Separation from Russell's Viper Venom of One Fraction Reacting with Factor X and Another Reacting with Factor V*

Sandra Schiffman, Ida Theodor, and Samuel I. Rapaport

ABSTRACT: The clotting activities in Russell's viper venom which react with either factor X or V have been separated from whole venom by chromatography on Sephadex G-200.

The fraction activating factor X, which has a molecular weight around 145,000, functions as a catalyst in a calcium-dependent reaction. The fraction reacting with factor V, which has a molecular weight around 10,000–20,000, is consumed in a reaction requiring no added cofactors. Neither factor V activating fraction nor whole venom appears to react with factor VIII.

Macfarlane and Barnett (1934) first described the dramatic clot promoting effect of Russell's viper venom. Macfarlane (1961) later presented data which strongly suggested that venom, in the presence of calcium ions, activated factor X in serum in a reaction in which the venom acted as an enzyme. Subsequently, Williams and Esnouf (1962) isolated a coagulant protein from Russell's viper venom by DEAE-cellulose chromatography. This protein functioned as an enzyme in activating a coagulation factor in bovine serum in the presence of calcium ions. These investigators (Esnouf and Williams, 1962) also isolated the coagulation factor from bovine serum and pointed out that its biological activity was similar to factor X.

Hjort (1957) reported that the reactivity of a bovine factor V preparation was increased on incubation with Russell's viper venom in the absence of added calcium ions. In retrospect, this was the first evidence that Russell's viper venom possessed a second clotting activity. Recently, Rapaport *et al.* (1966) presented data which fit the hypothesis that the reaction between Russell's viper venom and factor V is not enzymatic but stoichiometric. These authors suggested that Russell's viper venom may contain two separate clotting activities.

The following is a report of the clear separation of two clotting activities from whole venom: one activates factor X in a catalytic reaction; the other activates factor V in a reaction in which the venom activity is consumed.

Experimental Procedure

Materials

Russell's viper venom (whole RVV[1]) was obtained as a bulk powder from The Serpentarium, Miami, Fla. It was dissolved in citrate-saline in concentrations between 10 and 100 mg per ml for fractionation studies and in a concentration of 100 μg/ml for use as a whole RVV standard in clotting studies. The latter reagent was stored at −20° and kept in an ice–water bath after thawing. *Bovine thrombin* (Parke Davis) was adsorbed with $BaSO_4$ as described previously (Rapaport *et al.*, 1963). *Sephadex*, sizes G-200, G-100, G-50 fine, and G-25 medium, was obtained from Pharmacia Fine Chemicals. *Citrate–saline* was a solution of 0.017 M trisodium citrate and 0.125 M sodium chloride. *Barbital buffer* was a solution containing 0.028 M sodium chloride and 0.125 M sodium barbital adjusted to pH 7.25 with 0.1 N HCl.

Thromboplastin was a saline extract of human brain (Owren, 1949). *Cephalin*, an acetone–insoluble, ether-soluble extract of human brain which supplies an activity equivalent to platelet factor 3, was prepared and stored as a stock solution as described previously (Hjort *et al.*, 1955). *Cephalin–kaolin* was a barbital buffer suspension containing 10 mg/ml of kaolin powder (Mallinckrodt) and a 1:100 dilution of cephalin.

Clotting Reagents Derived from Blood Fractions. All clotting activities are expressed as per cent of the activity in a pooled plasma prepared from 14 normal donors which was stored at −20°. *Adsorbed ox plasma*, the source of factor V and fibrinogen for the substrate mixture of the factor X assay, was prepared by adsorbing oxalated beef plasma with 100 mg/ml of barium sulfate powder (Baker Chemical Co.). It contained over 300% factor V and 372 mg % fibrinogen. *Bentonite-adsorbed plasma*, a reagent supplying prothrombin free of significant factor X activity, was prepared from normal human plasma according to Hougie (1962). One preparation contained 13% prothrombin, another contained 20%; both contained less than 0.1% measurable factor X. *Artificial factor V deficient substrate* was

* From the Departments of Medicine and Biochemistry, University of Southern California School of Medicine, Los Angeles, California 90033. *Received December 16, 1968.* Supported by Grants HE 06128-07 and -08 from the National Heart Institute, U. S. Public Health Service.

[1] The following abbreviations will be used: whole RVV for whole, unfractionated Russell's viper venom; RVV-V for the activity in whole RVV or its fractions which activates factor V; RVV-X for the activity in whole RVV or its fractions which activates factor X; u for NIH units of thrombin. Clotting factors are designated by Roman numeral according to the convention adopted by the International Committee on Haemostasis and Thrombosis.

prepared from normal human plasma (Stormorken, 1957). It contained mean levels of more than 50% of prothrombin, factor VII, and factor X activities and about 100 mg % fibrinogen. *Natural factor V deficient substrate* was obtained from a patient with hereditary factor V deficiency.

Experiments to test the clotting activities of venom fractions required: (1) a factor V source free of factor X, (2) a factor X source free of factor V, and (3) a factor VIII source without significant factor V or factor X activity. *The factor V source* was citrated normal human plasma adsorbed twice with $Al(OH)_3$ gel (Cutter Laboratories). This reagent contained about 30% factor V and no measurable factor X. *The factor X source* was either oxalated serum from blood clotted with thromboplastin or Hjort's "proconvertin reagent" (Hjort, 1957). The former contained 60% factor X and the latter contained 40% factor X; neither contained measurable factor V. *The factor VIII source* was a lyophilized fraction I-O preparation obtained from Dr. Birger Blombäck. When reconstituted with distilled water it contained 1200 mg % fibrinogen, 188% factor VIII, 0.3% factor V, and 0.1% factor X.

Methods

Sephadex chromatography was carried out at room temperature using columns 90 cm high and 2.8 or 1.8 cm in diameter. Except as noted, the directions of the manufacturer were followed. Sephadex G-200 and G-100 were swollen by boiling for 20 min in 0.024 M sodium chloride solution, followed by cooling and several changes of citrate–saline. Citrate–saline was used for equilibration and elution. Columns were allowed to flow for 8–24 hr prior to application of 1 ml of a venom preparation; 4-ml samples were collected automatically. *Polyacrylamide disc electrophoresis* was carried out with chemicals and equipment from Canalco, Bethesda, Md., according to their directions. The sample gel contained a 1:8 dilution of the test material. *Absorbancy* was measured at 280 mμ in a Zeiss PMQ II spectrophotometer.

Clotting Procedures. Assays of RVV-X activity. Two assay techniques were used. In both, the venom source reacted with factor X and calcium ions in a test mixture for a measured time after which a subsample of the test mixture was added to a substrate containing all activities required for clotting initiated by activated factor X. In the *routine assay*, the *test mixtures*, containing equal parts of 30 mM calcium chloride, oxalated serum from thromboplastin-clotted blood diluted 1:5 in citrate–saline (source of factor X), and RVV test sample were incubated for precisely 5 min at room temperature. Then, 0.1 ml of this test mixture was added to a substrate mixture followed immediately by 0.1 ml of 40 mM calcium chloride. The *substrate mixture*, which had incubated at 37° for 3 min, contained 0.1 ml of cephalin diluted 1:100 in barbital buffer (platelet factor 3 substitute) and 0.1 ml of an equal mixture of adsorbed ox plasma (source of factor V and fibrinogen) and bentonite-adsorbed plasma (source of prothrombin). Standard curves were obtained by making test mixtures with *whole RVV* in concentrations from 0.05 to 1 μg per ml and plotting log substrate clotting time against log added RVV concentration. This yielded a straight-line reference curve for calibrating unknown RVV test samples, which were diluted as necessary to give clotting times within the limits of this line. For example, if a 1:200 dilution of a fraction from a Sephadex column gave the same clotting time as 0.5 μg/ml of whole venom, then that fraction was said to have an RVV-X activity of 100 μg/ml.

When whole RVV at a concentration of 1 μg/ml was added without factor X to a substrate mixture, the clotting time was shortened very slightly, but it fell far outside the range of the standard curve. When the curve was extrapolated, the clotting time was equivalent to that which would be expected from less than 0.001 μg/ml of whole RVV in a test mixture containing factor X. For assays designed to measure significant amounts of RVV-X activity, this effect was negligible. However, for experiments to study the *mechanism of action of RVV-X* we wanted to eliminate any uncontrolled effect of venom carried over from the test mixture. Therefore, in this assay a known excess amount of whole RVV was added to the substrate plasma by modifying the cephalin reagent to contain 2 μg/ml of whole venom. The incubation mixture was also modified by replacing the serum with Hjort's "proconvertin reagent," a more purified source of factor X. Details of the assay are given in the footnote to Figure 3. A standard curve was made from suitable dilutions of a test mixture containing 2 μg/ml of added RVV-X which had incubated for at least 20 min to assure full activation. This mixture retained full activity for at least 70 min more. The activity of a 1:5 dilution was designated as 100% venom-activated factor X. Clotting time plotted against per cent of fully activated mixture on log–log paper yielded a straight line from which clotting times of other test mixtures were converted into per cent venom-activated factor X activity.

Assays of RVV-V activity. Venom-activated factor V was generated in a test mixture containing a factor V source and RVV and was measured by adding a diluted subsample to a substrate containing all activities required for extrinsic clotting except factor V. In the *routine RVV-V assay* a test mixture of equal parts of $Al(OH)_3$-adsorbed citrated plasma and venom was incubated for 20 min at room temperature. Then, a subsample was diluted 1:50 in barbital buffer and 0.1 ml of the diluted subsample was added to the substrate followed by 0.1 ml of 60 mM calcium chloride. The *substrate*, which had incubated for 3 min at 37°, consisted of 0.1 ml of artificial or natural factor V deficient plasma and 0.1 ml of thromboplastin. Blank time was unaffected by direct addition to the substrate of 0.5 μg/ml of whole RVV. Standard curves were obtained from test mixtures to which 1–5 μg/ml of whole RVV had been added. Log RVV concentration plotted against log clotting time gave a straight line. Unknown venom samples were diluted to give clotting times within the limits of this line. All dilute RVV samples (less than 50 μg/ml) were kept in an ice–water bath prior to assay.

To establish the *mechanism of action of RVV-V* it was

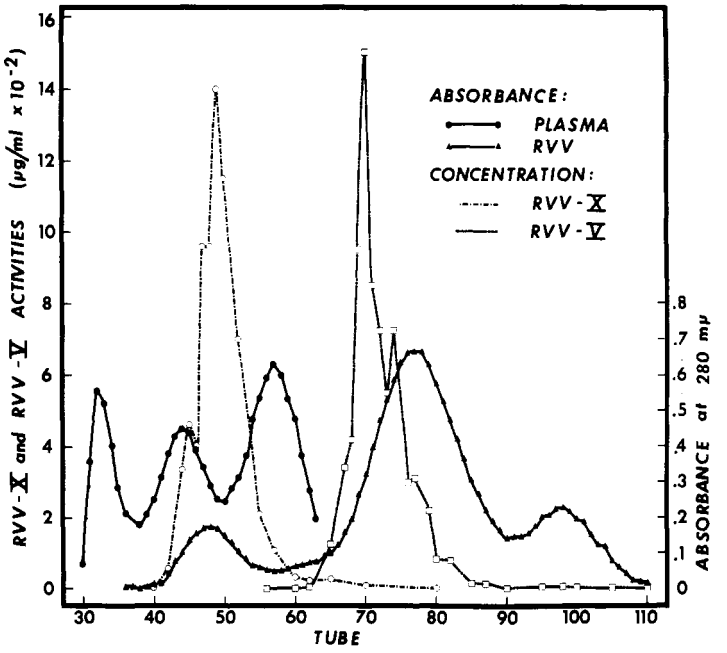

FIGURE 1: Sephadex G-200 chromatography of whole RVV. Whole venom (50 mg) was applied to the column.

necessary to expand the range of RVV-V concentration. However, at added RVV-V concentrations greater than 5 μg/ml, factor V activity did not increase proportionately. Therefore, the amount of factor V in the *test mixture* was increased to three parts of factor V source with one part of RVV-V. Test mixtures were incubated at room temperature. At intervals 0.1-ml aliquots were removed, diluted 1:20 in citrate–saline, and tested immediately in the substrate mixture described above. Standard curves were obtained from dilutions of a mixture containing either 10 or 20 μg per ml of RVV-V which had incubated at least 15 min before dilution to assure full activation. The activity of the undiluted mixture was stable for at least 30 min. The activity of a 1:20 dilution was designated 100% venom-activated factor V. Log clotting time plotted against log per cent of fully activated mixture gave a straight line from which clotting times of test mixtures were converted into per cent venom-activated factor V.

ASSAY OF FACTOR VIII REACTIVITY. A test sample, whole RVV, RVV-V, thrombin, or citrate–saline as a control, was incubated at room temperature with an equal volume of factor VIII source. At intervals, 0.1-ml samples were removed, diluted 1:50 in citrate–saline, and added as test substance to a substrate mixture. Each substrate tube contained 0.1 ml of 40 mM calcium chloride warmed to 37° to which the following were added without pause in the order listed: 0.1 ml of diluted test mixture; 0.1 ml of *either* citrate–saline *or* whole RVV, RVV-V, or thrombin in citrate–saline in a concentration that eliminated variation due to carryover of these activities from the test mixture; and 0.2 ml of a mixture of equal parts of cephalin–kaolin reagent and factor VIII deficient plasma which had been incubated together for 9 min at 37°. The time from the last addition to clotting was noted. This system allowed us to test the effect of venom upon factor VIII in concentrations that affected the substrate clotting time because a clotting mixture of an experiment and its control always contained the same final concentration of venom. However, in the control the venom was added to the substrate mixture just before clotting, whereas in the experiment the factor VIII source was incubated with venom 100 times stronger before it was diluted and added to the substrate.

Results

Sephadex Chromatography. Whole RVV was separated into three peaks by chromatography on Sephadex G-200 (Figure 1). RVV-X closely paralleled the first protein peak; RVV-V appeared in the leading edge of the second peak. Average recovery from four experiments was RVV-X, 71%; RVV-V, 74.5%. Maximum purification (average of three experiments) based on observed activity compared with absorption at 280 mμ was 7-fold for RVV-X and 3.8-fold for RVV-V. Comparison of the observed elution and void volumes with those reported by Andrews (1965) suggested a molecular weight of 145,000 for RVV-X and around 20,000 for RVV-V. Fractions containing RVV-V or RVV-X were pooled and lyophilized with little or no loss of activity. Similar elution patterns were obtained with Sephadex G-100.

Although RVV-X activity always paralleled the first protein peak, some of the pooled preparations were less homogeneous than others. The heterogeneous fractions could be further purified, about 1.3-fold, by rechromatography on Sephadex G-100. This also removed traces of RVV-V contamination.

As illustrated in Figure 1, the RVV-V activity was clearly not a uniform protein sample. However, rechromatography on Sephadex G-100, -50, or -25 did not result in significant purification. Data from

TABLE I: Effect of Time and Temperature on RVV-V and RVV-X Activities of Whole RVV.[a]

		Whole RVV Concentrations					
		1000 μg/ml		100 μg/ml		10 μg/ml	
Temp (°C)	Time	% RVV-V	% RVV-X	% RVV-V	% RVV-X	% RVV-V	% RVV-X
37	0 hr	100	100	100	100	100	100
	4 hr	96	85	82	76	69	71
	24 hr	78	84	61	76	30	56
Room temperature	0 hr	100	100	100	100	100	100
	4 hr	106	99	91	77	57	102
	24 hr	73	81	71	69	29	76
4	0 hr	100	100	100	100	100	100
	4 hr	109	86	89	80	65	107
	24 hr	81	80	69	77	46	66
	7 day[b]	91	78	66	76	65	65
−20	0 hr[b]	100	100	100	100	100	100
	7–14 day[b]	100	95	96	92	94	84

[a] Results are expressed as per cent of original activity in freshly prepared reagent. Except where noted the results represent the average of three experiments. [b] Average of two experiments.

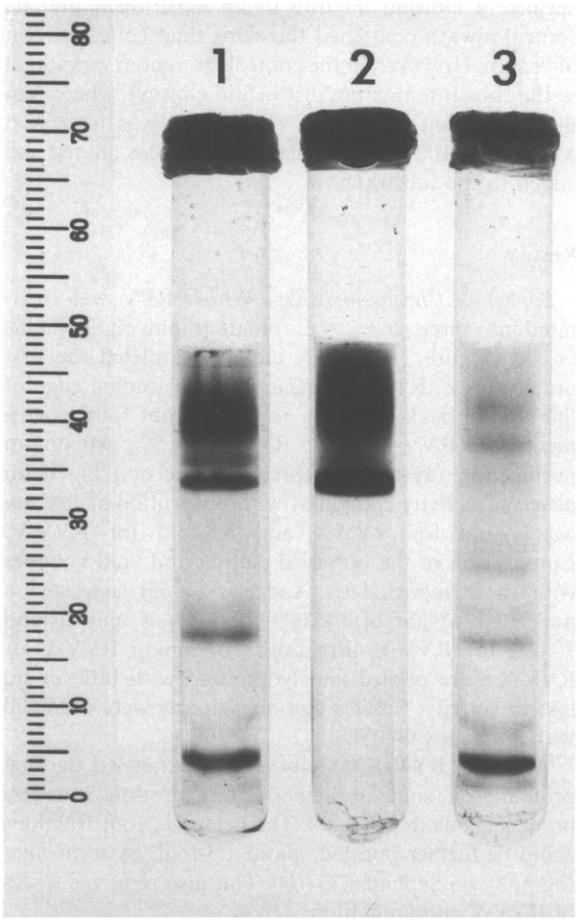

FIGURE 2: Polyacrylamide disc electrophoresis of whole RVV, RVV-X, and RVV-V. Tube 1 is whole RVV, tube 2 is RVV-X, and tube 3 is RVV-V.

Sephadex G-50 columns suggested that RVV-V was eluted later than the void volume, indicating a very small molecular weight, possibly closer to 10,000.

Polyacrylamide Disc Electrophoresis of Whole RVV and Partially Purified RVV-V and RVV-X. Electrophoresis of whole RVV produced ten distinct bands, as shown in Figure 2. Partially purified RVV-X (pooled, lyophilized, and reconstituted column fraction) appeared to contain only bands 1 and 3 identified in whole RVV. RVV-X activity from whole RVV or partially purified RVV-X could be eluted from the gel, and the activity corresponded to band 1. Partially purified RVV-V (pooled, lyophilized, and reconstituted column fraction) contained all bands found in whole venom; however, bands 1 and 3, so prominent in the RVV-X fraction, were markedly reduced. Despite repeated attempts, RVV-V activity from whole RVV could not be eluted from the gel.

Effect of Time and Temperature upon RVV-V and RVV-X Activities in Whole RVV. The effect upon RVV-V and RVV-X activities of incubating three concentrations of whole RVV for increasing intervals at different temperatures is summarized in Table I. At concentrations of whole RVV of 100 μg/ml or greater both activities were largely retained over 4 hr. About 20–30% of each activity was lost after 24 hr. Apparently, stability was unaffected by incubation temperature between 4 and 37°.

In dilute whole RVV solution (10 μg/ml), RVV-V activity was more labile than RVV-X activity. At all three temperatures studied, appreciable RVV-V was lost in 4 hr and less than 50% of original RVV-V activity was retained after 24 hr. Considerable RVV-X activity also disappeared in 24 hr, but at each temperature more RVV-V activity was lost than RVV-X activity.

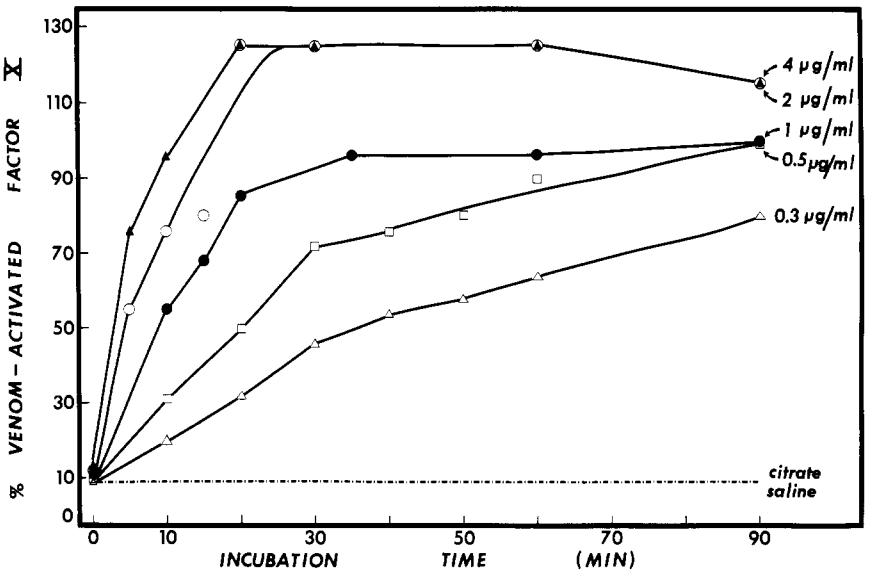

FIGURE 3: Activation of factor X by RVV-X. Test mixtures containing equal parts of Hjort's "proconvertin reagent" diluted 1:10 in citrate–saline (factor X), 30 mM calcium chloride, and either RVV-X or citrate–saline were incubated at 22°. At the intervals shown subsamples were removed, diluted 1:5 in citrate–saline–calcium solution, and 0.1 ml of the diluted test mixture was added to the substrate followed immediately by 0.1 ml of 40 mM calcium chloride. The substrate, which had been incubated for 3 min at 37°, consisted of 0.1 ml of 1:100 cephalin containing 2 μg/ml of whole RVV and 0.1 ml of an equal mixture of adsorbed ox plasma and bentonite-adsorbed plasma. Clotting times were converted into per cent venom-activated factor X as described in Methods.

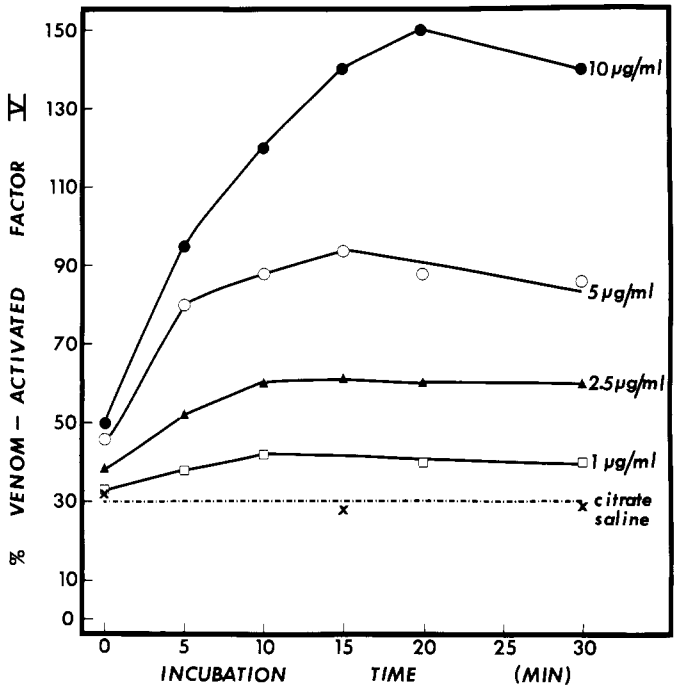

FIGURE 4: The effect of changing RVV-V concentration on the generation of venom-activated factor V. Three parts $Al(OH)_3$-adsorbed citrated normal plasma (factor V) were incubated with one part of either RVV-V or citrate–saline at room temperature. At the intervals shown subsamples were removed, diluted 1:20 in citrate–saline, and tested immediately in a thromboplastin factor V deficient plasma substrate. Clotting times were converted into per cent venom-activated factor V as described in Methods.

At all three concentrations both activities were stable in frozen solution for 7–14 days, and at a concentration of 100 μg/ml both activities were stable for at least 2 months.

Mechanism of Interaction of RVV-X and Factor X. Generation of venom-activated factor X was studied using an RVV-X sample purified about fivefold over the activity in whole venom by chromatography on Sephadex G-200 and rechromatography on Sephadex G-100. It contained 285 μg/ml of RVV-X activity and no RVV-V activity. Five test mixtures, containing equal concentrations of factor X, were incubated with RVV-X in concentrations between 0.3 and 4 μg per ml and tested as described in the legend to Figure 3. This was the lowest concentration range at which the RVV-X and the product could be maintained for sufficient experi-

mental time. As the concentration of RVV-X in the test mixture was increased, the rate of product formation increased, but in each mixture the maximum activity approached the same limit (Figure 3). These results indicate that RVV-X functioned as a catalyst.

Mechanism of Interaction of RVV-V and Human Factor V. Generation of venom-activated factor V was studied using an RVV-V sample from a Sephadex G-200 column which had been pooled, lyophilized, and rechromatographed on Sephadex G-25 to yield a total purification of twofold over whole venom. It contained 365 μg/ml of RVV-V activity and 3 μg/ml of RVV-X activity.

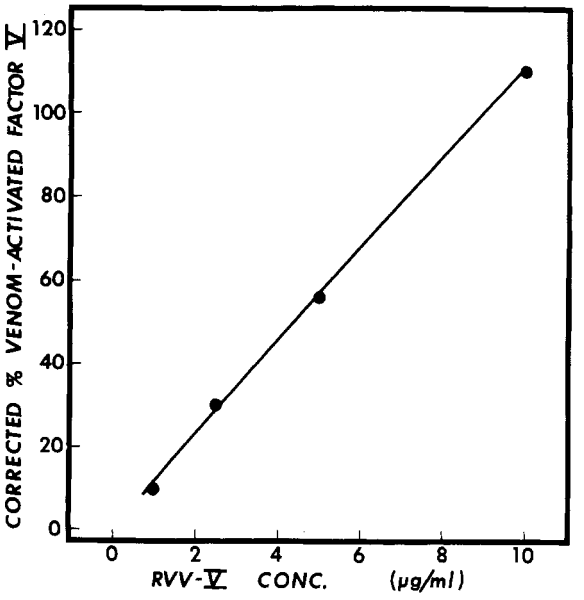

FIGURE 5: Relationship of corrected maximum venom-activated factor V to RVV-V concentration.

Four concentrations of RVV-V, ranging from 1 to 10 μg per ml, and citrate–saline as a control were incubated with a constant amount of factor V source as described in the assay for mechanism of action of RVV-V (see Methods) and generation of product was measured. The results are shown in Figure 4. In the absence of RVV-V (dashed line) the clotting time was equivalent to 30% of a fully activated mixture. As the concentration of RVV-V was increased, the maximum concentration of product generated also increased (solid lines). When the blank effect was subtracted from each observed maximum activity and the resultant "corrected" maximum activity was plotted against concentration of RVV-V added, the amount of product found was directly proportional to the concentration of RVV-V added (Figure 5). This suggested that RVV-V was consumed in the activation.

Further evidence that RVV-V was consumed in the reaction was obtained from experiments in which native factor V was added to a mixture of factor V and RVV-V which had reached maximum activation. As shown in curve B of Figure 6 no additional venom-activated factor V formed over time. Hence, no free RVV-V was available to react with the added factor V. The difference in apparent venom-activated factor V activity between curve B (factor V added) and curve C (citrate–saline added) reflects the effect of added native factor V on the assay substrate. Since no free RVV-V

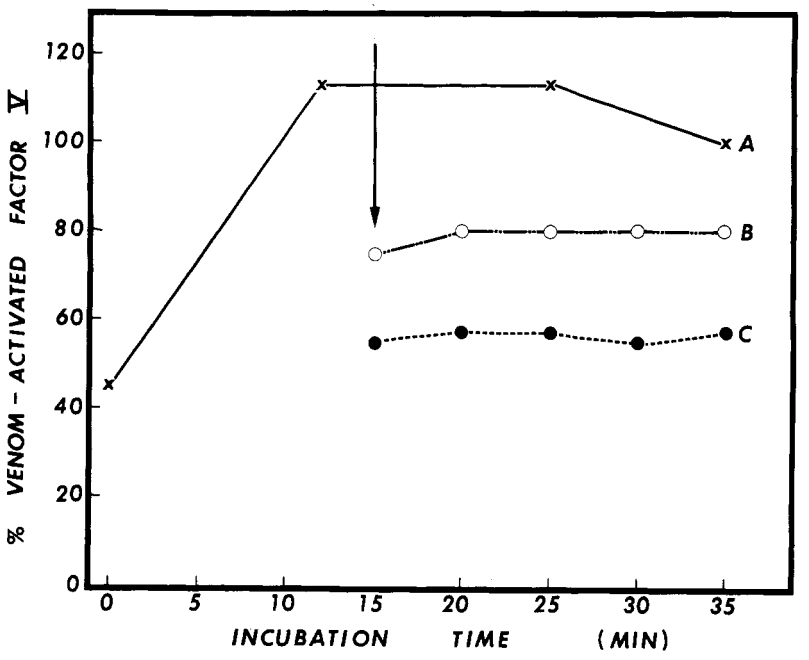

FIGURE 6: The effect upon a fully activated RVV-V reaction mixture of supplemental native factor V. After 15-min incubation at room temperature (arrow) a reaction mixture containing three parts of factor V source and one part of 20 μg/ml of RVV-V was divided into three fractions. Fraction A was a control to which nothing was added. Fraction B was diluted 1:2 in native factor V source. Fraction C was diluted 1:2 in citrate–saline. Samples of each were assayed at 5-min intervals.

TABLE II: Effect of RVV-V and Whole RVV on Factor VIII Activity.[a]

Test Substance	Concn in Incubation Mixture (μg/ml)	Concn added to Substrate (μg/ml)	Incubation Time (sec) 10	120	150	300	600	1200
			Clotting Time (sec)					
1. RVV-V	0.5	0.01	92		94	95	94	96
	0	0.01	94				93	95
	2.5	0.05	92		94	93	94	92
	0	0.05	94				94	93
	50	1.0	73		73	72	70	70
	0	1.0	74		74	75	73	73
2. Whole RVV	0.5	0.01	68		70	69	69	69
	0	0.01	68			69		68
	2.5	0.05	50			50	48	49
	0	0.05	50			48		48
3. Citrate–saline			94			95		94
4. Thrombin	0.05 u/ml	0.001 u/ml	95	90		75		

[a] Test substance and factor VIII source were incubated for the times shown, then tested in the factor VIII activation assay.

FIGURE 7: The effect of changing factor V concentration on the generation of venom-activated factor V. One part of RVV-V (50 μg/ml) was incubated with three parts of various dilutions of factor V source in citrate–saline (100% represents undiluted factor V source). At the intervals shown, subsamples were removed, diluted 1:50 in citrate–saline, and added immediately to a thromboplastin factor V deficient plasma substrate. Clotting times were converted into per cent venom-activated factor V as described in Methods.

was available in a fully activated mixture, and it could be demonstrated that RVV-V in the absence of factor V retained its activity under these experimental conditions, one must conclude that the RVV-V added to the original test mixture was consumed in the initial activation reaction.

The effect of factor V concentration upon the generation of venom-activated factor V was studied in the assay for mechanism of activation of RVV-V (see Methods) by incubating four concentrations of factor V source with 50 μg/ml of RVV-V, a level above which increased RVV-V concentration had no effect on product formation. This high RVV-V concentration was associated with a rapid decay of venom-activated factor V (see Figure 7) which made it impossible to determine precisely the maximum amount of venom-activated factor V generated. However, it is clear from Figure 7 that as the concentration of factor V source was decreased, the concentration of product formed also decreased. Thus, factor V also appears to be consumed during the reaction.

Lack of Effect of RVV-V on Rabbit Factor V. Since in earlier work, rabbit factor V would not react with whole RVV (Rapaport *et al.*, 1966), the RVV-V fraction used in the preceding experiments to activate factor V in human $Al(OH)_3$-adsorbed plasma was also checked for its ability to react with factor V in $Al(OH)_3$-adsorbed rabbit plasma. Three concentrations of rabbit plasma (undiluted, 1:10, and 1:20) and two concentrations of RVV-V (2 and 10 μg per ml) were used. Rabbit factor V activity failed to change over 20 min. When bovine thrombin (0.2 u/ml) replaced RVV-V in the mixture, factor V activity increased twofold over 20 min, which demonstrated that the rabbit factor V used was capable of activation by another agent.

Failure of RVV-V or Whole RVV to Activate Factor VIII. Since RVV and thrombin both activate human factor V (Rapaport *et al.*, 1966) and thrombin also

activates human factor VIII (Rapaport *et al.*, 1963), it seemed important to determine if either RVV-V or whole RVV could activate human factor VIII. The RVV-V fraction used in the experiments described above and whole RVV were incubated with the factor VIII source in the factor VIII activation assay (see Methods). The results are summarized in Table II, which is arranged so that each experiment (in which venom was added to the incubation mixture) is followed by its control (in which no venom was added to the incubation mixture but an amount giving the same final concentration was added to the substrate). As the data show, no time-consuming reaction could be demonstrated between factor VIII and either RVV-V (section 1) or whole RVV (section 2). Whole RVV did shorten the assay times, as did RVV-V at a high concentration. However, this stemmed from a direct effect upon the factor VIII deficient substrate as shown by the controls in which venom added directly to the substrate gave the same clotting times as venom incubated in much higher concentration for 5, 10, or 20 min with the factor VIII source. As shown in section 4, the factor VIII source could be activated by minute amounts of thrombin. Therefore, these data strongly suggest that neither RVV-V nor whole RVV can activate factor VIII.

Discussion

The experiments presented above clearly demonstrate that the clotting activities of whole RVV which activate factor X and factor V are different molecules readily separated by size. RVV-X appears to have a molecular weight in excess of 100,000 whereas RVV-V is quite small, with a molecular weight possibly as low as 10,000. Present techniques have yielded a 10-fold maximum purification of RVV-X and a 6.5-fold maximum purification of RVV-V. The gel electrophoresis experiments suggest that both activities can be further purified by techniques based upon charge differences, but better methods are first needed to stabilize the separated activities.

The isolated individual activities have been used to restudy the mechanism by which each activates its respective clotting factor. The data confirm that RVV-X catalytically activates factor X in a calcium-dependent reaction. This reaction mechanism has been demonstrated earlier for RVV-X in whole RVV (Macfarlane, 1961) and for RVV-X separated from whole RVV by DEAE chromatography (Esnouf and Williams, 1962). RVV-X obtained by DEAE chromatography (Williams and Esnouf, 1962) appears to have a slightly smaller molecular weight than indicated by our gel filtration data. However, both separation techniques yield data suggesting roughly the same amount of active material in whole RVV. Thus, Williams and Esnouf (1962) obtained a 12-fold purification whereas we obtained a 7-fold purification in a fraction appearing to contain two components, one of which contains the active material.

Our studies of the action of RVV-V show that the venom activity is consumed in the reaction, and that the amount of product formed is directly proportional to the concentration of RVV-V added. These data provide quantitative confirmation of the conclusions reached by Rapaport *et al.* (1966) from qualitative data.

It is established that factor V is also activated by thrombin (Ware and Seegers, 1948; Hjort, 1957; Rapaport *et al.*, 1966). Activation by thrombin resembles activation by RVV-V in that both thrombin and RVV-V are small molecules that react with native factor V in the absence of ionic calcium or other known cofactors. Moreover, prior activation of human factor V by thrombin appears to limit markedly the increase in factor V reactivity following its subsequent exposure to whole RVV (Rapaport *et al.*, 1966). However, the mechanism of the two activations apparently differs, for RVV-V clearly is consumed in the reaction with factor V whereas thrombin is said to activate factor V in an enzymatic reaction that reduces the molecular size of factor V (Papahadjopoulos *et al.*, 1964). In addition, thrombin activates factor VIII (Rapaport *et al.*, 1963) and clots fibrinogen, and our RVV-V fraction could neither activate factor VIII nor clot fibrinogen.

Since recent evidence suggests that factor V may not require preliminary activation to interact with activated factor X, lipid, and calcium ions in the formation of the prothrombin activator (Jobin and Esnouf, 1967; Barton *et al.*, 1967), the physiological significance, if any, of the increased factor V activity resulting from its incubation with agents such as RVV-V or thrombin is not clear. Obviously, however, the action of RVV upon factor V must always be taken into account in *in vitro* clotting systems. Whole venom added to a clotting mixture has the dual potentiality of activating factor X and increasing the activity of factor V, and each action could affect an experimental result. Since, as is shown herein, RVV-X can be readily separated from RVV-V on Sephadex G-200, it is now possible to use only the one specific clotting activity of whole venom desired in an experiment. It would appear particularly important to use RVV-X instead of whole RVV as the activator of factor X in experiments designed to study the interaction of activated factor X and native factor V in the generation of the prothrombin activator.

Acknowledgment

We wish to thank Miss Mary Jane Patch for preparing the figures for this paper.

References

Andrews, P. (1965), *Biochem. J. 96*, 595.

Barton, P. G., Jackson, C. M., and Hanahan, D. J. (1967), *Nature 214*, 923.

Esnouf, M. P., and Williams, W. J. (1962), *Biochem. J. 84*, 62.

Hjort, P. F. (1957), *Scand. J. Clin. Lab. Invest. 9, Suppl. 27*, 183.

Hjort, P., Rapaport, S. I., and Owren, P. A. (1955), *J. Lab. Clin. Med. 46*, 89.

Hougie, C. (1962), *Proc. Soc. Exptl. Biol. Med. 109*, 754.

Jobin, F., and Esnouf, M. P. (1967), *Biochem. J. 102*, 1966.

Macfarlane, R. G. (1961), *Brit. J. Haematol. 7*, 496.
Macfarlane, R. G., and Barnett, B. (1934), *Lancet 2*, 985.
Owren, P. A. (1949), *Scand. J. Clin. Lab. Invest. 1*, 81.
Papahadjopoulos, D., Hougie, C., and Hanahan, D. J. (1964), *Biochemistry 3*, 1595.
Rapaport, S. I., Hjort, P. F., and Patch, M. J. (1966), *Am. J. Physiol. 211*, 1477.
Rapaport, S. I., Schiffman, S., Patch, M. J., and Ames, S. B. (1963), *Blood 21*, 221.
Stormorken, H. (1957), *Scand. J. Clin. Lab. Invest. 9*, 273.
Ware, A. G., and Seegers, W. H. (1948), *Am. J. Physiol. 152*, 567.
Williams, W. J., and Esnouf, M. P. (1962), *Biochem. J. 84*, 52.

Studies on L-Amino Acid Oxidase. II. Dissociation and Characterization of Its Subunits*

Arie deKok and Allen B. Rawitch†

ABSTRACT: The quaternary structure of L-amino acid oxidase has been investigated by ultracentrifugation both in the presence of and in the absence of denaturing agent and by gel electrophoresis. Three variants of this enzyme (Wellner, D., and Meister, A. (1960), *J. Biol. Chem. 235*, 2013) are evident in starch gel electrophoresis experiments. While removal of the sialic acid residues of this glycoprotein results in an altered electrophoretic mobility, three bands are still observed. The native enzyme has been shown to be a noncovalent dimer, consisting of two subunits of molecular weight near 70,000. Two types of polypeptide chain occur in this enzyme system but in unequal amounts (a ratio of near 2.5:1). The three isozymes appear to be the result of the various combinations of these differing subunits to give native dimer. Peptide mapping studies are consistent with a high degree of homology between these two kinds of polypeptide chain. The chains differ, however, in both their electrophoretic mobility and amino-terminal residues.

Carboxymethylation studies indicate that the native enzyme contains both free sulfhydryl groupings and intrachain disulfide bridges.

L-Amino acid oxidase (EC 1.4.3.2) has been purified from *Crotalus adamanteus* venom by Wellner and Meister (1960). Upon crystallization, this enzyme was shown to be a glycoprotein containing approximately 5% carbohydrate and 2 moles of FAD/mole (130,000 g). While the enzyme appeared pure by several physical criteria, it could be resolved by both electrophoresis and ion-exchange chromatography into three components of equal specific activity.

As in the case of other flavoproteins, such as glucose oxidase (EC 1.1.3.4), lipoamide dehydrogenase (EC 1.6.4.3), and D-amino acid oxidase (EC 1.4.3.3) where two flavins per molecule of enzyme have also been found, the possibility of interaction between the two FAD moieties of L-amino acid oxidase during catalysis has been discussed (Wellner and Meister, 1961; Meister and Wellner, 1966; Massey and Curti, 1967; deKok and Veeger, 1967). While some spectral evidence has appeared which might suggest the possible role of an FAD–$FADH_2$ interaction during enzymatic catalysis, recent studies by Massey and Curti (1967) and by deKok and Veeger (1967) have attributed this spectral intermediate to a semiquinoid species of FAD and suggest independence of the FAD moieties. From a catalytic point o[view, the enzyme may therefore be considered to consist of two identical and independent active sties.

Because two similar FAD-containing enzymes, D-amino acid oxidase and lipoamide dehydrogenase, have been reported to consist of a pair of homologous or identical polypeptide chains (Massey *et al.*, 1962; Kotaki *et al.*, 1967), this has been suggested by Kotaki *et al.* as a characteristic of this group of flavoproteins. Because in these cases, an apparent dissociation to subunits was observed when the apoproteins were prepared, these authors suggest that FAD is the cementing force between the subunits. The existence of interchain disulfide bridges suggested by Massey *et al.* (1962) for lipoamide dehydrogenase has recently been questioned (Visser and Veeger, 1968). In the present paper we have studied L-amino acid oxidase under dissociating conditions both with and without prior reduction and alkylation. Information concerning the degree of homology between the peptide chains of

* From the Department of Biochemistry, Agricultural University, Wageningen, The Netherlands, and the Biochemistry Division, Department of Chemistry and Chemical Engineering, University of Illinois, Urbana, Illinois 61801. *Received October 23, 1968.* Supported by a research grant (GM 11223) from the U. S. Public Health Service, with partial financial aid from the Netherlands Organization for the Advancement of Pure Research (Z. W. O.).

† To whom inquiries should be directed at the University of Illinois.