occlusive effects of adventitious and applied currents is being studied. These indicate that the blood vessel wall does indeed expend energy in the active transport of ions, and it would seem to do so in order to keep the potential difference at the blood-intimal interface close to zero.

Equivalent experiments in which an injured piece of skeletal muscle was used as the source of injury current have been carried out. The vessels of the rat mesoappendix superimposed on dying muscle displayed occlusion. The control studies that showed that intact injured anoxic muscle has a maximum occlusive effect are enumerated.

ELECTROKINETIC CHARACTERISTICS OF HUMAN AORTAS

Aortas were retrieved from human subjects at autopsy, immediately immersed in cold modified Krebs solution, and analyzed both by visual examination and by pathological testing. They were graded as follows: Grade 0, Aortae retrieved from infants. No atheroma,

no pigmentation. Grade 1, Slight pigmentation of intima and plaquing. Grade 2, Gross atheroma, pigmentation and slight ulceration. Grade 3, Gross atheroma, increasing pigmentation, ulceration with beginning calcification. Grade 4, Gross atheroma, pigmentation, calcified plaques, loss of elasticity. Grade 5, Gross atheroma, hard calcified aortic pipe with an ulcerated intima.

Three sections were cut from each aortic specimen, one each from the aortic arch, descending thoracic, and abdominal aortas. These samples were kept in Krebs solution before and during the experiment.

The experimental procedure described earlier was closely followed. Experiments were conducted with all three regions of the aorta for each degree of atherosclerosis. All experiments started with a positive ionic current flow (current 10 milliamperes) from the adventitial (A) to the intimal (I) side. After 30 minutes the direction of the current was reversed.

Sixty-five increasingly atherosclerotic aortas were used in these experiments. Zeta potentials (ζ) were calculated according to the equation:

$$\zeta = (4 \pi K V / Di)$$

where K, ζ and D are the specific conductance, viscosity and bulk dielectric constant, respectively, of the electrolyte used for the electro-osmosis measurements; i is the current which flows across, and V is the rate of fluid flow across the membrane. The results, including standard deviations, are presented graphically for each degree of atherosclerosis and for the different sections of the aortic wall in FIG. 25. In FIG. 25 ζ potentials are shown for varying degrees of atherosclerosis without distinguishing between the direction of current flow.

FIGS. 25 and 26 show that ζ potentials of normal and atherosclerotic aortas are practically identical, except with studies of the aortic wall displaying the maximum degree of atherosclerosis. Because of the fairly high standard deviations in ζ potentials for the various cases, a rigorous statistical analysis of the results was made which yielded the same conclusion. No significant differences in ζ potentials were found with a reversal in the direction of current (that is, A-I or I-A).

Electro-osmosis is insensitive to small changes in the configuration of a porous medium. Changes can be expected only when one has lost the last statistically normal pore. The present experiments show that normal electro-osmotic behavior is observed until the maximum degree of atherosclerosis is attained. At this point, there is a sharp drop (loss of negative surface charge) in the ζ potential, which is indicative of some critical loss, both in normal porosity and in the pore surface charge of the negative surface charge of the blood vessels. The experimental findings and the conclusions drawn from these are consistent with the fact that the blood vessel wall is significantly resistant to thrombosis even in progressively atherosclerotic aortae until an advanced stage of atherosclerosis is produced. It lends further support to an electrochemical mechanism of intravascular thrombosis.

These findings explain the resistance to thrombosis of extremely atherosclerotic large blood vessels while normal blood pressures are applied to maintain the negative charge caused by electro-osmosis in spite of obvious loss of normal metabolic antithrombotic metabolisms. However, when blood pressure falls in these situations, thrombosis rapidly follows.

Table IX which follows below tabulates some of the substances screened in accordance with the above.

Drugs	Charge effect	Rat mesentery occlusion time, min.	Clotting time, min.	Blood destruction, hemoglobin
Control.....	0	30	2-4	0
SP 54 (Bene Chemie)...	(-)4	(+)4	(+)2	+2
Flavanoid 700 (Zyma)...	(+)1	+2	(+)1	
Flavanoid 748 (Zyma)...	(+)1	+4	(+)2	
Chondroitin sulfate...	(-)2	+6	(+)2	
Aspirin.....	(+)1	+1		
Ascorbic acid.....	(-)1	+1	(+)1	
Heparin.....	(-)4	+4	(+)0=00	0
Protamine.....	(+)2	-2	-2V+2	0
Dimethyl amino ethanol.....	(-)2	+2	(+)2	0
Diethyl amino ethanol.....		+1-2		
Dimethyl amino ethanol HCl.....				
Cholesterol.....	0			
N-ethyl maleimide.....	(+)1 _{very}	+1	(+)1	+1
Potassium SP G (old).....	(-)4	-4	(+)4	+4
Polyquaternary.....	(+)4	-4	??+2	+2
Di-threo-erythritol.....	0		+2	+1
Chondroitin sulfate.....	(-)3	+4	+4	0
Dextran.....	-2		-1	0

NOTE.—The reference marks used in Table IX are explained as follows:

- Charge effect:
 (-) = more negative I.F.
 (+) = more positive I.F.
 Rat mesentery occlusion time:
 (+) = Longer, good.
 (-) = Shorter, poor.
 Clotting time:
 (+) = Longer coagulation time.
 (-) = Shorter coagulation time.
 Blood destruction:
 (+) = More destruction.
 (-) = Less destruction.

There have now been set forth above a wide variety of tests employed in screening chemical agents and compounds in accordance with the invention. This involves measuring the effect of such substances on streaming potential in blood vessels or circulatory systems both in vivo and in vitro. Also involved is the measurement of the effect of such substances on electro-osmosis, as well as the effect thereof on the charge of blood cells in electrophoresis. In further accordance with the screening of the invention, the effect of the substance on the act of transport of ions across blood vessel walls is measured, as is the sorption and desorption of ion with respect to the pores and intimal surface of the circulatory system. Still further, the invention requires measuring the effect of the substance undergoing test on blood cell change as well as on coagulation characteristics and the evaluation of the effect of such substances on a test animal mesentery.

The above tests permit one to distinguish between the antithrombotic and anticoagulant characteristic of a substance. The capability of distinguishing between these characteristics is important for the reasons enumerated hereinabove. It will, however, be understood that varying degrees of these two characteristics are possible and that it is not the intention of the tests herein to label a substance as being either anticoagulant or antithrombotic to the exclusion of the other characteristic.

What is claimed is:

1. A method of screening a substance for use in the treatment of circulatory system diseases, said method comprising determining the effect of the substance on streaming potential in circulatory systems by measuring the change in potential between an electrode pair inserted into the systems; determining the effect of the substance on electro-osmosis in the circulatory systems

by measuring the rate at which a fluid containing the substance can be pumped by a constant current of known polarity through blood vessel walls from said systems; determining the effect of the substance on the charge of blood cells by measuring the cataphoretic mobility of cell samples containing the substance; determining the effect of the substance on the transport of ions through walls of the circulatory systems by measuring the change in rate of ion isotope diffusion across the walls in the presence of said substance; determining the effect of the substance on the sorption and desorption of ions in the circulatory systems by measuring the change in rate of ion desorption from walls of the systems produced by said substance; determining the effect of the substance on blood destruction; and determining the effect of the substance on test animal mesentery thrombosis by measuring the change in rate of the mesentery vessel occlusion after administration of said substance.

2. A method as claimed in claim 1, wherein the effect of streaming potential is determined *in vivo*.

3. A method as claimed in claim 1, wherein the effect on streaming potential is determined *in vitro*.

4. A method as claimed in claim 1 comprising measuring the change of rate of the deposition of blood cells and thrombus on an electrode immersed in a fluid containing the substance.

5. A method as claimed in claim 1 comprising evaluating said effects to distinguish an anticoagulant from an antithrombotic agent based on said change in rate of occlusion.

6. A method as claimed in claim 1, wherein the test animal mesentery is examined *in vivo* by extracting the mesentery from the animal, passing a light through the mesentery, passing an electrical current through the mesentery, and observing the occlusion of blood vessels in the mesentery.

7. A method as claimed in claim 6, wherein the current is in the order of 10-20 microamperes.

8. A method as claimed in claim 7, wherein the mesentery is bathed in a gelatin Ringer's solution.

9. A method as claimed in claim 1, wherein the streaming potential is examined with a segment of the circulatory system *in vitro* by placing the segment in a bathing solution having the same ionic composition as blood and establishing flow rates through the segment with an inert gas.

10. A method as claimed in claim 9 comprising measuring the streaming potential across the ends of the segment by placing the electrodes at said ends and connecting the electrodes to an electrometer.

11. A method as claimed in claim 10, wherein the downstream electrode is connected to the positive terminal of the electrometer.

12. A method as claimed in claim 1, wherein the streaming potential is examined with a segment of the circulatory system *in vivo*, comprising inserting tubes into spaced positions in said system and connecting the said positions to electrodes which are, in turn, connected to an electrometer.

13. A method as claimed in claim 1, wherein the transport of ions is examined by separating two chambers having bathing solutions therein with a segment of said system, and adding a radioactive substance to one of said chambers.

14. A method as claimed in claim 13, wherein the transport is examined under aerobic conditions.

15. A method as claimed in claim 13, wherein the transport is examined under anaerobic conditions.

16. A method as claimed in claim 1, wherein ion elution is determined by placing a segment of said system into a solution containing an isotope and then removing the segment and placing the same for periodic intervals in isotope-free aliquots of solution, the rate at which the isotope escapes from the segment being a measure of ion turnover rate.

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