A SIMPLE METHOD FOR PURIFYING AN ACTIVATOR OF PROTHROMBIN (ANTIHEMOPHILIC FACTOR?)

by

L. LORAND AND K. LAKI*

Department of Physiology and Pharmacology, College of Medicine, Wayne University, Detroit, Mich. and National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Md. (U.S.A.)

Several years ago, a joint effort of the authors[1,2] resulted in finding an apparently simple way of purifying an accelerator of the prothrombin-thrombin conversion believed to be lacking in hemophilia. The main feature of the procedure for preparing the activator is its adsorption on kaolin. The substance has so far been isolated from human, bovine, and canine blood collected with a variety of anticoagulants (citrate, oxalate, ethylenediaminetetra-acetic acid). It should be added that not only fresh plasma, but also plasma stored in the frozen state for some time (*c.* 2 months), may be used as a fairly adequate source for preparing the material. Details of the method of isolation run as follows:

Adsorption on kaolin. To 100 ml plasma 80 ml 0.9 % (w/v) NaCl and 20 ml pH 5.9, *M*/5 phosphate buffer are added, and the mixture is stirred for 15 minutes, preferably in the cold, with 5 g kaolin (Mallinckrodt, N.F. VI. Colloidal). The adsorption of the factor on kaolin is greatly dependent on the pH; thus for a fair yield the pH of the medium should be between pH 5.8 and 6.0. It is also useful to wash the kaolin first with a solution of 0.9 % (w/v) NaCl containing *M*/50 pH 5.9 phosphate buffer.

Elution from kaolin. After the adsorption step, the kaolin is separated by centrifugation and homogenized at room temperature for about 5 min with a mixture of 30 ml *M*/20 Na-tetraborate and 20 ml 0.9 % (w/v) NaCl. The kaolin is centrifuged and homogenized for a second time (15 min) with a 50 ml solution of 0.1 % (w/v) $(NH_4)_2CO_3$. The kaolin is then removed by centrifugation. The opaque supernatant contains the active principle. In our earlier work, borate was used for both elutions, but now we find that the weak solution of $(NH_4)_2CO_3$ is more suitable for the purpose, because during further steps of concentrating the material the salt can be volatilized. In course of the isolation an appreciable amount of active principle is lost to the first eluate (which also contains some inhibitors of the prothrombin-thrombin conversion), but when purity is preferred to yield only the second eluate is carried through further steps of fractionation. Should, however, yield and not purity of the product be the major consideration, the two eluates may be pooled at this stage. If this is required, $(NH_4)_2CO_3$ should be used also for the first elution.

Concentration of the eluate and fractionation with $(NH_4)_2SO_4$. The pH of the eluate is adjusted to about pH 7.4 by addition of *N*-HCl and the solution is concentrated by rapid lyophilization. The material is then dissolved in 5 ml distilled water and remaining kaolin particles are removed by high-speed centrifugation or filtration; thus a water-clear solution results. Practically all the activity of this solution can be obtained in the fraction precipitated by ammonium sulfate between 0.41 and 0.5 saturation. The precipitate is dissolved in 1 ml distilled water and the preparation is stored in the frozen state.

Preliminary experiments (in collaboration with Dr. W. R. CARROLL, National Institutes of Health, Bethesda, Md.) show that the final solution gives mainly one component in electrophoresis with a mobility resembling that of β_2-globulins; this component possessing the platelet co-factor[3] activity. The ultracentrifuge shows one major and two smaller peaks.

As judged by the platelet co-factor test, using a modification of the assay of JOHNSON *et al.*[3] (to be described later), the final product has more than two-hundred-fold activity per mg protein over that of the initial plasma. Since the material, obtained as above, acts jointly with platelets as an activator of purified prothrombin**, it behaves as the antihemophilic factor[4].

Some of our fractions were also tested directly on hemophilic blood**. Addition of the isolated

* Visiting Scientist of the National Institute of Arthritis and Metabolic Diseases.

** Thanks are due to Dr. W. H. SEEGERS (Wayne University, Detroit, Mich.) for a gift of purified prothrombin, to Dr. G. BRECHER (National Institutes of Health, Bethesda, Md.) for providing platelets, and to Dr. S. A. JOHNSON (Wayne University, Detroit, Mich.) for testing one of our preparations for platelet co-factor activity. We are also indebted to Drs. A. AXELROD, J. RUTZKY and E. DEUTSCH (Detroit, Mich.) for their help in assaying our fractions on hemophilic blood.

material to the patient's blood reduces not only the clotting time, but also normalizes the prothrombin consumption as measured by SOULIER's[5] procedure one hour after clotting. A similar normalizing effect is exerted when the factor is added to oxalated hemophilic plasma and clotting is initiated by recalcification. Though there is no doubt that our preparations possess appreciable antihemophilic activity, more rigorously quantitative tests will have to be employed to prove the identity of the isolated platelet co-factor with the antihemophilic principle. Such a research is in progress.

It is interesting to note that the platelet co-factor in the partially purified form precipitates between 0.4 and 0.5 ammonium sulfate saturation, and not around 0.33–0.36 saturation as in the case when the factor is isolated from plasma[3,6]. This could be explained in terms of co-precipitation of the factor with other plasma proteins or by assuming two manifestations of the factor.

Whether the platelet co-factor (antihemophilic principle?) will turn out to be a catalyst or a stoichiometric partner in the prothrombin-thrombin conversion, its amount will represent only a trace fraction of the totality of the plasma proteins. We feel that our method for isolating the factor can be included easily in the large-scale plasma fractionation schemes (alcohol, ether) as a starting operation. Indeed, in general, it may be worthwhile to consider the possibility of removing important trace proteins from plasma by adsorbents (such as *e.g.* the platelet co-factor by kaolin, an antithrombin by $Al(OH)_3$[7], etc.), and then start with the precipitation techniques for preparing larger fractions.

REFERENCES

1 K. LAKI AND L. LORAND, *Hung. Acta Physiol.*, 2 (1949) 12.
2 L. LORAND, *J. Hung. Med. Ass. (Orvosi Hetilap)*, 8 (1949) 230.
3 S. A. JOHNSON, W. M. SMATHERS AND C. L. SCHNEIDER, *Am. J. Physiol.*, 170 (1952) 631.
4 K. M. BRINKHOUS, *Blood clotting and allied problems*, Trans. First Conf., New York. Josiah Macy, Jr., Foundation, p. 39 (1948).
5 J. P. SOULIER, *Ann. Biol. Clin.*, 7 (1949) 305.
6 K. LAKI, *Studies Inst. Med. Chem. Univ. Szeged*, 3 (1943) 5; *Schweiz. Med. Wschr.*, 74 (1944) 13.
7 K. LAKI AND L. LORAND, *Experientia*, 2 (1946) 412.

Received January 18th, 1954

EFFECT OF MELANOPHORE-DISPERSING HORMONE ("B") ON LACTATING MAMMARY GLAND SLICES *IN VITRO*

by

T. R. BRADLEY AND S. J. FOLLEY

National Institute for Research in Dairying, University of Reading (England)

AND

F. W. LANDGREBE AND G. M. MITCHELL

Welsh National School of Medicine, Cardiff (Wales)

If mammary gland slices from lactating rats are incubated in the Warburg apparatus with acetate + glucose dissolved in KREBS-bicarbonate-saline in equilibrium with 95% O_2 + 5% CO_2, the overall pressure curve (composite curve) has a positive slope because the R.Q. is greater than 1[1]. BALMAIN AND FOLLEY[2] showed that addition of prolactin to the medium increased the slope of the composite curve of mammary gland slices taken from rats in early lactation. We have confirmed this with eight prolactin preparations of potency varying from 11–22 i.u./mg and have shown that the effect can be obtained with tissue from rats at all stages of lactation but not in late pregnancy or after weaning. The response was also given by mammary gland slices from lactating mice but not from guinea pigs, rabbits or sheep in early lactation. Liver slices from lactating rats gave no response to prolactin. However, the threshold concentrations were always rather high, the lowest being about 25 μg/ml and moreover there was no relation between prolactin potency and ability to increase the slope of the composite curve of rat mammary slices. These facts suggested that the metabolic effect might be due to some contaminant in the prolactin.