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Committee:

WALTER H. SEEGERS, ELWOOD A. SHARP, PAUL HALICK

An Estrogenic Preparation and Certain Coagulation Factors. Johnson Frederic, J., Department of Physiology and Pharmacology, Wayne State University, Detroit.

Intravenous estrogens are known to be effective for the control of hemorrhage from the upper respiratory tract, and other areas as well. An investigation was therefore undertaken to determine if there were any alterations of certain plasma factors involved in coagulation coincident with this procedure. Following intravenous administration of the estrogens in the dog and also in human subjects, there is a sharp, rapid rise in the plasma concentrations of prothrombin and Ac-globulin. Accompanying this rise there is a fall in the antithrombin activity of the plasma. The changes would tend to enhance blood coagulation. Other factors, such as autoprothrombin I and II, platelet cofactor I, and fibrinogen, were also studied and slight changes in their plasma concentrations were noted. All the alterations accompanying the use of the estrogens persisted for several hours, paralleling the observed period of their clinical usefulness.

Relation of Stress to Hemorrhage and Prothrombin Time Following Anticoagulants. Lowenthal, J., Fisher, L. M. and Jaques, L. B., University of Saskatchewan, Saskatnoon.

Rabbits were subjected to stress (insulin shock, hypertonic saline, frostbite) with and without anticoagulant pretreatment (dicumarol, phenylindanedione, heparin). Pretreatment with indirect anticoagulants (dicumarol, phenylindanedione), but not with heparin, increased significantly the incidence of hemorrhagic death compared to control groups receiving anticoagulant alone or subjected to stress only. Incidence of hemorrhagic death was found to correlate with a fall in hematocrit values. The increase in mortality from hemorrhage was found also in stressed rats when pretreated with anticoagulants. In rats, stress alone caused an increase in the one-stage prothrombin time. Addition of human serum or $BaCO_3$ adsorbed bovine serum to the plasma of stressed rats with a prolonged prothrombin time returned the values to normal. The failure of heparin to increase the incidence of hemorrhagic death (in rabbits) has been investigated. Heparin has been shown to interfere with some of the peripheral responses to stress.

Application of Thymol Turbidity Test for the Prevention of Serum Hepatitis. Jennings, E. R., Hindman, W. M., Zak, B., Reed, J. and Brines, O. A., Wayne State University and Detroit Receiving Hospital, Detroit.

During the past 30 months thymol turbidity tests have been performed on the serum of blood donors at the Detroit Receiving Hospital and the blood has been used without regard to

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the results of the test. The critical level of serum thymol turbidity which has been used in this study is 8 Shank-Hoagland units. Donors whose serum thymol turbidity value has been in excess of 8 Shank-Hoagland units have been called "high thymol" donors. There have been follow-up examinations on 193 recipients of "high thymol" blood. In this group there have been 10 cases of serum hepatitis and 5 cases of possible hepatitis. In the control series of 603 recipients (recipients of blood from donors whose serum thymol turbidity value was normal or unknown), there has been no serum hepatitis and two cases of possible hepatitis. These data clearly indicate that the hazard of serum hepatitis is increased if blood is used from donors whose thymol turbidity value is in excess of 8 Shank-Hoagland units. The feasibility of the use of this test for the screening of blood donors was discussed.

Sphingosine Discoverd as Physiological Inhibitor of Blood Clotting. Hecht, E., State University of Utrecht, Utrecht, Holland.

Two different investigations led to the discovery of sphingosine as a physiological inhibitor of blood clotting. (1) The endeavors to separate with the help of the paper chromatography the active principle from a lipid mixture, called lipid activator. The lipid activator gives before hydrolysis with phenol-water, 4 positive reactions with ninhydrine, after hydrolysis with sulfuric acid, however, only 3, which were identified as glutamic acid, serin and ethanolamin. By treatment of the residue from the hydrolysis with NaOH, then, by chromatographic investigation, we obtained a substance that gives a positive reaction with ninhydrin with an RF-value of 90. On the same place before hydrolysis, a positive reaction was obtained with ninhydrin and we supposed that the substance in question formed an insoluble sulfate during the hydrolysis. The eluate corresponding with RF: 90, has inhibitor activity. (2) The second investigation was to verify the assertion of Chargaff that sphingomyelins are inhibitors of blood clotting. This was indeed so, but only with sphingomyelins giving a positive reaction with ninhydrin. On the basis of their chemical formulas, this was not expected. Purified sphingomyelins, giving no positive reaction with ninhydrin were indifferent in relation to clotting. The impure sphingomyelin chromatographically investigated gave a positive reaction with ninhydrin with RF: 90 and the eluate had also a clotinhibiting effect. After hydrolysis, the positive reaction with ninhydrin disappeared. On the ground of these observations, we concluded that the substances, giving the 4th spot on the chromatogram of the lipid activator and the impurity of the sphingomyelins were the same. We began to suspect to suspect sphingosine which is closely related to several lipids such as sphingomyelins or cerebrosides. Sphingosine possesses a free NH_2-group and forms an insoluble sulfate. Indeed, chromatographic investigation of sphingosine, prepared from physiological material (cerebron and sphingomyelin of pig brain), gave with ninhydrin the same reaction with RF: 90.

The identification of the inhibiting substance as sphingosine was proved: 1. On the ground of the same RF-values with ninhydrin in consequence of a chromatographic comparison with 5 different developing solutions. 2. By the same characteristic clotting reactions with chicken plasma. 3. By the agreement of these reactions with physiological and synthetic preparations.

By investigations with 9 different derivatives of sphingosine, it could be demonstrated that the complete molecule with the double bond and free NH_2- and OH-groups is required to give the following typical reaction with chicken plasma: Sphingosine, in very small concentrations prolongs the clotting time to 40 hours or more. In the presence of the lipid activator, we observed with sphingosine clotting times, which are at most a little shorter than those with the lipid activator in optimal concentrations alone. The last named clotting times are however shortened to a sixth and sometimes to a tenth by addition of the lipid activator to chicken plasma incubated previously with sphingosine. This result is not obtained after incubation of the lipid activator with sphingosine so that a contribution from the plasma must be presumed.

A comparative investigation with the antithromboplastin of Tocantins, which he believes is increased abnormally in hemophilia, suggested that the essential principle of the antithromboplastin is sphingosine.

The Effects of Products of Thermal Denaturation of Thrombin Preparations on the Acceleration and Inhibition of Various Phases of Coagulation. Klein, E., Farber, S. and Djerassi, I., From the Children's Cancer Research Foundation and The Tumor Therapy Group of the

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Division of Laboratories and Research, The Children's Medical Center and the Department of Pathology, Harvard Medical School.

The thermal denaturation of preparations of bovine thrombin has yielded a water soluble material which promotes the early phases of coagulation. The material does not clot fibrinogen in the manner of unmodified thrombin. In the presence of high concentrations of the degradation product, unmodified thrombin is inhibited. The degradation product which represents 4.5 per cent of the weight of the starting material is non-dialyzable, contains glucosamine-polysaccharide and peptide bonds, and is precipitated by alcohol from aqueous solution. A single peak is observed in the analytical ultracentrifuge. On free-boundary electorphoresis a pattern is obtained, which resembles that of the starting material in terms of the general relation of the major peaks to each other, but the rate migration is considerably reduced in the modified preparation as compared to that of the starting material.

The observations indicate that the activities of thrombin in the earlier phases of coagulation can be separated from its effect on fibrinogen. It is also suggested that modification of the enzyme preparation can result in a material which will inhibit the activity of the starting material.

This investigation was supported by the Atomic Energy Research Contract AT (30—1) 1275 and Grant # C—937 from the National Cancer Institute.

Cinemicrography of the Formed Elements in the Microcirculation. Fulton, George P. and Berman, H. J., Department of Biology, Boston University, Boston, Massachusetts.

The dynamic characteristics of the formed elements of blood have been studied *in vivo* at magnifications as great as 2000 X by means of transillumination applied to the thin membranous cheeck pouch of the hamster and retrolingual membrane of the frog. The circulation of the formed elements is shown in normal preparations and in pathological conditions. Motion picture records have been edited for presentation of significants findings.

Extravascular components such as vasomotor nerve plexus, vascular smooth muscle, and perivascular tissue mast cells are shown as important factors which may regulate or modify the distribution of the formed elements.

The erythrocytes are shown in blood circulating through arterioles, capillaries and venules, thus providing a comparative indication of rates of flow. Adaptability to deformation is illustrated by the squeezing of individual erythrocytes passing through narrow vascular sphincters or through the endothelium during extravasation and petechial formation. Red cells often strike against portions of vessel walls, especially at "sharp corners" of branching arterioles. Changes in the flow characteristics of erythrocytes are recorded in conditions of anemia, after injection of moccasin snake venom, during adrenalectomy, and at terminus following lethal total body x-irradiation. The mechanics of petechial formation are demonstrated by the popping of red cells, one by one, through the endothelium without evidence of actual openings. The importance of the venous side of the circulation is emphasized by the vulnerable nature of venous junctions with respect to petechial formation and by the comparatively large surface area of the venules. A few hours before death from lethal irradiation or cortisone poisoning, the erythrocytes circulate in aggregates resembling "sludged blood", but the aggregates do not form occluding thrombi and break up readily at narrow capillary junctions.

The appearance and behavior of leukocytes is photographed in normal preparations and under conditions such as infection, malignancy, during anticoagulant therapy, and during infusion with dextran. An increase in adhesiveness of leukocytes to the vessel walls is documented in traumatic states and after intravascular injection of various substances such as heparin, x-ray contrast media, or dextran. The presence of coatings of immobile white cells is shown, especially in venules.

The platelets are photographed within living blood vessels, especially in eddies, stationary or slowly moving plasma, and in circulating blood near the endothelial wall at times when the flow is slow. During spontaneous rotation, the bayonet-like edges and oval surface aspect are apparent in the unagglutinated platelet *in vivo*. The role of platelets in hemostasis is demonstrated by the formation of platelet aggregates at points of injury in blood vessels.

Furthermore, the mechanics of platelet thromboembolism are shown by motion pictures of the entire process.

Test procedures for susceptibility to platelet thromboembolism, vascular fragility, and bleeding tendency are demonstrated, and results are presented.

Studies on Cold Precipitable Fibrinogen (Cryofibrinogen). Glueck, Helen I. College of Medicine, University of Cincinnati and The Cincinnati General Hospital, Cincinnati.

The present studies of cold precipitable fibrinogen, „cryofibrinogen“ were prompted by finding such a protein in the plasma of a patient with a congenital aneurysm of the iliac artery. There had been several attempts at surgical therapy of the lesion. At first, the aneurysm had been wrapped in cellophane. Steel wire has been threaded into the sack of the aneurysm one and three years before his last hospital admission.

Blood was collected in the usual concentration of citrate, oxalate or heparin, centrifuged and the resulting plasma stored at 4° C. A flocculant precipitate began to appear after 4—6 hours, increasing in amount as storage continued. On rewarming, the precipitate contained in the plasma dissolved at 37° C. No precipitate appeared if the plasma was maintained at room temperature. Serum stored at 4° C., did not contain the precipitate. The cryofibrinogen separated by centrifuging the plasma at 4° C., was washed twice with cold saline, and redissolved in saline at 37° C. It was insoluble in water at 37°. A fibrin clot formed on addition of thrombin to the solution of cryofibrinogen. The supernatent above this clot was incoagulable. Clots formed from cryofibrinogen were insoluble in 30% urea. On heating the solution of cryofibrinogen at 56 ° for ten minutes a precipitate formed, which did not clot on the addition of thrombin. The characteristics of the cryofibrinogen resembled that noted by Korst and Kratochvel. („Cryofibrinogen in a case of Lung Neoplasm associated with Thrombophlebitis Migrans.“ Blood *10:* 945 [1955]).

The plasma, serum and cryofibrinogen solution were studied by paper electrophoresis. The factor appeared to migrate with the fibrinogen fraction. It could be prepared as a single component by repeated washing and resuspension. Serum did not contain the factor. The cryofibrinogen was present in all samples tested preoperatively. It varied in amounts from 25 to 55% of the total fibrinogen which preoperatively was quite low (160 mg%). Preceding, during and following surgery the patient received 5 units of blood and 7 grams of fibrinogen. Nevertheless, shock and anuria developed following ligation of the aneurysm. The total fibrinogen rose to 310 mg% on the third post operative day; of this 140 mg precipitated at 4° C. The protein disappeared on the 13th post operative day and did not reappear after three months of observation.

Dogs were used in attempting to produce the protein experimentally. Preoperatively no cryofibrinogen was noted in one dog. It appeared three days after wiring of the iliac artery and has persisted for ten months. A „non wired“ dog served as a control. Initially, cryofibrinogen was absent in this dog's plasma. The protein appeared spontaneously (following 14 venosections) during the tenth week of observation. It has persisted for additional twenty weeks. The significance of this observation is unknown.

Cryofibrinogen was not found in the plasma of 21 normal students. Nineteen patients with normal pregnancies have been studied. In five, small amounts of the protein have appeared intermittantly. Four pregnant patients with clinically diagnosed thrombophlebitis have been observed. Cryofibrinogen was present in considerable amounts in the plasma of all. The quantity of the precipitate and its time of appearance have not always correlated with the activity of the phlebitis. The precipitate disappeared in all four patients on the second to fourth post partum day. In one patient with hypofibrinogenopenia associated with abruptio placenta the precipitate was present during labor, but disappeared 24 hours after delivery.

The protein has been seen in three patients following massive hemorrhage, and in one with myeloid metaplasia.

These observations are of a preliminary nature. The origin of cryofibrinogen, its relation to previously observed „intermediate“ fibrinogens, and its clinical significance aro still under study.

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I wish to thank Dr. Louis Herrmann and James Helmsworth for the opportunity to study this patient and for their help in subsequent experimental surgery.

Studies on the Nature of the Antihemophilic Activity of Normal Human Plasma. Surgenor, D. M. and Steele, B. B., Department of biological Chemistry, Harvard Medical School.

A protein fraction which contains antihemophilic factor activity has been obtained from fresh platelet-poor human resin plasma by the following procedure. After quantitative removal of prothrombin and the other proteins which interact with barium sulfate, over 80% of the AHF activity was precipitated into a fraction similar to Fraction I. This was then redissolved and treated with a high molecular weight dextran sulfate (DSO_4 — 0.01 to 0.02 l/g protein). The insoluble fibrinogen -DSO_4 complex was removed, leaving a solution which contained only traces of fibrinogen but good AHF activity. On dialysis, the AHF activity precipitated with a euglobulin fraction. The product was dried and remains stable for months at —20 ° C.

This purified AHF was free of other demonstrable clotting factors; it reduced the clotting time of hemophilic plasma, but not of plasma from a PTC deficient patient. It contained several electrophoretic components, the major component being β-globulin. At 0 ° C, solutions are relatively stable at pH 5.5—7, but are very unstable at alkaline reactions. The activity is destroyed by heating for 20 minutes at 56 ° C.

The behavior of this material in isolated systems confirms the activity toward hemophilic plasma. In the thromboplastin generation test it exhibits good accelerator activity with cephalin. In a thrombin generating system, using human platelets or extracted portions there of, it is active in ug. concentrations. Dextran sulfate, if used in even slight excess, is strongly inhibitory, particularly in the thromboplastin generating system; considerable care must be used in carrying out the separation from Fraction I.

Recent Observations on the Clotting of Plasma by Coagulase and on the Nature of the Coagulase Reacting Factor (CRF). Tager, M., Emory University, Georgia.

The demonstration of CRF activity in purified human prothrombin of Seegers and the preparation of purified CRF essentially lacking in prothrombin activity, has led to studies which have sought to reconcile these apparently conflicting findings. Prothrombin has been altered to determine whether the two activities might be dissociated. It has been found that „autoprothrombin" of Seegers still exhibits CRF activity, while in essence demonstrable prothrombin function is lost. Further, human thrombin, in which presumably prothrombin has been totally converted, still possesses the ability to react with staphylocoagulase and accelerate clotting. The evidence presented suggests that the CRF activity represents a fraction of prothrombin; thus the intact prothrombin molecule has both functions, while the purified CRF fraction, and the prothrombin derivatives tested have only CRF activity. Studies have been continued to determine the enzymatic activity of staphylocoagulase. It has been found that purified coagulase changes the suspension stability of egg yolk. Observations carried out with Miss Margaret Drummond have indicated that this is not a lecithinase effect, but is a lipase which splits tributyrin and other similar substrates. The relation of this action to the blood clotting phenomenon is under further study with inhibitors and specific antibodies.

Immunologic Studies on Serum Lipoproteins. Brown, R. K. and Lawrence, L., Division of Laboratories and Research, New York State Department of Health, Albany.

Investigation of the immunologic relationship of the various lipoproteins may aid their quantitation and clarify their metabolic significance. Rabbit antiserum to a low-density lipoprotein was studied by single and double diffusion in agar and by the precipitin and complement-fixation reactions. It was immunochemically homogeneous after absorption with human serum freed from low-density lipoproteins by ultracentrifugation. All flotation classes of low-density lipoproteins examined cross reacted with the antiserum but the highdensity lipoproteins did not. This antigenic similarity implies structural similarity. It would be difficult, if not impossible, to prepare antisera for the estimation of specific classes of low-density lipoproteins.

Similar studies showed that it is difficult to prepare an immunochemically homogeneous antiserum to high-density lipoproteins. Only one of ten antisera gave one line by single and double diffusion in agar against whole serum. The recovery of added high-density lipoprotein

cholesterol in the immune precipitate and the lack of reaction with low-density lipoproteins define the specificity of this antiserum. This antibody was more reactive with aged lipoprotein than fresh and provided a means of studying the changes that occur during preparation and storage. Preparative ultracentrifugation at high salt concentrations caused partial dissociation of lipid and protein, a change distinct from aging. The partially reactive protein portion became more reactive after addition of lipid. Sera from patients with xanthomatous biliary cirrhosis contain much lipoprotein reacting with antiserum to high-density lipoproteins although little such lipoprotein is present by physico-chemical studies. Perhaps the protein portion of these lipoproteins is similar to that of normal high-density lipoproteins and the increased lipid content causes altered flotation characteristics. The immunochemical distinctness of these types of lipoproteins agrees with chemical studies of their protein portions. All low-density lipoproteins examined have similar amino acid compositions and an N-terminal glutamic acid. High-density lipoproteins have a characteristic amino acid composition and a N-terminal aspartic acid.

Light Scattering Studies on the Fibrinogen Polymerization. Sheppard, E., Cornell University, New York.

Early work in our laboratory has demonstrated that heparin and other ionic anticoagulants will increase the electronegativity of the fibrinogen molecule's surface. It was of interest to see if this increase in electrical surface charge was sufficient in magnitude to interfere with the conversion of purified fibrinogen. Light scattering studies were done to follow the polymerization of fibrinogen in the presence of heparin as a function of ionic strength, concentration and pH. It has been demonstrated that heparin can block or retard the rate and degree of fibrinogen conversion *in vitro* due to repulsive forces. However, in the presence of serum, heparin's anticoagulant activity in our experimental system is due to the formation of antithrombin II arising from the interaction of heparin and its cofactor. The properties of the heparin-cofactor complex, and in particular, the effect of heat and ether treatments as gleaned from our studies will be described.

Using heparin as the prototype, a variety of similar polyanionic compounds has been studied. These compounds exhibit anticoagulant properties which *in vitro* are due to electrostatic forces. Evidence will be presented to show that the activation of a heparin-like cofactor can occur and that this is a function of he molecular weight of the polyanion.

Antithrombin and Heparin Cofactor from Human Plasma. Brambel, Ch. E., University of Notre Dame, Notre Dame, Indiana.

Monkhouse, France and *Seegers (Circulation Research 3:* 397 [1955]) have prepared potent antithrombin concentrates from heat defibrinated bovine plasma. In our studies, antithrombin with heparin-cofactor activity was isolated as a concentrate from oxalated human plasma which was partially defibrinogenated, deprothrombinized and ether extracted.

Defibrinogenation was accomplished by the cold precipitation method of *Seegers* and coworkers *(Arch. Biochem. 13:* 232 [1947]). The defibrinogenated plasma was then adsorbed with barium sulphate to remove prothrombin and associated clotting factors. The resulting supernatant plasma was extracted three times with equal volumes of ethyl ether containing 10% ethanol for the purpose of removing antithrombin.

No antithrombin *(Schaefer* et al., *J. Appld. Physiol. 8,* 300 [1955]) or heparin-cofactor activity was found in the residue from ether-ethanol extracts.

On standing for 16 hours at 32° to 35° F a precipitate forms in the plasma-ethanol-ether mixture. The precipitate was removed by centrifugation. This fraction was rich in antithrombin and heparin-cofactor activity. Subsequent purification of this concentrate resulted in no separation of heparin cofactor from antithrombin activity. The successive stages of isolation were followed with electrophoretic analysis.

Antithrombin and heparin cofactor activity was demonstrated by the prolongation of thrombin times using purified fibrinogen. The activities were checked with the TAME assay *(Sherry* and *Troll, J. biol. Chem. 208:* 95 [1954]).

When thrombin times were used, a greater effect was noted with the addition of heparin to the concentrate. These data may suggest that additive effects are obtained, or, that multiple

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mechanisms are involved. On the other hand, heparin had no effect on the TAME assay, which is a measure of hydrolytic activity. Thus, a dual function for antithrombin suggests itself: 1) the destruction of thrombin; and 2) in the presence of heparin, interference with the thrombin-fibrinogen reaction.

The foregoing data show that heparin with its cofactor (in this case, antithrombin) interferes with the thrombin-fibrinogen reaction but does not alter the esterase activity of thrombin.

Data will be presented to show that this concentrate („antithrombin-heparin cofactor") also interferes with the conversion of prothrombin to thrombin in human plasma. Inhibition of Ac-globulin as a probable mechanism will be presented. These findings indicate a third function for antithrombin.

Studies on Antithrombin and Heparin-Cofactor. Monkhouse, F. C., University of Toronto, Toronto.

The antithrombin potency of defibrinated plasma or serum is the result of two distinct types of action; (a) destruction of thrombin, and (b) interference with the action of thrombin on fibrinogen. In this paper the term antithrombin activity will be used to indicate a plasma constituent which destroys thrombin activity. The term heparin-cofactor activity will be used to refer to a plasma constituent which in the presence of heparin prevents thrombin from clotting fibrinogen. With the assumption that a better knowledge of the relationship between antithrombin and heparin-cofactor, and an understanding of the part they play in blood coagulation, can only be obtained by a study of more purified factors, we have attempted to isolate the active materials.

The original antithrombin concentrates prepared by *Monkhouse, France* and *Seegers (Circulation Research 3:* 397 [1955]) were obtained by adsorbing the active material on aluminium hydroxyde and eluting with phosphate buffer. The eluate was further fractionated by ammonium sulphate. The activity was contained in the fraction soluble at 40 per cent saturation and insoluble at 70 per cent saturation with ammonium sulphate. This fraction contained mainly alpha globulins and albumins. Electrophoretic techniques have been employed in an attempt to determine which protein is most closely associated with activity. Patterns on starch and on paper indicate that both antithrombin and heparin-cofactor activities are found mainly with the alpha globulins or associated lipoproteins. The most active material has been obtained by the use of a vertical curtain electrophoretic apparatus. In terms of activity per mg of nitrogen, antithrombin has been purified 7.5-fold and heparin-cofactor 130-fold over the concentrates prepared by ammonium sulphate fractionation of phosphate eluates.

Though this represents some progress the question of whether or not antithrombin and heparin-cofactor are distinct plasma proteins remains unanswered. Fractions with antithrombin activity but without demonstrable heparin-cofactor activity have been obtained by the use of the vertical curtain electrophoretic technique. Nevertheless, it would be unwise to conclude from these results that they are separate proteins. Many fractions contain material which inhibits the action of heparin and thus interferes with heparin-cofactor assay. Further it has not been possible to prepare any fraction exhibiting heparin-cofactor activity which did not also show antithrombin activity.

The exact relationship between activity and lipoprotein content of the fractions has not been determined in this series of experiments. It has been observed, however, that the concentrates of antithrombin and heparin-cofactor consistently have a high lipid content. In one experiment an antithrombin concentrate was adjusted to a specific gravity of 1.063 and then centrifuged for 24 hours at 100,000 g. This resulted in sedimentation of both antithrombin and heparin-cofactor and a similar concentration of their activities. This would indicate that if the active material is associated with lipid it is most likely in the form of a high density lipoprotein.

Reactions of a Blood Trans-α(1 ⟶ 4)-Glucosylase. Miller, K. D. and Copeland, W. H., Division of Laboratories and Research, New York State Department of Health, Albany.

A highly purified enzyme preparation was obtained from bovine plasma which specifically attacks terminal α (1 ⟶ 4) linkages between D-glucopyranose residues of the amylosaccharides, maltose, amylose, amylodextrin, and glycogen. Glucose is the common end-product

produced from these substrates. With maltose as substrate oligosaccharide synthesis also occurs. Products of the enzyme maltose reaction, when quantitatively separated and analyzed, demonstrate one mole of glucose formed from two moles of maltose. One of the synthetic oligosaccharides isolated was identified as amylotriose. Therefore it is indicated that maltose acts as both donor and acceptor substrate and that the enzyme functions by a transglucosylation process.

The enzyme does not alter diglucose compounds containing the α (1 → 6) link (isomaltose), the β (1 → 4) link (cellobiose), the β (1 → 6) link (gentiobiose), and the α, α[1] (trehalose). Neither does it affect glucose-1-phosphate or the α- and β-methylglucosides. That terminal linkages of amylopolysaccharides are attacked is indicated not only by the production of glucose as the sole reducing product but also by failure of the enzyme to alter the cyclic Schardinger α- and β-dextrins.

Properties of the enzyme reactions were studied in order to establish sensitive assay procedures. Both a high molecular weight substrate (amylose) and a low molecular weight substrate (maltose) were employed. Maximum activity on amylose was achieved at pH 7.2, in 1 per cent amylose solution which was 0.05 *M* with respect to Ca^{++}. Under these conditions the initial reaction velocity is directly related to the enzyme concentration. This enzyme-amylose reaction is insensitive to the type of anion or the changes in ionic strength as adjusted with NaCl.

The enzyme-maltose reaction was studied manometrically by superimposing on it the glucose oxidase system, thus measuring the glucose liberated specifically. At pH 6.0 in 0.015 *M* maltose the initial reaction velocity was directly related to the enzyme concentration. The Ca^{++} concentration had no effect on this reaction. *Michaelis* constant for the maltose reactions is 0.0022 *M*.

Products of the enzyme-amylose reaction are different when ionic calcium is present and absent. In absence of Ca^{++} glucose is the only reducing end-product while a homologous series of compounds are split from the polysaccharide with Ca^{++} present. Since Ca^{++} does not influence the enzyme-maltose reaction, the calcium is considered to reduce the high specificity of the enzyme for terminal α (1 → 4) glucosidic linkages, allowing it to couple at other sites on the substrate. Such a mechanism for Ca^{++} action may prove valuable in understanding the function of this action in many biologic systems.

In Vitro, Animal and Clinical Studies on Lyophilized Platelet Material. Djerassi, I., Farber, S. and Klein, E., From The Children's Cancer Research Foundation and The Tumor Therapy Group of the Division of Laboratories and Research, The Children's Medical Center and the Department of Pathology, Harvard Medical School.

Lyophilized platelet material has been shown to correct increased vascular fragility and to promote hemostasis in patients with secondary thrombocytopenia due to acute leukemia and aplastic anemia. These effects were not associated with an increase of the peripheral platelet count. This suggested that platelet compounds could contribute to vascular integrity in the absence of intact platelets.

A number of fractions have been obtained from lyophilized platelet material. The differences in the physical chemical properties of the various fractions have been demonstrated by free-boundary electrophoresis and in the analytical ultracentrifuge. Two of these preparations contained apparently protein-bound carbohydrate. Various activities, such as antithrombin, antiheparin and antihyaluronidase have been related to different fractions.

Lyophilized platelet material and products of its fractionation have been investigated in regard to vascular fragility and hemostasis in thrombocytopenic animals.

Materials with some of the chemical characteristics and biological activities of platelet preparations have been obtained from biological sources other than platelets.

This investigation was supported by the Atomic Energy Research Contract AT (30—1) 1275 and Grant # C-937 from the National Cancer Institute.

Genesis of Prothrombin Activity by Cellular Constituents. Barnhart, Marion I., Wayne State University, Detroit.

Recently there have been two independent reports that mitochondria have the ability to generate prothrombin activity. The present study on genesis of prothrombin activity represents

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an attempt to define the chemical entity responsible for activity of the mitochondria. conditions were established for obtaining high yields of prothrombin activity from an 'inactive' prothrombin preparation through use of mitochondrial systems. Moreover, it was observed that cathepsin, a group cellular proteinases, possessed the power of regenerating prothrombin activity. Preparations containing all of the cellular cathepsins as well as preparations more potent in cathepsin B and C were utilized. Cathepsin B, which is largely localized on mitochondria, appeared to be the most effective proteinase in genesis of prothrombin activity in solutions of pH 7.5 to 8.1, the range for similar activity with mitochondrial preparations. When acid conditions prevailed, mitochondria and cathepsin accelerated destruction of prothrombin activity. Thus, the ability of cathepsin concentrates and mitochondria to inactivate prothrombin or to generate the activity from 'inactive' prothrombin depends on the conditions selected. Since parallel results were obtained with mitochondria and with cathepsin in these protein reactions, it is possible that cathepsin of the mitochondrion is the active agent in generation of prothrombin activity from a precursor.

Lipids in Blood Clotting. Sarkar, N. K., Chaterjee, G. and Banerjee, R., Physical Chemistry Department, University of Calcutta and All India Institute of Biochemistry and Experimental Medicine, Calcutta.

Oxalated rabbit plasma coagulates when Russell's viper (RV) venom and Ca^{++} are simultaneously added to it but loses this property after successive treatments with petroleum ether (b.p. 40—60 ° C). This lipid-free (LF) plasma can be coagulated in the presence of brain or lung thromboplastin, because both of the materials contain a sufficient amount of lipid; thus the addition of either one of these agents is equivalent to the addition of the protein and lipid parts of the active thromboplastin molecule. RV venom is free of lipids and by itself cannot coagulate the LF-plasma.

In studying the nature of the lipid part of the thromboplastin molecule, RV venom was used as the protein portion of the thromboplastin molecule. The lipid part was replaced by purified phospholipids obtained from ox-brain, beef heart, egg yolk, etc. in our reconstituted system.

Among the phospholipids studied, cephalin but not lecithin, was found to induce thromboplastic activity in RV venom. Phosphatidic acid (cardiolipin) can to a great extent replace cephalin. Among the fractions of cephalin prepared according to the Folch procedure phosphatidyl ethanolamine and inositol containing phosphatide were most active. Phosphatidyl serine was a poor activator while fractions II and IV were more active than phosphatidyl serine; they were far less active than phosphatidyl ethanolamine.

Chargaff's purified lung thromboplastin, even after heating at 70—75 ° C for 30 minutes, was found to coagulate the LF-plasma in the presence of RV venom and Ca^{++}. Similarly, platelets either heated (100° C for 30 min.) or unheated can coagulate the LF-plasma under identical conditions. These experiments clearly demonstrate the importance of lipids in blood coagulation.

Experiments with synthetic glycerides showed that only triglycerides can replace cephalin in inducing thromboplastic activity in RV venom. Diglycerides were extremely poor activators and monoglycerides were completely inactive.

The Preparation and Activation of Human Plasminogen. Sgouris, J. T., McCall, K. B. and Inman, J. K., Division of Laboratries, Michigan Department of Health, Lansing.

We have fractionated seven lots of dried Fraction III by the procedure developed by *Kline* (*J. Biol. Chem. 204:* 949 [1953]) and found that the potency of the final preparations varied from 12.6 to 26.6 proteolytic units (P.U.'s)/mg. N by the caseinolytic assay of *Remmert* and *Cohen* (*J. Biol. Chem. 181:* 431 [1949]). These values are considerably lower than the value of 60 P.U.'s/mg. N reported by *Kline*. In order to determine if this was due to the use of aged Fraction III, we prepared a dried Fraction III from relatively fresh, frozen Fraction II + III paste. Upon subsequent fractionation this material yielded plasminogen with a potency of 35.5 P.U.'s/mg. N. Dried Fraction III-3 was also investigated as a source of plasminogen. It was prepared by Method 9 of *Oncley, et al.* (*J. Am. Chem. Soc. 71:* 541 [1949]), with the

omission of the final lysis step. This fraction proved to be an excellent starting material for the *Kline* procedure. In this case, the final product had a potency of 80—90 P.U.'s/mg. N

Plasminogen prepared by the *Kline* procedure from dried Fraction III was heated for ten hours at 60° C. at pH 3.5 in order to destroy possible viral contaminants. Losses in activity of less than five per cent were observed.

We have investigated the conversion of plasminogen to the active component of the fibrinolytic system (plasmin) by the following means: (1) spontaneous activation, (2) use of a placental activator, (3) streptokinase activation and (4) urokinase activation. Spontaneous activation by the method of Alkjaersig and Sherry did not occur with all preparations. Plasminogen which had been heated for ten hours at 60° C. did not show any spontaneous activation even in the presence of small amounts of plasmin. We consider the use of streptokinase undesirable because of its pyrogenic and antigenic properties. Human urokinase does not present these disadvantages. In fact it is feasible to consider the value of a final plasmin product containing an activator. We have prepared a potent, non-pyrogenic urokinase, free of direct proteolytic activity, from human urine by a method involving clarification, isoelectric precipitation and barium sulfate adsorption (129th National Meeting of the American Chemical Society, Dallas, Texas, April 1956). The activation of plasminogen by urokinase was studied at 0 to + 2° C. in the absence of casein and at pH 7.4. The proteolytic activity resulting from streptokinase activation was used as an index of maximal plasmin activity. This material has a relative potency of 1/17 that of streptokinase. These studies demonstrated the enzymatic nature of urokinase. Urokinase is labile below pH 5.0 with an optimal stability between pH 5.0 and 10.0.

This work was done at the request of the Director of the American National Red Cross Blood Program under an agreement between the American National Red Cross and the Michigan Department of Health Laboratories.

Studies on the Activation of Human Plasminogen (Profibrinolysin). Alkjaersig, Norma and Sherry, Sol, The Jewish Hospital and Washington University, St. Louis.

Several different types of activation of human plasminogen were investigated.

Spontaneous activation. Purified human plasminogen preparations, devoid of significant amounts of antiplasmin, will spontaneously activate to completion, without significant loss of enzyme activity, in the presence of 50% glycerol. The activation appears to be autocatalytic in nature. Temperature, ionic strength, metallic ions, and pH affect the activation rate. Plasmin preparations of high purity and free of activators have been obtained by this procedure.

Trypsin activation. Studies of the kinetics of trypsin activation of plasminogen confirm the catalytic nature of the activation. The activation is relatively slow and is non-competitively inhibited by benzoyl arginine methyl ester. These observations suggest that the affinity of trypsin for plasminogen is of a relatively low order.

Urokinase activation. Urokinase activates plasminogen rapidly and catalytically. Highly purified urokinase digests casein, and arginine and lysine esters. Since the synthetic substrates competitively inhibit the urokinase activation of plasminogen, the esterase activity appears to be an integral part of the activating function.

Streptokinase activation. Recent studies have indicated that the streptokinase activation of plasminogen is a two-step reaction: 1) streptokinase interacts with a plasma factor to form an activator, and 2) the activator catalytically converts plasminogen to plasmin. The catalytic nature of this activation is apparent only when streptokinase is removed from the system prior to assay. The isolated plasmin has similar properties to spontaneously activated plasmin. The activator formed from streptokinase and the human plasma factor (probably plasminogen itself) appears to be a proteolytic enzyme with lysine and arginine esterase activity. Competitive inhibition of the streptokinase activation of plasminogen by arginine and lysine esters, supports this view.

During the activations studied 25—30% of the plasminogen nitrogen becomes TCA soluble. Furthermore in each instance the activation appears to be accomplished by an enzyme capable of digesting casein and the synthetic substrates, suggesting that the conversion from plasminogen to plasmin involves a proteolytic step.

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Physicochemical Studies on Profibrinolysin (Plasminogen) and Fibrinolysin (Plasmin). Shulman, Sidney, Alkjaersig, Norma and Sherry, Sol, Department of Bacteriology and Immunology, University of Buffalo School of Medicine, Buffalo, and Jewish Hospital, St. Louis, Missouri.

A number of preparations of human plasminogen and plasmin have been studied by means of ultracentrifugal and electrophoretic analyses. The plaminogen was prepared from Fraction III by the *Kline* procedure and was activated either spontaneously or by streptokinase.

Examination in the ultracentrifuge showed the plasminogen to be quite homogeneous, with a sharp main peak and a small, broad, slightly faster component. A plot of sedimentation constant versus concentration (in g./dl.) for the main component in 17 runs, using 3 different preparations, gave a line with the equation, $s_{20, 10} = 4.28\ S - 0.4\ c$. The spontaneously-active plasmin showed a similar sedimentation pattern. A plot for 7 runs, using 2 different preparations, gave the line, $s_{20, 10} = 3.56\ S - 0.7\ c$. Several plasmin samples showed a very slow peak with a sedimentation rate approximately 1 S. This component was never seen in plasminogen.

The diffusion constant for plasminogen was determined to be 2.96×10^{-7} cm^2 sec.$^{-1}$. The intrinsic viscosity was also measured, resulting in a value of 0.070 dl./g.

Examination by electrophoresis revealed the plasminogen to consist of a pair of very poorly resolved boundaries, while the spontaneously-active plasmin seemed quite homogeneous. These observations were made in glycine buffer, ionic strength 0.10, pH 2.2. A series of runs at various pH values between 2.2 and 4.2 have been made in order to establish the plot of mobility versus pH and to locate the isoelectric point of plasmin. Difficulties are encountered in the isoelectric region because of the very low solubility of the material but a value of approximately 7 can be tentatively established by extrapolation.

The content of tyrosine, tryptophan, and carbohydrate have also been determined in both plasminogen and plasmin, and will be reported and discussed.