# INFLUENCE OF THE PHYSIOLOGICAL BLOOD CLOTTING PROCESS ON THE COAGULATION OF BLOOD BY STAPHYLOCOAGULASE

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A significant advance in the knowledge of the mechanism of staphylocoagulase action was the discovery by Smith and Hale (1) of a factor in plasma and in tissues with which the coagulase reacts. This agent was named "activator" by the British investigators, and the "coagulase-reacting factor," or "C.R.F." by others (2–4). Evidence has been presented that C.R.F. is distinct from any of the blood components known at present to be essential for physiological clotting (2, 3). Separation from prothrombin has been accomplished by Seitz filtration, and C.R.F. has been identified with the globulin moiety of plasma (3). Contrary claims, linking C.R.F. with albumin (5) and fraction IV-$_4$ of Cohn (6) resulted from the use of too weak coagulase preparations which permitted a coagulase inhibitor to mask the C.R.F. activity of globulins, and from reliance on ammonium sulfate fractionation which fails to eliminate C.R.F. from albumin.

C.R.F. of plasma, titrated against a standardized coagulase preparation (7), remains constant for many months at ordinary ice box temperatures. It has often been observed (8), however, that the conversion of plasma to serum may lead to significant changes in the C.R.F. All previous standardizations, therefore, were based on the use of plasma rather than serum. It was further noted (8) that the strength of the coagulase was critical: thus, with highly active coagulase preparations, plasmas consistently had more C.R.F. than the derived serum. When weaker coagulase preparations were used, however, and the concentrated levels of plasma and serum tested, the clotting time of serum might actually be more rapid than that of plasma.

Recent unpublished observations with Dr. A. B. Stavitsky have indicated striking fluctuations in the C.R.F. of serum under certain experimental conditions. Rabbits were sensitized with a specific antigen, and at suitable time intervals sera and plasmas were collected after a challenge inoculation with the same antigen. Plasma C.R.F. titers remained constant, while striking changes were noted in the C.R.F. content of the sera. This suggested that either C.R.F. is actively influenced by the physiological clotting process, or that it is passively adsorbed on the fibrin clot. The recent report of Duthie and Lorenz (9) implicating calcium ions in the loss of C.R.F. in serum points to the former alternative, namely that the C.R.F. changes result from an active interaction with the agents concerned in physiological clotting.

The fact that the coagulase-reacting factor of plasma is actively influenced by physiological clotting assumes special interest in that it indicates some in-

teraction between the participants of two diverse clotting systems. Whether this reaction is fortuitous, or actually has some significance in physiological clotting, is at present undetermined. The object of this report is to elucidate the mechanism of C.R.F. loss when plasma is converted to serum.

## *Materials and Methods*

*Coagulase.*—*Staphylococcus aureus* No. 104 was grown in 200 ml. lots in 1 liter Roux bottles in a brain-heart infusion medium (Difco) modified by the addition of a mixture of ions (7). After 5 days at 37°C., the cells were removed by centrifugation, and the supernatant solution was acidified to a pH of 5.2, using 5 M acetic acid. The solution was chilled to 0°C., and cold absolute methyl alcohol was added to a final concentration of 20 per cent, maintaining the temperatures below +1°C. at all times. After forming in a cold bath at −5°C. for 12 to 18 hours, the precipitate was centrifuged in the cold, and was resuspended and partially dissolved in 0.15 M sodium acetate, followed by the cautious adjustment of the pH to 7.4 with 1 M NaOH. The insoluble precipitate was again centrifuged, and active coagulase eluted with further small increments of 0.15 M sodium acetate. The active supernatant solutions were pooled, and then diluted with an equal volume of distilled water. The pH was adjusted to 5.4 with 1 M acetic acid, and precipitation carried out with methyl alcohol to a final concentration of 17 per cent, observing the same temperature precautions as before. The precipitate was then handled as after the first alcohol treatment. If necessary, a third cycle of alcohol precipitation was carried out at a pH of 5.3 in 0.075 M sodium acetate buffer and at a concentration of methyl alcohol of 20 per cent. This procedure generally gave purification of about 125 times over the original cell-free culture supernatant, and entailed a loss of 50 per cent of the initial coagulase activity. Purification was determined by activity to nitrogen ratios. The coagulase, now a water-clear solution, was either lyophilized after dialysis, or maintained in the deep freeze unit at about −15°C. The use of methyl alcohol here is analogous to the procedures developed by Pillemer and his associates (10) for the purification of bacterial toxins. This method was preferred to a scheme of coagulase purification previously developed (11) because of its greater simplicity and because the significantly higher purification attained by the older method was not necessary for the present purposes. This coagulase clotted an equal volume of "non-inhibitory" plasma (7) in 20 seconds, or, when diluted 1:10,240, the coagulase still clotted an undiluted, non-inhibitory plasma in 24 hours. Reaction of this coagulase with an equal volume of fibrinogen and plasma diluted 1:4500 generally led to coagulation within 15 minutes. In the tests, the coagulase was generally diluted 1:4 or 1:5 before use.

*Thromboplastin.*—Prepared from acetone extracts of rabbit brain essentially as developed by Quick (12). 0.3 gm. of the powder was suspended in 10 ml. of veronal buffer (v.i.), heated at 50–55°C. for 10 minutes, and the supernatant material used.

*Veronal Buffer.*—0.1 M sodium diethylbarbiturate was adjusted to a pH of 7.05 with 0.1 M HCl, and 5.67 gm. of NaCl was added per liter of buffer. This solution was then diluted with an equal volume of physiological saline solution (13).

*Prothrombin.*—A purified product, lot 470509, containing 11,500 units per mg. of tyrosine, prepared from bovine plasma, and kindly supplied by Dr. Walter H. Seegers, was used in a concentration of 0.2 mg. per ml. of veronal buffer at a pH of 7.05.

*Factor V.*—In the main, the technique of Owren (13) was followed. 100 ml. of freshly drawn oxalated bovine plasma was freed of prothrombin activity by 5 Seitz filtrations. The plasma was then diluted with an equal volume of distilled water, and 100 ml. of saturated ammonium sulfate was added (70.6 gm. of ammonium sulfate plus 100 ml. of water at 0°C. was taken as saturation). After removal of the precipitate by centrifugation, a further incre-

ment of 100 ml. of saturated ammonium sulfate was added to the supernatant solution to make a solution saturated 50 per cent with respect to ammonium sulfate. The precipitate was centrifuged, dissolved in saline, and thoroughly dialyzed against distilled water. After dialysis, the solution in the casing was acidified to a pH of 5.3, and the precipitate which formed was collected by centrifugation and dissolved in 20 ml. of veronal buffer at pH 7.05 and preserved at −15° to −20°C. For use one part of this was diluted with 9 parts of veronal buffer.

*Fibrinogen.*—Armour's fraction I, bovine fibrinogen, was made up at a concentration of 250 mg. per cent in veronal buffer at a pH of 7.05.

*Buffer for C.R.F. Titrations of Diluted Postconversion Samples.*—Veronal buffer (see above) containing 0.005 M sodium citrate and 2 mg. of bovine albumin (Armour) per ml.

*General Method of Measuring Residual C.R.F. after Prothrombin Conversion.*—(For specific details and modification, see the individual protocols.)

The various materials employed, *e.g.* fresh plasma, thromboplastin, factor V, etc., were maintained in the deep freeze unit at −15°C. and fresh dilutions were made for each day's experiments. The preliminary incubation mixture consisted of the plasma and various added factors involved in prothrombin conversion made up to a constant volume of 1 ml. in veronal buffer. Controls, containing the same ingredients, but no added calcium, were set up in the same manner. Such clots as formed were shaken loose and the mixtures allowed to stand in the 37° bath for 1 to 2 hours, after which time thrombin had disappeared and no further change in residual C.R.F. levels could be detected.

The reacted samples were then further diluted in veronal-citrate-albumin buffer to give plasma or serum dilutions of 1:200, 1:400, and 1:1000. To 0.5 ml. portions of these mixtures, 0.5 ml. fibrinogen solution and 0.5 ml. coagulase were added, and the tubes incubated under constant observation at 37°C., the appearance of the first wisp of fibrin being taken as the C.R.F. clotting time. Thrombin controls, omitting coagulase, were always carried out simultaneously with the test.

## RESULTS

### *The Effect of Blood Coagulation on C.R.F.*

The object of this experiment was to follow the changes in C.R.F. as freshly drawn blood was allowed to clot.

Venous blood was collected with a siliconized[1] needle and syringe, and 2 ml. lots were distributed in a series of lusteroid tubes. The first of these tubes contained 0.2 ml. of 0.1 M sodium citrate and served as the plasma control. At various intervals of time, the same amount of citrate was added to the other tubes to stop further prothrombin conversion. The cells were removed by centrifugation immediately, and the supernatant solutions diluted with veronal buffer, in a ratio of 0.1 ml. of serum to 1.9 ml. of buffer. From these master dilutions, two further dilutions were made for the C.R.F. tests and for the prothrombin determinations.

(*a*) For C.R.F. determination, the master dilution was further diluted to a final level of 1:320 with veronal-citrate-albumin buffer, and then reacted with coagulase and fibrinogen using 0.5 ml. of each reagent in the final test mixture. The controls for thrombin were negative.

(*b*) For the prothrombin determination, the master dilution was further diluted 1:3 in the thromboplastin-calcium mixture. After 3 and 6 minutes respectively, 0.4 ml. of this sample was added to 0.1 ml. of fibrinogen in 10 × 75 mm. tubes, and the clotting times were measured.

[1] Silicone dri-film No. 9987 kindly supplied by General Electric Co.

The results are summarized in Table I. It will be noted that there is a progressive loss of C.R.F. as prothrombin is converted, so that the clotting time was four times longer in the sample citrated 1 hour after the blood was drawn than in the control sample. Other similar experiments using a variety of dilutions of serum were confirmatory of these findings. It was also found that heparin, like citrate, will prevent C.R.F. loss when added to the sample immediately.

TABLE I

*The Effect on C.R.F. Clotting Time of the Addition of Citrate at Various Time Intervals to Freshly Drawn Blood*

| Time elapsed before citrating | CRF clotting time | Prothrombin time (modified two stage) |
|---|---|---|
| *min.* | *min.* | *sec.** |
| 0 | 4½ | 27 |
| 5 | 4¾ | 31 |
| 10 | 5 | 37 |
| 15 | 5½ | 57 |
| 20 | 5½ | 95 |
| 30 | 8¾ | 352 |
| 40 | 12¼ | 704 |
| 60 | 19 | — |
| 120 | 19 | — |

* Average of 3 and 6 minute readings.

### *The Effect of Plasma Age, Factor V, and Calcium on C.R.F. Clotting Time after Plasma to Serum Conversion*

The age of the human plasmas used in this study varied considerably, since for the most part these were blood bank discards. It therefore seemed possible that the age of the plasmas might play a role in the variations encountered in C.R.F. activity from sample to sample upon the conversion of plasma to serum. This even seemed probable from the observation that the serum C.R.F. of freshly drawn blood was often considerably lower than that of older samples.

To test the possibility further, a freshly drawn sample of blood was citrated, the cells were removed by centrifugation, and the plasma divided into two portions: one of these was immediately placed in a deep freeze unit at about −15°C., while the other sample was maintained in the ice box at 5°C. The plasmas were kept for 3 weeks, and the sample maintained at −15° will be referred to as "fresh" plasma, while the specimen kept at 5° for 3 weeks will be called "old."

It has been shown that Owren's factor V may lose activity when stored at ordinary ice box temperatures (13). It therefore seemed possible that variations in the activity of factor V might be implicated in the C.F.R. loss upon the conversion of plasma to serum, and might therefore be involved in the differences of behavior of plasmas of varying age. Factor V was therefore prepared from fresh bovine plasma (see Methods) and its effect on the C.R.F. re-

sidual studied. Parallel titrations were carried out with and without the presence of added calcium in the prothrombin conversion mixture.

The results are summarized in Table II. A comparison of the C.R.F. clotting times of test A when calcium ions were added to the preliminary incubation mixture with the C.R.F. clotting times of control B in which calcium ions were omitted from the incubation mixture, immediately makes evident the essential nature of these ions for the subsequent C.R.F. loss. The hypothesis that the age of the plasma is involved was substantiated, since the serum derived from the aged plasma showed significantly greater C.R.F. activity than that from the fresh plasma (clotting time of 8 minutes *vs.* 17.5 minutes).

TABLE II

*The Effect of Plasma Age, Factor V, and Calcium on the C.R.F. Clotting Time after Plasma to Serum Conversion*

| Test No. | Prothrombin conversion incubation mixture* | | | | Residual C.R.F. clotting time at final plasma dilutions below: | | | |
|---|---|---|---|---|---|---|---|---|
| | Aged plasma | Fresh plasma | Factor V | Thrombo-plastin | Test A (with calcium)‡ | | Control B (no calcium) | |
| | | | | | 1:600 | 1:1200 | 1:600 | 1:1200 |
| | *ml.* | *ml.* | *ml.* | *ml.* | *min.* | *min.* | *min.* | *min.* |
| 1 | 0.1 | 0 | 0 | 0.1 | 8 | 12¾ | 3½ | 4¾ |
| 2 | 0.1 | 0 | 0.1 | 0.1 | 26½ | 41 | 3½ | 4½ |
| 3 | 0.1 | 0 | 0.2 | 0.1 | 31 | 53 | 3½ | 4¾ |
| 4 | 0.1 | 0 | 0.3 | 0.1 | 31 | 57 | 3½ | 4¾ |
| 5 | 0 | 0.1 | 0 | 0.1 | 17½ | 29 | 3½ | 4¾ |
| 6 | 0 | 0.1 | 0.2 | 0.1 | 37½ | 52½ | 3½ | 4¾ |

* All volumes made up to 1 ml. with veronal buffer.

‡ 0.05 ml. of 0.1 M $CaCl_2$ added to the prothrombin conversion incubation mixture.

Thrombin controls of A and B: no clot at end of 1 hour when coagulase omitted.

However, when factor V was added to the aged plasma before the conversion, the serum thus obtained had much less residual C.R.F. activity.

It is of interest to note that the variations in the components of the prothrombin conversion mixture were not of sufficient magnitude to produce any effect on the one stage prothrombin clotting time of these samples, which fell between 50 and 55 seconds. In spite of this, however, striking effects were produced on the residual C.R.F. after the plasma to serum conversion.

### *The Effect of the Preliminary Heating of Plasma and the Restoration of Factor V on the Residual C.R.F. after Plasma to Serum Conversion*

Since factor V is known to be more thermolabile than prothrombin (13, 14) or C.R.F. (3), advantage was taken of this property to determine the extent to which the C.R.F. residuals may be affected by heating fresh plasma before

its conversion to serum. The plasma was therefore heated at 51°C. for various times up to 30 minutes, a treatment which leaves C.R.F. and prothrombin activity virtually unimpaired. Although the C.R.F. titrations were carried out in the usual manner, the results have been expressed in terms of per cent activity by extrapolating arithmetically several clotting times. It will be noted (Chart 1) that heating the plasma for 15 to 30 minutes virtually abolished C.R.F. loss after conversion.

This system, further, lent itself to assaying quantitatively the sensitivity of the C.R.F. system to various amounts of factor V. One unit of factor V consisted of 0.005 ml. of the preparation. It is evident that the addition of 2 units of factor V resulted in a C.R.F. loss of over 80 per cent after conversion, while increasing factor V to 10 units resulted in a 95 per cent C.R.F. loss.

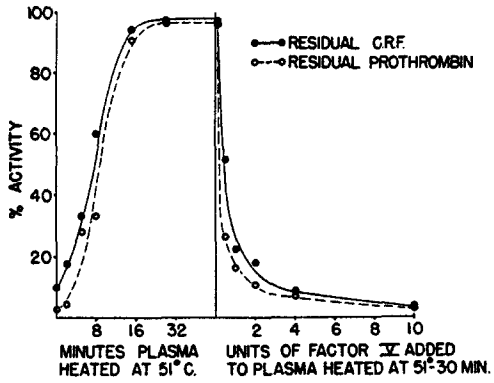


CHART 1. C.R.F. and prothrombin residual activity after heating plasma and restoring factor V.

Other titrations were carried out in which factor V was heated and then added to unheated and to heated plasma samples. The results were entirely confirmatory of the data here presented.

The prothrombin consumption studies indictated on Chart 1 will be discussed below.

### *The Relation of Thromboplastin to C.R.F. Loss after Plasma to Serum Conversion*

Since calcium is required for the C.R.F. loss during serum formation (9), and since factor V exerts a profound effect on this loss in the presence of an otherwise complete conversion system, it was of interest to investigate the role of thromboplastin in this reaction. A satisfactory test system for this purpose was furnished by a plasma which had been previously clotted with a large amount of thrombin (300 units per ml. of purified bovine thrombin[2]). Ware

[2] The purified thrombin was obtained through the courtesy of Dr. Walter H. Seegers.

and Seegers (15) have shown that such treatment of a plasma results in a loss of AC globulin (or factor V), while prothrombin is not similarly affected. Studies here have shown that the C.R.F. content is likewise unimpaired. The data of Table III show the effects of recalcifying such a sample with the addition of factor V and thromboplastin alone and in combination. The addition of thromboplastin alone (No. 4) caused no C.R.F. loss, since this sample was now deficient in factor V. While the restitution of factor V alone (No. 5) caused only a slight change in the clotting time, from 3 to 4½ minutes, the combination of the two factors (No. 6) resulted in a residual C.R.F. clotting time of 45 minutes, indicating the almost complete removal of C.R.F. It should be noted that the original untreated plasma apparently contains its full complement of these conversion factors since the addition of calcium alone (No. 2)

TABLE III

*The Relation of Thromboplastin to C.R.F. Loss after Plasma to Serum Conversion*

| No. | Prothrombin conversion incubation mixture* | | | | | Residual C.R.F. clotting time at final plasma dilutions below | | |
|---|---|---|---|---|---|---|---|---|
| | Untreated plasma | Thrombin-clotted plasma | Thrombo-plastin | Factor V | $CaCl_2$ 1 M | 1:600 | 1:1200 | 1:3000 |
| | *ml.* | *ml.* | *ml.* | *ml.* | *ml.* | *min.* | *min.* | *min.* |
| 1 | 0.5 | 0 | 0 | 0 | 0 | 3¾ | 5¼ | 8½ |
| 2 | 0.5 | 0 | 0 | 0 | 0.025 | 40 | — | — |
| 3 | 0 | 0.5 | 0 | 0 | 0 | 3 | 4¾ | 7¾ |
| 4 | 0 | 0.5 | 0.25 | 0 | 0.025 | 3 | 4½ | 7¼ |
| 5 | 0 | 0.5 | 0 | 0.1 | 0.025 | 4½ | 7 | 13 |
| 6 | 0 | 0.5 | 0.25 | 0.1 | 0.025 | 45 | — | — |

* All mixtures made up to 1 ml. total volume with veronal buffer.
Thrombin controls on test, by omitting coagulase: no clots in 1 hour.

was sufficient to potentiate a comparable C.R.F. loss (clotting time 40 minutes).

It would thus appear that not only was factor V greatly reduced by the thrombin treatment, but the thromboplastic activity was impaired, since only the restoration of both these substances permits the system to regain its full potential as far as C.R.F. consumption is concerned.

### *The Effect on C.R.F. Loss of the Removal of Prothrombin by Seitz Filtration and of the Restoration of Prothrombin to the Preliminary Incubation Mixture*

For the present, the best established function of factor V is its capacity to accelerate prothrombin conversion. The participation of calcium, factor V, and of thromboplastin in this reaction makes it increasingly obvious that it is linked to prothrombin conversion. Removal of prothrombin should abolish

the effect on C.R.F. This problem was studied by the use of Seitz-filtered plasmas from which demonstrable prothrombin activity had been eliminated but which retained substantial amounts of C.R.F. and some factor V. Restoration of prothrombin was then accomplished by the addition of highly purified bovine prothrombin of Dr. Seegers, which is essentially free of C.R.F. The results, summarized in Table IV, indicate that indeed no C.R.F. loss occurs when prothrombin is unavailable (Nos. 1 and 2). The addition of prothrombin led to over a twofold prolongation of the residual clotting time (Nos. 3, 4, and 5), since unquestionably some factor V activity was present in the plasma. When, however, both prothrombin and factor V were added, over five-fold prolongation of the clotting time resulted (No. 6).

TABLE IV

*The Effect on C.R.F. Loss of the Removal of Prothrombin by Seitz Filtration and the Restoration of Prothrombin to the Preliminary Incubation Mixture*

| No. | Prothrombin conversion incubation mixture* | | | | Residual C.R.F. clotting time at final plasma dilutions below | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Test A (with calcium)‡ | | | Control (no calcium) | | |
| | Plasma (Seitz) | Prothrombin | Factor V | Thromboplastin | 1:600 | 1:1200 | 1:3000 | 1:600 | 1:1200 | 1:3000 |
| | *ml.* | *ml.* | *ml.* | *ml.* | *min.* | *min.* | *min.* | *min.* | *min.* | *min.* |
| 1 | 0.1 | 0.1 | 0 | 0.1 | 2½ | 4 | 7 | 2½ | 4 | 7 |
| 2 | 0.1 | 0 | 0.2 | 0.1 | 2¼ | 3¾ | 7¼ | 2¾ | 4½ | 8¼ |
| 3 | 0.1 | 0.1 | 0 | 0.1 | 5½ | 9 | 19 | 2½ | 4¼ | 7 |
| 4 | 0.1 | 0.2 | 0 | 0.1 | 5¾ | 9 | 19 | 2½ | 4 | 7¼ |
| 5 | 0.1 | 0.4 | 0 | 0.1 | 5¾ | 9 | 20 | 2¾ | 4 | 7 |
| 6 | 0.1 | 0.1 | 0.2 | 0.1 | 12½ | 20 | — | 2¾ | 4½ | 8¼ |

* All mixtures made up to total volume of 1 ml. with veronal buffer.

‡ 0.05 ml. of 0.1 M $CaCl_2$ added to the prothrombin conversion mixture.

Thrombin controls of A and B: no clot at the end of 1 hour when coagulase omitted.

### *Prothrombin Consumption in Relation to C.R.F. Consumption after Prothrombin Conversion*

Since the process of prothrombin conversion is implicated in the residual C.R.F. levels, it became of interest to establish whether any parallelism could be demonstrated between prothrombin and C.R.F. consumption. The experiment is presented in Chart 1.

The prothrombin consumption test was based on a modified two stage method. The incubation mixture consisted of 0.1 ml. of a suitable dilution of plasma or test mixture, 0.2 ml. of thromboplastin, 0.04 ml. of 0.02 M $CaCl_2$, 0.2 ml. of factor V containing 20 units per ml., and buffer to a final volume of 2 ml. The serum or plasma concentrations were adjusted to give clotting times from 15 to 100 seconds. This mixture was incubated for 2, 4, 6, 8, and 10 minutes respectively, and was reacted in a volume of 0.2 ml. with an equal volume of fibrinogen to determine the clotting times. The maximum thrombin time was taken to be the

lowest constant value obtained. The per cent of prothrombin activity of the sera was calculated from a base line determined by plotting several dilutions of the original plasma against the clotting time.

It is apparent that under the conditions of the test, a close parallelism between prothrombin and C.R.F. consumption has been demonstrated.

*The Effect of the Addition of a Prothrombin Conversion Mixture in Various Stages of Completion to a Seitz-Filtered Plasma as the C.R.F. Source*

In preceding experiments, prothrombin, ancillary conversion factors, and the C.R.F. were incubated together, and the effect on the C.R.F. clotting time determined. The present experiment was designed to evaluate the effect of

TABLE V

*Effect of the Addition of a Prothrombin Conversion Mixture at Various Stages to C.R.F.*

| Preincubation time of conversion mixture before addition of plasma | Thrombin times of conversion mixture at dilutions below | | | Residual C.R.F. clotting time at final plasma dilutions below | |
|---|---|---|---|---|---|
| | 1:20 | 1:40 | 1:100 | 1:600 | 1:1200 |
| *min.* | *min.* | *min.* | *min.* | *min.* | *min.* |
| 0 | 45 | — | — | 12 | 21 |
| ½ | 45 | — | — | 13 | 22 |
| 1 | 45 | — | — | 14 | 21 |
| 2 | 24 | 45 | — | 12 | 19 |
| 5 | 5 | 8½ | 21 | 12 | 20 |
| 10 | 4½ | 7½ | 16 | 5¼ | 11½ |
| 20 | 2¾ | 4½ | 10 | 4 | 5¾ |
| 40 | 2¾ | 4½ | 10½ | 3 | 5¼ |
| Control* | — | — | — | 2½ | 3½ |

* No calcium added to conversion mixture.

the developing prothrombin conversion on C.R.F. by sampling from the conversion mixture at intervals of time and adding these samples to a prothrombin-free, Seitz-filtered plasma containing the C.R.F.

The reaction mixture was composed of 1 ml. of Seegers' bovine prothrombin, 1 ml. of factor V, 2 ml. of thromboplastin, 0.5 ml. of 0.1 M calcium chloride, and 4.5 ml. of veronal buffer. As the mixture incubated at 37°C., 0.9 ml. lots were withdrawn at the indicated time intervals (Table V), and were added to 0.1 ml. of a prothrombin-free, Seitz-filtered plasma. These were then incubated for 2 hours, and tested for C.R.F. by the usual addition of coagulase and fibrinogen at a final dilution of 1:600 and 1:1200 of the plasma in veronal-citrate-albumin buffer. Thrombin times were determined on the same dilutions by the addition of fibrinogen, but omitting the coagulase.

The data of Table V indicate that as the reaction of the preincubation mixture is allowed to go to completion, there is a diminishing effect on C.R.F. loss in the subsequent test system. As the prothrombin-converting process ap-

proaches completion, as indicated by the piling up of thrombin and the consequent shortening of the thrombin time, the subsequent C.R.F. loss progressively diminishes. It will be noted that a rather sharp break took place after 5 minutes (from 5¼ to 12 minutes at 1:600), while after 40 minutes of preincubation the C.R.F. clotting time approaches that of the control in which calcium was withheld to prevent any prothrombin conversion from taking place.

*The Effect of the Fibrin Clot on C.R.F. Loss after Plasma to Serum Conversion*

The possibility was considered that the C.R.F. loss in the course of serum formation might be due to adsorption on the fibrin clot. That this is not the case is evident from Table III. It can be seen (Nos. 1 and 3) that the C.R.F. activity is quite unimpaired after the plasma has been clotted by a large amount of thrombin; indeed, it is slightly enhanced probably because of the removal of some inhibitors. It is not until this defibrinated plasma (No. 6) is treated with the prothrombin converting reagents (calcium, factor V, and thromboplastin) that the loss of C.R.F. occurs, although no clot formation intervenes in this process.

Other experiments in which plasma was defibrinated with smaller amounts of thrombin and in which purified fibrinogen was restored to the system, confirmed these observations.

## DISCUSSION

There is ample evidence to support the view that staphylocoagulase action is distinct from the physiological mammalian blood-clotting process. In favor of this contention, one may cite the following observations:—

1. Anticoagulants, such as citrate and oxalate which depress ionized calcium, do not block coagulase activity (16).

2. Coagulase acts in the presence of heparin, which is assumed to have antiprothrombic activity as well as the ability to block thrombin-fibrinogen interaction (17–19).

3. C.R.F. has been dissociated from prothrombin by Seitz filtration of plasma (3, 9, 20, 21). If C.R.F. is available, coagulase will still clot blood even though all conventional tests of prothrombin activity are negative.

4. There is a natural dissociation of prothrombin and C.R.F. in some animal hosts (1, 3). This may be because of an inherent lack of C.R.F., or may represent a masking of the coagulase-C.R.F. reaction by inhibitors.

5. Attempts to identify C.R.F. with any of the known blood components which function in physiological clotting have been unsuccessful.

In the light of some evidence (1, 22) that an intermediate complex may be formed, one may tentatively consider that the coagulase reaction includes the following steps:—

Staphylocoagulase + C.R.F. = intermediate complex (called "coagulase thrombin" by Miale (22))

Intermediate complex + fibrinogen = fibrin

The present observations, as well as those of Duthie and Lorenz (9), bear directly on the problem of the relation of the two clotting processes. These studies indicate an interaction between the two systems in so far as prothrombin conversion exerts a profound effect on residual C.R.F. titers. Any system favoring effective prothrombin conversion favors maximal C.R.F. loss; conversely, factors which may hinder prothrombin conversion will reduce this C.R.F. loss. Obviously, all the components required for prothrombin conversion must be on hand, and a deficiency in any of the essential components of prothrombin conversion or interference with their function will be reflected in the residual C.R.F. clotting times. The most marked C.R.F. loss (from 90 to 98 per cent) was observed following the conversion of fresh human plasma to serum, and in the presence of an effective concentration of accelerator globulin (factor V), provided adequate amounts of thromboplastin and calcium were at hand. If prothrombin is removed, the subsequent C.R.F. loss is abolished. No support was found for the possibility that adsorption on the fibrin was significantly implicated in the C.R.F. loss.

An increasing number of factors which accelerate prothrombin conversion have been described, although some of these may prove to be identical. Alexander (23, 24) has described a plasma component SPCA (serum prothrombin conversion accelerator) which he regards as distinct from Seegers' AC globulin and Owren's factor V, but which accelerates prothrombin conversion only in the presence of plasma AC globulin. In unpublished observations, utilizing a partially purified sample of SPCA kindly supplied by Dr. Alexander, it was found, as might have been anticipated from the reported properties of SPCA, that this agent alone had only little effect on the C.R.F. titers after prothrombin conversion, an effect which might well have been due to trace contamination with AC globulin. This is consistent with Alexander's claim that SPCA alone is relatively inert when acting on purified prothrombin.

Milstone (25, 26) has presented a three stage analysis of blood clotting, involving (*a*) the conversion of prothrombokinase to thrombokinase, (*b*) the conversion of prothrombin to thrombin, and (*c*) the reaction of thrombin with fibrinogen to yield fibrin. Calcium is of importance for the first two stages, and evidence has been presented further that "thrombokinase" may be essential for both the conversion of prothrombokinase to thrombokinase as well as for the conversion of prothrombin to thrombin, rather than that thrombin or lipoid thromboplastin normally functions in this regard. In the present studies, the thromboplastin has been used to activate prothrombin conversion, and the three stage scheme of Milstone has not been applied. Nevertheless, it appears probable that the C.R.F. loss is related to Milstone's second phase rather than the first, if for no other reason than that an incubation mixture containing everything necessary for conversion except prothrombin does not lead to C.R.F. loss.

At present, the functions of C.R.F. are not known other than its reaction

with staphylocoagulase. Since C.R.F. is apparently consumed during effective prothrombin conversion, further studies of the functional significance of this globulin in physiological clotting and in disease may prove of interest. In so far as this reaction is a sensitive indicator of disturbances in prothrombin conversion, it offers a new variable which might be put to use in the study of disturbances of prothrombin conversion.

SUMMARY

The conversion of plasma to serum results in a variable loss of the coagulase-reacting factor (C.R.F.) of the plasma.

The C.R.F. loss is incurred during the process of prothrombin conversion: conditions which favor the most effective prothrombin conversion result in maximal C.R.F. loss, while factors which interfere with prothrombin conversion spare the C.R.F.

In a system containing an adequate concentration of calcium, thromboplastin, and prothrombin, the C.R.F. loss reflects the amount of prothrombin conversion–accelerating substances (factor V or AC globulin).

If fibrin clots are produced directly by thrombin, and prothrombin conversion is excluded, there is no significant C.R.F. loss.

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