Accelerator-Globulin and Prothrombin: Preparation of Accelerator-globulin Free Plasma. (17799)

JOHN R. CARTER AND E. D. WARNER.

From the Department of Pathology, College of Medicine, State University of Iowa, Iowa City.

In recent years, considerable clarification of the conversion phase of the blood clotting mechanism has resulted from the introduction of the concept of a "convertibility" factor (1-3), and more recently, from the conclusive demonstration of the existence of an accessory clotting factor which accelerates the conversion of prothrombin to thrombin(4-7). Many discrepancies which have been observed in different species, under slightly modified experimental conditions, and with different methods of quantitative prothrombin estimation, have been reconciled, in part, by an understanding of the role of the accelerator factor in the coagulation of blood.

The present investigation was undertaken to elucidate further the qualitative and quantitative nature of the accelerator factor. Plasma was subjected to the action of chemical, physical, and enzymatic agents. With such agents, denaturation, adsorption, precipitation, and inhibition phenomena were studied and evaluated with respect to the activity of the accelerator factor. During the course of our studies, a method was found by which accelerator-globulin could be removed from bovine plasma without concomitant removal of the prothrombin. This has provided an accelerator-globulin-free solution of plasma prothrombin which has proved very useful as a reagent for studies of the clotting mechanism.

Methods. Throughout this work, the method of Ware and Seegers(8) for the assay of accelerator-globulin activity was used to check our results.* The careful studies of Flynn and Standley(9) have led them to conclude that all of the "accessory factors" described manifest virtually the same effect, and are probably one and the same. However, since we have used the techniques of Ware and Seegers and compared our results only with theirs, we feel justified in this paper in referring to the accelerator factor as Ac-globulin. Prothrombin concentrations were determined by the modified two-stage technic (10-11). Purified thromboplastin, prepared after the method of Chargaff *et al.*(12), slightly modified by Layton,† and centrifuged at 5000 r.p.m. to remove the particulate material, was used. This product is stable, gives consistent, reproducible results, and does not manifest Ac-globulin activity. Armour bovine fibrinogen was used exclusively. Our data indicate that it is free of Ac-globulin.

Results. The data obtained when oxalated bovine plasma was subjected to the action of agents which involved basic chemical, physi-

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* We are indebted to these investigators for the generous supply of their purified prothrombin.

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cal, and enzymatic reactions, showed great variations in the response of Ac-globulin and prothrombin to such agents. Agents used included ultraviolet light; buffer solutions over a wide range of pH values; denaturing reagents such as urea, alcohol, detergents and phenol; adsorbents such as salts of barium and phosphorus, and silicon compounds; a variety of salts of heavy metals; and proteolytic enzymes such as trypsin, pepsin, and papain. Variable degrees of destruction occurred, and with most of the agents, a decrease in Ac-globulin activity did not occur without comparable loss of prothrombin. With some agents, the prothrombin, although only moderately reduced quantitatively, was so altered qualitatively that its reactivity with respect to the conversion phase of clotting was unpredictable. A few agents were effective in reducing prothrombin concentration without greatly altering Ac-globulin activity. During the course of these investigations, it was recognized that a plasma, containing prothrombin, but free from Ac-globulin would be very useful in the study of certain components of the clotting system, and particularly, as a source of prothrombin in the assay of Ac-globulin. None of the agents tested were sufficiently selective to quantitatively remove either Ac-globulin or prothrombin without effecting some concomitant loss of the other.

Method. Because of the complexity of the variables in the clotting system, the method we have used for preparing Ac-globulin free plasma is a compromise and a balance of many factors, and does entail some loss of prothrombin.

1. To each 100 ml of oxalated beef plasma, add 17 g of kaolin (NF powder); mix thoroughly, and immediately place kaolin-plasma at –20°C to –60°C; swirl every few minutes until completely solidified.

2. Store kaolin-plasma at –20°C to –60°C for 24-48 hours.

3. Thaw out the kaolin plasma at room temperature, swirl once or twice, and centrifuge for 15 minutes at 5000 r.p.m. at 5°C.

4. Decant the plasma, and to it add slowly, with constant stirring, an equal volume of 1.5% $HgCl_2$ solution. Do not centrifuge.

5. To 100 ml of the $HgCl_2$-plasma add immediately 20 g of Bacto-Egg Albumin, Coagulated.‡ The egg albumin is first moistened by the addition of water, followed by centrifugation and decantation.

6. Stir mixture gently for 5 minutes.

7. Centrifuge the mixture for 30 minutes at 5000 r.p.m. at 5°C.

8. Decant supernatant; again add moistened coagulated egg albumin in a concentration of 20 g per 100 ml, and stir as before.

9. Centrifuge as in step 7. Decant and filter (filter paper) supernatant.

10. To approximately 1 ml of the final product, add a small crystal of $SnCl_2$. If a white milky precipitate results, the mercuric ions have been removed, if the precipitate is black, mercuric ions are present, and further treatment with coagulated egg albumin is necessary.

11. Put 1.2-1.3 ml amounts of $HgCl_2$-plasma in separate capillary tubes, and store at –20°C to –60°C. Use a fresh tube for each Ac-globulin determination. Do not use until the $HgCl_2$-plasma has been frozen for at least 1 hour.

12. Fibrinogen, small amounts of which may be present in the final preparation, precipitates on thawing, and may be removed readily.

The product, which henceforth we shall refer to as $HgCl_2$-plasma, has a prothrombin concentration of 50-60 units per ml depending upon the initial prothrombin concentration of the native beef plasma. The $HgCl_2$-plasma has a pH 7.2, no specific buffer having been added. The amount of prothrombin adsorbed by the kaolin is fairly constant, being approximately 46%. The prothrombin concentration of the kaolin treated plasma again is reduced by one-half because of the dilution effected by the addition of an equal volume of 1.5% $HgCl_2$ solution. The $HgCl_2$ precipitates an additional 2-3% prothrombin. Therefore the prothrombin concentration of the $HgCl_2$-plasma is approximately one-fourth that of the original beef plasma. Although the quantitative yield of prothrombin is only approximately one-half qualitatively it apparently is unaltered by the procedure. The

‡ Difco Laboratories, Detroit, Mich.

$HgCl_2$-plasma does not manifest Ac-globulin activity, and the antithrombin titer is extremely low. The prothrombin concentration of the $HgCl_2$-plasma when the latter is stored at −20°C to −60°C for 4 months remains unaltered.

In all prothrombin determinations of the $HgCl_2$-plasma, the modified two-stage method of assay was used(11). However, because no Ac-globulin is present in the $HgCl_2$-plasma, it is necessary to add a larger amount of beef serum (Ac-globulin) to the incubation mixture than is normally used. We have found that a 1-400 total dilution of beef serum insures complete conversion, but even with this concentration, the conversion rate is slower than it is in normal plasma.

The method of assay of Ac-globulin, as well as studies on the kinetics of the conversion phase of the clotting system, will be presented in detail in a subsequent paper. A modification of the 2-stage procedure(8) has been devised. It is sufficient to present here only the tenets on which the quantitative estimation is based. The amount of Ac-globulin in any given plasma is manifested as a function of 3 variables: (1) the time when prothrombin conversion begins, (2) the rate of prothrombin conversion, and (3) the yield of thrombin. Therefore a quantitative measure of Ac-globulin can be represented by an area which is circumscribed by the activation curve (clotting time plotted against incubation time) and by straight lines through a constant, arbitrarily selected point on each axis. This area can be determined by the square counting method, one of the quadrature methods, or more simply with a planimeter. The amount of Ac-globulin of an unknown is determined by comparing the area of the unknown with that of a normal control. By referring the area to a standard reference curve, the Ac-globulin activity can be expressed in percent of normal. The prothrombin concentration is adjusted so that only small quantities of plasma are required for the measurement of Ac-globulin, thereby greatly minimizing the effect of antithrombin on the conversion rate and thrombin yield. A concerted effort was made to devise a one-stage technic for the quantitative determination of Ac-globulin. The results of such an attempt were unsatisfactory because the three variables, noted above, could not be isolated, and no one variable could be interpreted as being specifically related to Ac-globulin activity.

Summary. The chemical and/or physical relationship between prothrombin and Ac-globulin apparently is very close as evidenced by the inability to denature, adsorb, precipitate or otherwise inactivate completely, and at times partially, the one, without completely or partially removing or inactivating the other. A method is described whereby oxalated bovine plasma may be rendered free from Ac-globulin, but not from prothrombin.

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Relation of Oxygen to Photoreactivation of Bacteria After Ultraviolet Radiation.* (17800)

F. H. JOHNSON, E. A. FLAGLER, AND H. F. BLUM.

From the Department of Biology, Princeton University, Princeton, N. J., and the National Cancer Institute, Bethesda, Md.

The loss of capacity for cell division resulting from exposure to ultraviolet radiation has been shown in some instances to be restored by subsequent illumination with visible light

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