CELLULAR SOURCE AND CHARACTER OF A HEAT-STABLE BACTERICIDAL PROPERTY ASSOCIATED WITH RABBIT AND RAT PLATELETS*

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Donaldson and Marcus (1) have recently demonstrated that a heat-stable bactericidal property of rabbit serum for *Bacillus subtilis* is formed during coagulation of blood. They observed that addition of sufficient heparin to inhibit blood coagulation resulted in a reduction of bactericidal effect of plasma, while equivalent amounts of heparin added to serum had no inhibitory effects. Hirsch (2) has shown that platelet-poor plasma has a reduced bactericidal activity and that restoration of platelets to such plasmas restores bactericidal activity. However, he was unable to demonstrate any bactericidal effect of saline suspension of platelets.

These data suggest that the property of serum which is bactericidal for *B. subtilis* is elaborated or activated at some point during the complex reaction of blood coagulation. The present investigation attempts to delineate the mechanism for the production of this bactericidal factor. Our data indicate that platelets contain two components which, together with bicarbonate ions, constitute a system that is lethal for *B. subtilis*. Both components are present in rabbit and rat platelets, but neither is present in normal human platelets. These two components are released into plasma by platelet disruption and are reponsible for the bactericidal effects of rat and rabbit sera.

Methods

Determination of the Bactericidal Strength of Sera or Platelets.—A stock culture of *B. subtilis* is inoculated into an infusion broth (Baltimore Biological Laboratory) containing 1.0 per cent dextrose. After incubation at 37°C. for 16 hours, 0.2 ml. of this culture are added to a number of 10 ml. volume tubes containing 5.0 ml. of a 1.0 per cent dextrose infusion broth. These tubes are stored at 4°C. and serve as stock cultures. In preparation for an experiment, the stock culture tube is incubated for 16 hours at 37°C. 0.5 ml. of the culture are then added to a tube containing 10 ml. of a 1.0 per cent dextrose infusion broth and the sample is incubated at 37°C. for 4 hours. The bacteria are then sedimented by centrifugation. After decanta-

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tion of the supernatant broth, the bacteria are resuspended in 2 ml. of a solution which contains three parts of a 0.85 per cent sodium chloride and one part of a 1.0 per cent dextrose infusion broth.

Tenfold dilutions of this bacterial suspension are made in the saline and infusion broth diluents. 0.1 ml. aliquots of each concentration of bacteria (bacterial count usually 30 to 50 $\times 10^6$ in the most concentrated and 300 to 500 in the most dilute sample) are then placed in a number of wells in a plastic disposable tray.[1] 0.1 ml. aliquots of the mixture to be tested are then added to each dilution of bacteria. After mixing the samples thoroughly by rocking the tray, incubation is carried out at 37°C. for 2 hours. At the end of the incubation period, a subculture of each sample is made by streaking a standard loop of the material (2 mm. in diameter) on nutrient agar. The subcultured mixtures are then incubated 16 to 24 hours at 37°C. and the end-point of the bactericidal effect is determined as the dilution of bacteria in which no growth occurs.

RESULTS

The data shown in Table I demonstrate the results obtained when rabbit serum was investigated for bactericidal activity by this method. Whole rabbit serum destroyed *B. subtilis* effectively until a high concentration of bacteria had been added. A progressive diminution of bactericidal effect occurred as the rabbit serum was diluted, indicating that a stoichiometric relation may exist between quantity of bacteria and the bactericidal factor.

Determination of Components 1 and 2.—Myrvik (3) has shown that the bactericidal property of serum for *B. subtilis* is dependent on two proteins, a fraction designated as component 1 which precipitates with 16 per cent ethanol, and a second fraction (component 2) which precipitates after the addition of 30 to 40 per cent ethanol. Neither component has bactericidal properties alone but when combined, a system that is lytic for *B. subtilis* results.

We have found component 2 in sheep, horse, bovine, rabbit, rat, and normal human sera. Component 1 is present in high concentration in rabbit and rat sera but is either absent or present in very low concentration in sheep, bovine, horse, and normal human sera. This fact permits one, after suitable dilution and combination of sera of different species, to estimate the concentration of each component. Thus, rabbit serum diluted to 1:60 has no bactericidal effect for *B. subtilis* as shown in Table II. If one adds a non-bactericidal aliquot of bovine serum to the diluted rabbit serum, a bactericidal effect is produced. When a 16 per cent ethanol precipitate of rabbit serum, which by itself is non-bactericidal as shown in Table II, is added to bovine serum, a bactericidal effect also results. It is evident that the diluted rabbit serum and the 16 per cent ethanol fraction supply a factor lacking in bovine serum.

When rabbit serum is serially diluted in bovine serum that has been diluted 1:3, the end-point of bactericidal effect occurs at a much higher dilution than when the rabbit serum is diluted in saline-bicarbonate diluent. This observation

[1] Disposo Trays obtained from Linbro Chemical Co., New Haven, Conn.

indicates that bovine serum supplies a component (component 2) which has been lost by dilution from the rabbit serum, when the latter has been diluted 1:60 in saline-bicarbonate diluent. Therefore, when one uses bovine serum as a

TABLE I

Dilution of Rabbit Serum

Increasing concentrations of *B. subtilis* were added to different dilutions of rabbit serum. A stoichiometric bactericidal effect is observed. Bacterial growth is graded from 0, indicating no growth to 4+ growth where no inhibition is present.

Inoculum	Undil.	1:10	1:20	1:40	1:60	1:80	1:100
33×10^6	4+	4+	4+	4+	4+	4+	4+
33×10^5	0	4+	4+	4+	4+	4+	4+
33×10^4	0	0	4+	4+	4+	4+	4+
33×10^3	0	0	10 col.	3+	3+	3+	3+
33×10^2	0	0	3 col.	29 col.	2+	2+	2+
33×10	0	0	0	0	16 col.	2+	2+

TABLE II

The bactericidal effect for *B. subtilis* with combinations of different sera is shown. The first column shows bovine serum (B.S.), the second rabbit serum (R.S.) diluted 1:60 and the third demonstrates the combination of these two sera. In column four a 16 per cent alcohol precipitate of rabbit serum, redissolved in NaCl-$NaHCO_3$ diluent shows little bactericidal activity. In column five this precipitate added to inactive bovine serum shows bactericidal activity. In the last column is shown the activity of the untreated rabbit serum from which the precipitate was derived. Bacterial growth is recorded as 0, no growth, to 4+ growth where no inhibition is found.

Inoculum	0.1 ml. B.S. 0.1 ml. NaCl 0.1 ml. bacteria	0.1 ml. R.S. diluted 1:60 0.1 ml. bacteria	0.1 ml. B. S. 0.1 ml. R.S. 0.1 ml. bacteria	0.1 ml. precipitate 0.1 ml. NaCl 0.1 ml. bacteria	0.1 ml. precipitate 0.1 ml. B.S. 0.1 ml. bacteria	0.1 ml. R.S. 0.1 ml. NaCl 0.1 ml. bacteria
50×10^6	4+	4+	4+	4+	4+	4+
50×10^5	4+	4+	3+	4+	0	0
50×10^4	4+	3+	17 col.	2+	0	0
50×10^3	4+	2+	0	2+	0	0
50×10^2	2+	2+	0	3 col.	0	0
50×10	1+	1+	0	0	0	0

diluent, a constant amount of component 2 is supplied to the system so that one can now determine the end-point of component 1 effect by dilution.

Conversely, if one employs a 1:60 dilution of rabbit serum as a diluent, one has a constant amount of component 1 which is still present in effective concentration, but component 2 has been diluted to the point of non-effectiveness. Non-bactericidal sera such as those obtained from sheep and human beings

can now be analyzed for concentration of component 2 by making progressive dilutions of these sera in the diluted rabbit serum. The concentration of component 2 is estimated at the dilution in which the loss of the bactericidal effect occurs.

TABLE III

Serum Dilution

The bactericidal activity of rat serum for *B. subtilis* added to saline, bovine and rabbit serum diluents is shown. None of these diluents had any bactericidal activity. The augmented effect of rat serum diluted in bovine serum indicates a high concentration of component 1 in rat serum.

Inoculum	Control	1:30	1:60	1:90	1:120	1:240	1:360	Test system
31×10^3	Saline 4+	0	0	6 col.	2+	3+	2+	Rat serum diluted in $NaCl$-$NaHCO_3$ solution.
31×10^3	Bovine serum 1:3 4+	0	0	0	0	0	2+	Rat serum diluted in 1:3 bovine serum.
31×10^3	Rabbit serum 1:60 4+	0	0	1 col.	3+	3+	3+	Rat serum diluted in 1:60 rabbit serum.

An analysis of rat serum which employs the above principle is shown in Table III.

In this experiment, constant amounts of bacteria were added to 0.1 ml. aliquots of rat serum diluted respectively in sodium chloride—sodium bicarbonate solution, in bovine serum previously diluted 1:3, and in rabbit serum previously diluted 1:60. (None of these diluents have any bactericidal effect for *B. subtilis*.) After incubation for 2 hours, subcultures of the serum and bacterial mixtures were made as described above and the end-point of bactericidal activity was determined.

It will be observed in Table III that the rat serum in the bicarbonate-saline solution had measureable bactericidal activity in a dilution of 1:60 but no activity at higher dilutions. However, when the bovine serum was employed as a diluent (to supply component 2), the bactericidal effect of the rat serum increased to a titre of 1:240. When the rat serum was diluted in a 1:60 dilution of rabbit serum, the same end-point of bactericidal effect was obtained as when bicarbonate-saline solution was used as the diluent. This indicates that the concentration of component 2 in rat serum was exhausted at a 1:60 dilution and that addition of the rabbit serum containing only component 1 in effective

concentration did not augment the bactericidal effect of the rat serum. The addition of the 1:3 dilution of bovine serum markedly enhanced the bactericidal effect of the rat serum by supplying component 2.

Effect of Coagulation on the Bactericidal Property of Rabbit Serum.—It is clear that some event occurs during blood coagulation which activates or releases a substance that is bactericidal for *B. subtilis*. When heparin is added to unclotted blood or when platelets are removed from plasma, these plasmas lose lytic activity for *B. subtilis*. Since Hirsch (2) could not find any substance in platelets which had bactericidal activity, attempts were made to determine

TABLE IV

The effect of blood coagulation on release of bactericidal effect is shown. Analysis of component 1 was made by diluting sera or plasmas in a 1:3 dilution of bovine serum. Component 2 was assayed by diluting sera or plasmas in a 1:60 dilution of rabbit serum. The titres shown in this table indicate the end point of bactericidal activity.

	Inhibition of serum or plasma	Concentration component 1	Concentration component 2
A. Prothrombin-deficient plasma spun 2900 R.P.M. (clotted)	<1:15	<1:15	<1:15
B. Serum from prothrombin deficient rabbits	1:60	>1:600	1:60
C. Prothrombin-deficient plasma spun 600 R.P.M. (clotted)	1:60	1:480	1:60
D. Normal rabbit serum	1:60	>1:600	1:60
E. Plasma A plus RBC phospholipid to accelerate coagulation	<1:15	<1:15	<1:15
F. Plasma A plus rabbit platelet phospholipid to accelerate coagulation	<1:15	<1:15	<1:15
G. Plasma A plus brain thromboplastin	<1:15	<1:15	<1:15

whether some chemical events may occur during coagulation which might release a bactericidal factor.

Male rabbits weighing 3 to 4 kg. were injected twice subcutaneously with 25 mg. of coumadin with an interval of 36 hours between injections. Twelve hours after the last injection, blood was withdrawn by cardiac puncture into cold, silicone-treated syringes. The blood samples were then transferred into chilled silicone-treated glass tubes and immediately centrifuged at 2900 R.P.M. for 20 minutes in a refrigerated centrifuge at 4°C. The plasma was carefully removed and kept at 4°C. until used in the bactericidal studies. The clotting time of these plasmas was prolonged to 6 to 12 hours when incubated at 37°C. In addition, aliquots of clotted whole blood and citrated blood were collected from the same rabbits. Citrated plasma samples were found to have a marked reduction of prothrombin.

Table IV shows that the lack of prothrombin had no effect on the bactericidal system, since the serum from the prothrombin-deficient rabbits (Table IV,

B) had the same concentration of components 1 and 2 as the serum of untreated animals (Table IV, D). It thus appears that prothrombin plays no role in the bactericidal activity of serum derived from clotted blood. However, the prothrombin-deficient plasma obtained by centrifugation at 2900 R.P.M. (Table IV, A) is deficient in both components 1 and 2, a finding which indicates that these components may be removed along with the platelets.

Experiments were also done in which highly bactericidal sera were adsorbed with $Al(OH)_3$ and $BaSO_4$ to determine whether some of the blood coagulation proteins which are known to be removed by these procedures, might effect the bactericidal property. No reduction of bactericidal effect resulted from such adsorption procedures.

There is a possibility that the loss in bactericidal activity caused by platelet deficiency is due simply to an interference with coagulation, due to a lack of coagulating factors present in the platelets. To test this possibility, a phospholipid active in clotting was prepared from red blood cells (Table IV, E) and from rabbit platelets[2] (Table IV, F). These preparations were added to the prothrombin and platelet-deficient plasmas. Both of these phospholipids accelerated coagulation but caused no release of bactericidal activity. The same experiment was then repeated except that platelet-deficient plasma was used. Identical results were obtained with platelet-deficient plasmas and with plasmas deficient both in prothrombin and platelets. A preparation of brain thromboplastin was similarly tested in these plasmas and this also failed to release any bactericidal activity even though a marked acceleration of clotting occurred.

A more penetrating investigation of the role of platelets in the formation of the bactericidal substance of serum was next undertaken.

Rabbit blood was collected by cardiac puncture as described above, using chilled, silicone-treated glassware. As an additional measure to prevent clotting, 3 ml. of a 1.5 per cent solution of dipotassium ethylenediaminetetraacetate (EDTA) was added to the rabbit blood. The blood was centrifuged at 900 R.P.M. for 20 minutes in a refrigerated centrifuge at 4°C. The supernatant plasma was carefully removed, placed in another centrifuge tube and again centrifuged at 2900 R.P.M. for 20 minutes. The plasma was decanted. The pellet of platelets deposited at the bottom of the tube was then resuspended in a 0.85 per cent saline solution and again was centrifuged at 2900 R.P.M. Several more washings of the platelets were made to remove any residual plasma components. Microscopic examination of the final platelet suspensions revealed many intact platelets, only a rare leukocyte or erthrocyte being observed Platelet counts, done on the saline suspensions diluted to the same volume as the original blood sample, ranged from 300,000 to 800,000 per cubic ml.

Certain samples of the saline suspension of platelets were ground in a Dounce glass homogenizer. In other experiments homogenates of platelets were prepared by disrupting the platelets in a Raytheon ultrasonic apparatus. Bactericidal studies of both types of platelet homogenates were then made.

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[2] Generally supplied by Dr. Claude Reed, Dept. of Medicine, University of Rochester School of Medicine, Rochester, New York.

In this work the experimental results described by Hirsch were confirmed, since intact platelets or platelet homogenates added to platelet-poor plasma caused these plasmas to develop an enhanced bactericidal effect, whereas platelet homogenates suspended in 0.85 per cent NaCl solution had no bactericidal activity.

The heat stabilities of bactericidal systems of platelets and serum were then investigated. When a platelet-poor plasma was heated at 56°C. for 30 minutes, the plasma was found to develop bactericidal activity after a platelet homogenate made with the homogenizer was added. Moreover, adding a platelet homogenate that itself had been heated to 56°C. for 30 minutes still caused a

TABLE V

Platelet homogenates were prepared and different solutions were added to the saline suspensions. It will be observed that the ultrafiltrates of bovine and rabbit serum supply an activating material. Bicarbonate but not calcium or pH effect seem to be critical for activation of the platelet bactericidal property. Bacterial growth is shown as 0 when no growth is obtained and as 4+ when no bactericidal effect is present.

Inoculum	Rabbit platelets in 0.85 per cent NaCl	Rabbit platelets in in 0.1 per cent $NaHCO_3$ and 0.95 per cent NaCl pH 8.5	Rabbit platelets in bovine serum ultrafiltrate	Rabbit platelets in rabbit serum ultrafiltrate	Rabbit platelets in 0.01 M NaOH at pH 8.5	Rabbit platelets in 0.02 M $CaCl_2$
30×10^6	4+	4+	4+	4+	4+	4+
30×10^5	4+	0	0	0	4+	4+
30×10^4	3+	0	0	0	4+	4+
30×10^3	2+	0	0	0	3+	1+
30×10^2	2+	0	0	0	11 col.	1+
30×10	8 col.	0	0	0	5 col.	0

restoration of the bactericidal activity of the plasma. These experiments indicated that both the plasma and the platelet components of the bactericidal system are stable to heating for 30 minutes at 56°C.

Myrvik has shown (3) that bicarbonate ions are necessary to reactivate the bactericidal property of dialyzed rabbit serum. It has also been shown that calcium ions (4) are required for activation of the bactericidal system of human serum. It is possible, therefore, that the inactivity of platelet homogenates relates to a lack of some cofactor present in serum.

In order to test this possibility, ultrafiltrates were prepared from bovine and rabbit sera and these were added to platelet homogenates prepared with the homogenizer. It will be observed that these ultrafiltrates caused the platelet homogenates to develop a marked bactericidal effect (Table V). A 1.0 per cent sodium bicarbonate solution had a similar activating effect, but the addition of calcium ions had no activating effect.

Since it seemed possible that the activating property of bicarbonate ions might be related to a pH effect, a number of buffers ranging in pH from 5.7 to 10.0 were tested for their ability to activate the bactericidal factors of platelets. None of the buffers showed any activating effect except an ammediol buffer of pH 9.0, with which the effect was not constant. Since it was observed that a freshly prepared ammediol buffer produced no activation of platelet homogenates while a stored buffer develops this property, it is likely that this buffer becomes contaminated with CO_2, and that its activating effect may therefore be related to bicarbonate ions rather than to pH or to a specific

TABLE VI

The supernatant solutions of platelets prepared by glass homogenization and by ultrasonic disintegration are compared. It will be seen that component 2 is not released into solution when platelets are not made nearly completely soluble.

Inoculum	Supernatant of glass-homogenized platelets (A) with $NaHCO_3$	Supernatant A added to bovine serum diluted 1:3	Supernatant A added to rabbit serum diluted 1:60	Supernatant of ultrasonic treated platelets with $NaHCO_3$
35×10^6	4+	4+	4+	4+
35×10^5	4+	2+	3+	1+
35×10^4	3+	0	3+	0
35×10^3	2+	0	2+	0
35×10^2	2+	0	1+	0
35×10	1+	0	12 col.	0

property of ammediol. When platelet homogenates prepared in the homogenizer were treated with dilute sodium hydroxide to bring the pH to 8.5, corresponding to the pH of the platelet homogenates suspended in bicarbonate solution (Table V), no bactericidal activity developed.

Homogenates of rat platelets were prepared in the same manner as has been described for the rabbit platelets. It was found that saline homogenates prepared in the homogenizer have no bactericidal activity, but the addition of bicarbonate produced an active bactericidal property as in the case of rabbit platelets. The rat platelets also resembled the rabbit platelets in that neither calcium ions nor adjustment of pH with sodium hydroxide had any activating effect on the homogenates.

Human platelets, prepared with the homogenizer in the same manner as described for rabbit platelets, were also examined for bactericidal activity. No activity was found in homogenates made in saline solution, nor was any bactericidal activity found after the addition of bicarbonate, serum ultrafiltrate or calcium ions. Sera, prepared from the same human subjects from which the platelets were obtained, also had no bactericidal activity.

In order to investigate the extractability of bactericidal factors from platelets,

rabbit platelets suspended in saline were disrupted in a Dounce homogenizer and then centrifuged to remove particulate material. A sizeable pellet was deposited after centrifugation. Examination of the supernatant solution revealed that no bactericidal activity was present (Table VI), but the sediment, when resuspended in a saline-bicarbonate solution, was strongly bactericidal. The inactive supernatant solution was added to a 1:3 dilution of bovine serum. It can be seen from Table VI that the latter mixture produced a bactericidal effect. However, when the supernatant solution from the homogenized rabbit platelets was added to a 1:60 dilution of rabbit serum, no bactericidal activity for *B. subtilis* was observed.

These data indicate that component 1 can be extracted from rabbit platelets under the conditions described above, while component 2 remains insoluble and is sedimented with the broken platelets. Platelet extracts obtained by ultrasonic treatment have a different property. High speed centrifugation of these homogenates causes but little sediment to be deposited, and the supernatant solution is fully bactericidal. This indicates that complete disruption of the platelets causes component 2 as well as component 1 to become soluble.

RECAPITULATION AND DISCUSSION

These experimental results identify platelets as the source of the bactericidin in rabbit and rat serum. The lack of bactericidal activity of platelet-poor plasma is thus accounted for.

The data reveal that platelets of the species mentioned contain two components which together with a bicarbonate cofactor, constitute a heat-stable, lytic system for *B. subtilis*. Neither calcium ions nor alterations of pH over the range of 5.7 to 10.0 appear to influence the activation of the two components either when they are present in platelet homogenates or in dialyzed sera. The stability of platelet homogenates on heating at 56°C. for 30 minutes, considered together with other properties of the platelet bactericidin, indicate that it is identical with the serum bactericidin. It thus appears that disruption of platelets during blood coagulation results in a release of both components 1 and 2 into the serum.

Identification of components 1 and 2 in platelet homogenates or in serum is simplified by employing a system of diluted serum of different species. Bovine serum has little or no bactericidal activity for *B. subtilis*. A 16 per cent alcohol precipitate obtained from rabbit serum, designated by Myrvik as component 1, also has no bactericidal effect, but a combination of these two materials produces a bactericidal effect. Thus component 2 of bovine serum, in conbination with the component 1 contained in the 16 per cent ethanol precipitate from rabbit serum, causes destuction of *B. subtilis*.

Rabbit sera which have a high concentration of component 1 can be made non-bactericidal in a dilution of 1:60. It is at this point that component 2 is

sufficiently diluted so that the bactericidal properties of the serum disappear. When an aliquot of non-bactericidal bovine serum and non-bactericidal, diluted rabbit serum are combined, a significant bactericidal effect results. Using similar systems, we have demonstrated that platelets obtained from rabbits and rats contain both components 1 and 2. Component 1 is easily soluble in saline extracts, whereas component 2 is insoluble in saline solution and remains attached to platelet fragments. When platelets are completely disintegrated by ultrasonic treatment, however, component 2 as well as component 1 becomes soluble.

There is little evidence that serum bactericidin for *B. subtilis* is directly related to any of the proteins which are important in blood coagulation. Factor V is heat-labile, whereas bactericidin is heat-stable. Normal rabbits and rabbits with marked hypoprothrombinemia have the same level of bactericidin in platelets and serum. Factor VII is heat-labile and can be adsorbed by $BaSO_4$ and $Al(OH)_3$, whereas serum bactericidin is not adsorbed by these salts. Phospholipids prepared from human erythrocytes and from rabbit platelets have no bactericidal activating effect when added to platelet-poor plasma even though they promote active thromboplastin generation. Tissue thromboplastin added to platelet-poor plasmas also has no bactericidal effect.

Several possibilities can be suggested to explain the presence of the bactericidal components in platelets. Components 1 and 2 may be synthesized in megakaryocytes and remain a part of platelet structure until the platelets undergo viscous metamorphosis during the early stages of blood coagulation. On the other hand, it is possible that components 1 and 2 are produced elsewhere in the body. It is known that platelets have adsorptive properties enabling them to adsorb not only a number of plasma proteins (5) but also less complex molecules such as serotonin (5-hydroxytryptamine) and histamine (6). It follows that components 1 and 2 might become adsorbed to platelets and be transported in this way in the circulation until the platelets are destroyed. Whatever the case, further work will be required to find the origin of the bactericidal components.

SUMMARY

Rabbit and rat platelets contain two non-dialyzable components (analogous to Myrvik's components 1 and 2 of serum) which, together with bicarbonate ions, produce a potent bactericidin for *B. subtilis*. These platelet factors appear to be identical with the serum bactericidal substances, suggesting that disruption of platelets during blood coagulation releases the platelet factors into serum. Normal human platelets contain no measurable component 1 or 2.

Since bovine serum contains component 2 but little or no component 1, estimation of the concentration of component 1 in sera or platelet suspensions of other species can be made by diluting the materials to be tested in bovine

serum. The level at which a bactericidal effect is lost represents the end-point of the titration of component 1.

Rabbit serum has a high concentration of component 1 and a lower concentration of component 2. It can be diluted to a point at which bactericidal activity is lost, owing to a depletion of component 2, but at which component 1 is still present in effective concentration. At this point the addition of another serum or a platelet homogenate causes a restoration of the bactericidal activity and permits an estimate of the concentration of component 2 in the added material.

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