dissimilation of gluconic acid by the 3 cell preparations was similar to that of glucose. Acid gluconate-grown cells also oxidized 6-phosphogluconate and fructose 1,6-diphosphate in a pattern that varied from that of the other two cell preparations. The observed differences in the response of the cell preparations is not due to the pH of the growth medium alone, since cells grown on acidified (pH 4.0) glucose medium oxidized the various substrates in a manner similar to that of the neutral glucose-grown cells.

Summary. Endogenous oxygen consumption of *Serratia marcescens,* grown on a medium containing glucose at pH 4 and 7 or gluconate at pH 6, continues unchanged during oxidation of glucose and several other substrates. Correcting total oxygen consumption in the presence of a substrate for the endogenous oxygen consumption, affords an accurate representation of the course of oxidation of the substrates. *S. marcescens* grown in gluconate-containing medium at pH 4 has a higher endogenous oxygen consumption than cells grown on the other media. At one stage of the endogenous metabolism a sharp increase in rate of oxygen consumption occurs. Such sharp increase in oxygen consumption does not occur during oxidation of glucose and several other substrates. Hence correction of total oxygen consumption in the presence of the substrates for endogenous respiration of the cells may lead to an erroneous interpretation of the data.

1. Cochrane, V. W., and Gibbs, M., *J. Bacteriol.,* 1951, v67, 305.

2. Wilner, B., and Clifton, C. E., *ibid.,* 1954, v67, 571.

3. Doudoroff, M., *Enzymologia,* 1940, v9, 59.

4. Wiame, J. M., and Doudoroff, M., *J. Bacteriol.,* 1951, v62, 187.

5. Barker, H. A., *J. Cell. Comp. Physiol.,* 1936, v8, 231.

Received June 11, 1957. P.S.E.B.M., 1957, v95.

Inhibition of Prothrombin Activation in Sodium Citrate Solutions.* (23339)

WALTER H. SEEGERS AND RICARDO H. LANDABURU

Department of Physiology and Pharmacology, Wayne State University College of Medicine, Detroit, Mich.

In 25% sodium citrate solution purified prothrombin slowly transforms to thrombin (1). The discovery of this transformation cleared the view that prothrombin, as a single molecular entity, contains all of the structural material required in the thrombin molecule (2). The activation is autocatalytic and is initiated or accelerated by the addition of thrombin. This fundamental consideration alerts us to the possibility that prothrombin activation under physiological conditions may also be autocatalytic, that thrombin may be the only enzyme in prothrombin activation and that other factors are accessory.

The autocatalytic activation of purified prothrombin in sodium citrate solution can be accelerated with an organic compound of known structure such as 3-chloro-4,4'-diaminodiphenyl sulfone(1). By substituting an amino group for the chlorine atom and thus having 3,4,4'-triaminodiphenyl sulfone the autocatalytic activation is inhibited. In a similar way soybean trypsin inhibitor stops the autocatalytic process(3). We thus have a greatly simplified view of prothrombin activation that may actually be giving us an accurate concept of the minimal chemical requirements for prothrombin activation and its inhibition. The inhibitors 3,4,4'-triaminodiphenyl sulfone and soybean trypsin inhibitor are however not to be anticipated in plasma or serum but apparently no one has inquired whether other inhibitors of prothrombin activation in sodium citrate solution might be found in plasma or serum. With this idea in mind we conducted many experiments and

* This investigation was supported by research grant from N.I.H., U.S. Public Health Service.

have found that plasma and serum do indeed function as an inhibitor even if considerably diluted. At a 12-fold dilution of serum the autocatalytic transformation of prothrombin to thrombin can virtually be reduced to zero. To a certain extent this property of serum is adsorbed on barium carbonate.

Methods. Purified prothrombin was prepared from bovine plasma as described by Seegers and Alkjaersig(4). These prothrombin preparations were also used to obtain purified biothrombin by the methods described by Seegers and Alkjaersig(4). Prothrombin activity and thrombin activity were measured as previously described(5,6). Barium carbonate eluate is a preparation obtained from bovine serum as described by Seegers, Johnson, Fell and Alkerjaersig(7). In principle, barium carbonate is added to serum, removed by centrifugation, washed and mixed with a 5% sodium citrate solution. This elutes protein which can be fractionated with ammonium sulfate. After dialyzing the protein is obtained by drying from the frozen state. We suppose this preparation to be rich in Factor VII (SPCA, convertin, cothromboplastin, stable conversion factor, β-thromboplastin), platelet cofactor II (PTC, Christmas factor) and other factors. Whatever this preparation may contain it has only been characterized accurately by a description of its preparation and the specific conditions under which it exhibits activity. As previously, we use the term barium carbonate eluate when referring to this preparation. Bovine serum was shaken with an equal volume of ether for 3 minutes. The ether was removed and the process repeated twice. This removes much of the antithrombin activity.

Results. Our observations are considered in terms of 3 experiments all performed several times and with variations. In one of these a solution of purified prothrombin was mixed with a solution of thrombin so that the final concentration was 3,000 and 240 units/ml respectively. Immediately sodium citrate was added to give a 25% solution. At room temperature thrombin activity increased during a period of 12 hours with a final yield of about 66% of the potential thrombin that could theoretically be expected from the original prothrombin added (Fig. 1). The autocatalytic activation of prothrombin was typical of that previously reported.

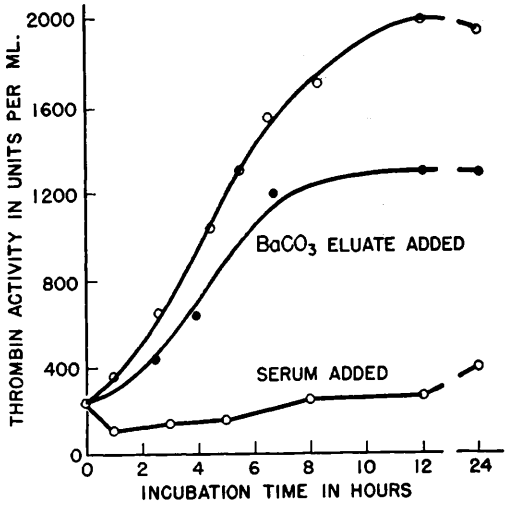

FIG. 1. Activation of purified prothrombin in 25% sodium citrate solution, and its inhibition with serum diluted 1:12 or barium carbonate eluate at a concentration of 2.5 mg/ml.

In a second experiment bovine serum was diluted 12-fold and the amount of prothrombin, thrombin, and sodium citrate added was the same as in the previous experiment. We observed that the thrombin concentration in the mixture actually dropped during the first hour but then increased slowly during the next 24 hours. At most about 10% of the prothrombin eventually was converted to thrombin (Fig. 1). Serum possesses inhibitory properties. We also found that serum adsorbed with barium carbonate, as in the preparation described for barium carbonate eluate(7) still possessed the power to inhibit the formation of thrombin. The same was observed when barium sulfate was used in place of barium carbonate, but in either case we made no attempt to see whether more barium carbonate or sulfate would diminish the power of serum as an inhibitor.

In a third experiment we wanted to see whether prothrombin activation would be retarded with material adsorbed on barium carbonate and eluted with sodium citrate. For this purpose prothrombin and thrombin were again mixed in 25% sodium citrate solution just as in the first 2 experiments. The dry barium carbonate eluate was then added to

give a concentration of 2.5 mg/ml. Prothrombin was not converted to thrombin quite as rapidly as without the barium carbonate eluate and the thrombin yield was only 43% (Fig. 1). The eluate was an inhibitor of the interactions. To obtain even less thrombin more of the barium carbonate eluate could be added. Similar properties were found in another eluate prepared with the use of barium sulfate instead of barium carbonate. Seitz filtration of the barium carbonate eluate did not destroy or remove the hypothetical inhibitor. As another variant in our experiments the barium carbonate eluate was fractionated. For this purpose it was dissolved in water. The pH was brought to 5.4 and a precipitate formed which was removed. The supernatant solution was further acidified to pH 4.6. Another precipitate formed just as occurs in the final fractionation steps in the purification of prothrombin(4). It was taken up in water, neutralized and dried from the frozen state. This dry precipitate used in the same quantities as the original barium carbonate eluate inhibited the interactions to the same extent as the original barium carbonate eluate. We further determined that an active barium carbonate eluate could be made from plasma just as well as from serum.

Discussion. The autocatalytic activation of prothrombin in 25% sodium citrate solution is not only inhibited with 3,4,4'-triaminodiphenyl sulfone or soybean trypsin inhibitor but also by plasma, serum and protein adsorbed on barium carbonate. We postulate that there is at least one inhibitor in plasma and serum that can account for our experimental results. Although we find the adsorbed serum still possesses the hypothetical substance(s) we do not know whether this is on the basis that more barium carbonate might adsorb all or whether the material not adsorbed is qualitatively different from that which is adsorbed.

In our purification of prothrombin the inhibitor(s) is evidently not concentrated with the prothrombin otherwise it would not be possible to obtain thrombin in 25% sodium citrate solution. During the purification there are several possibilities for the elimination of the inhibitor(s) including that adsorption on magnesium hydroxide may not occur. On the other hand it is likely that the adsorption on barium sulfate or barium carbonate in the purification of prothrombin and Factor VII as described for example in the work of Duckert, Koller and Matter(8) could carry the inhibitor through into the final product. Such prothrombin or Factor VII would then be "contaminated" and require different conditions for activation than more highly purified preparations or even preparations of low purity but not possessing the postulated inhibitor. Deutsch and Schaden(9) reported that their Factor VII preparation has not been converted to thrombin in 25% sodium citrate solution. The preparation was obtained by adsorbing the active material on barium sulfate and eluting with sodium citrate. These are the conditions under which we obtained our inhibitor. Probably Factor VII may after all be converted to thrombin in 25% sodium citrate when the conditions for its concentration exclude the inhibitor. This is even more likely in view of the fact that prothrombin can be obtained from Factor VII preparations (Ba adsorbed and citrate eluted) with the use of mitochondria (10). If a Factor VII preparation made with the use of barium adsorption can first yield prothrombin and then thrombin there must be some reason why it does not yield thrombin directly in 25% sodium citrate solution. Moreover the methods used in our purification of the prothrombin employed in this work have been applied for obtaining a prothrombin conversion accelerator from serum. This preparation *does* yield thrombin from a non-prothrombin precursor in 25% sodium citrate(11).

The significance of prothrombin activation in 25% sodium citrate solution may not be much appreciated if the view is taken that "the conditions are so unphysiological." However, this activation is the first unequivocal demonstration of the autocatalytic activation of prothrombin. In our work we are considering the possibility that all prothrombin activation is autocatalytic or stated in another way that thrombin is the only enzyme concerned with the activation of prothrombin whether this occurs in sodium citrate or any

other solutions.

Summary. Activation of purified prothrombin in 25% sodium citrate solution can be inhibited by use of a small amount of serum or plasma or barium carbonate eluate. It is postulated that plasma and serum contain an inhibitor(s) that can retard autocatalytic activation of prothrombin.

1. Seegers, W. H., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 677.

2. ———, *Advances in Enzymology,* 1955, v16, 23.

3. Alkjaersig, N., Deutsch, E., and Seegers, W. H., *Am. J. Physiol.,* 1955, v180, 367.

4. Seegers, W. H., and Alkjaersig, N., *Arch. Biochem. and Biophysics,* 1956, v61, 1.

5. Ware, A. G., and Seegers, W. H., *Am. J. Clin. Path.,* 1949, v19, 471.

6. Seegers, W. H., and Smith, H. P., *Am. J. Physiol.,* 1942, v137, 348.

7. Seegers, W. H., Johnson, S. A., Fell, C., and Alkjaersig, N., *ibid.,* 1954, v178, 1.

8. Duckert, F. D., Koller, F., and Matter, M., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 259.

9. Deutsch, E., and Schaden, W., *Biochem. Z.,* 1953, v324, 266.

10. Lasch, H. G., and Roka, L., *Hoppe-Seyler's Z. physiol. Chem.,* 1953, v294, 30.

11. McClaughry, R. I., *Am. J. Physiol.,* 1956, v186, 335.

Received June 10, 1957. P.S.E.B.M., 1957, v95.

Effect of CA^{++} and MG^{++} on Anticomplementary Sera in Complement-Fixation Tests. (23340)

GLADYS M. GNESH (Introduced by Gilbert Dalldorf)

Division of Laboratories and Research, N. Y. State Department of Health, Albany

The observation that mouse liver powder reduces anti-complementary (AC) activity of certain human sera(1) prompted a search for the active principle in the powder. Data have been obtained that indicate that Ca^{++} and Mg^{++}, both of which activate complement (2-8), may be involved.

Materials and methods. Since anticomplementary properties have been attributed to a relative increase in gamma globulin(9-15), 3 lots of commercial gamma globulin were used in the present study. Normal mouse liver powder was prepared and used as described previously(1). The chelating agent for removing Ca^{++} and Mg^{++} from liver powder was an isotonic aqueous solution of ethylene diamine tetra acetate (EDTA), pH 7.8(6). Calcium chloride and magnesium sulfate were used to prepare varying concentrations of Ca^{++} and Mg^{++}. All the reagents were diluted with 0.85% sodium chloride solution(16a). The technic for the complement-fixation test was unchanged(1). AC activity of gamma globulin was measured by its effects on one, two, and three 50% units of complement. According to our standards(1), 85% hemolysis with two 50% units of complement indicates AC activity. Titrations were also made to determine the effect of Ca^{++} and Mg^{++} on the 50% unit of complement(16b). Preliminary incubation for 24 hours at 3-6°C was followed by 30 minutes at 37°C for hemolysis.

Results. Fifty mg of mouse liver powder were shaken with 1-ml amounts each of distilled water, 0.85% sodium chloride solution, alcohol, ether, and acetone for one hour at room temperature to determine the solubility of the active principle. The mixtures were centrifuged, and the sediment and supernate of each dried in a vacuum desiccator over $CaCl_2$. Commercial gamma globulin solution was diluted 1:128 and 1 ml of this dilution, which was known to be AC, was shaken for one hour at room temperature with each of the dried materials, centrifuged, and tested for AC activity. The saline extract was the most effective in removing AC properties from the gamma globulin (Table I).

The active substance in the saline extract was dialyzable through a cellulose membrane.* Passage through an ion exchange

* Visking cellulose sausage casing, purchased from Visking Corp., Chicago, Ill.