# The Mechanism of Activation of Bovine Factor IX (Christmas Factor) by Bovine Factor $XI_a$ (Activated Plasma Thromboplastin Antecedent)†

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ABSTRACT: Bovine factor IX (Christmas factor) is a coagulation protein (mol wt 55,400) present in plasma in a precursor or zymogen form. It is a glycoprotein composed of a single polypeptide chain with an amino-terminal sequence of Tyr-Asn-Ser-Gly-. During coagulation, factor IX is converted to an enzyme, factor $IX_a$ (mol wt 46,500), by factor $XI_a$ (activated plasma thromboplastin antecedent). The activation reaction occurs in a two-step reaction. In the first step, an internal peptide bond in factor IX is cleaved, leading to the formation of an intermediate containing two polypeptide chains held together by a disulfide bond(s). This intermediate, which has no enzymatic activity, is composed of a light chain (mol wt 16,600) with an amino-terminal sequence of Tyr-Asn-Ser-Gly- and a heavy chain (mol wt 38,800) with an amino-terminal sequence of Ala-Glu-Thr-Ile-. In the second step of the activation reaction, an activation peptide (mol wt 9000) which contains about 50% of the carbohydrate present in factor IX is split from the amino-terminal end of the heavy chain, giving rise to factor $IX_a$. Thus, factor $IX_a$ is composed of two chains, a heavy chain (mol wt 27,300) with an amino-terminal sequence of Val-Val-Gly-Gly- and a light chain (mol wt 16,600) with an amino-terminal sequence of Tyr-Asn-Ser-Gly-. Other properties of factor $IX_a$ including its clotting activity and chemical composition are also described.

Factor IX (Christmas factor)[1] is a plasma glycoprotein which participates in the middle phase of intrinsic blood coagulation (Davie and Kirby, 1973). In individuals with factor IX deficiency (Christmas disease or hemophilia B), this protein is inactive or absent, resulting in a coagulation disorder characterized by intermittent bleeding into the joints and soft tissues. Like classic hemophilia, factor IX deficiency is an X-linked recessive disease which affects primarily males.

During coagulation, factor IX is converted to an enzyme, factor $IX_a$, by factor $XI_a$ (activated plasma thromboplastin antecedent) (Soulier *et al.*, 1958; Waaler, 1959; Ratnoff and Davie, 1962; Schiffman *et al.*, 1963; Nossel, 1964). Factor $IX_a$ then converts factor X (Stuart factor) to factor $X_a$ in the presence of factor VIII (antihemophilic factor), calcium ions, and phospholipid (Lundblad and Davie, 1964; Biggs and Macfarlane, 1965; Barton, 1967; Schiffman *et al.*, 1966; Hougie *et al.*, 1967). These reactions are shown in the following equations.

$$\text{factor IX} \xrightarrow{\text{factor } XI_a,\ Ca^{2+}} \text{factor } IX_a$$

$$\text{factor X} \xrightarrow{[\text{factor } IX_a\text{-factor VIII-}Ca^{2+}\text{-phospholipid}]} \text{factor } X_a$$

It has been suggested that factor $XI_a$ activates factor IX by limited proteolysis since factor $XI_a$ is a serine esterase which is inhibited by diisopropyl phosphofluoridate (Ratnoff and Davie, 1962; Kingdon *et al.*, 1964). This proposal is consistent with the fact that partially purified preparations of factor IX and factor $IX_a$ show different elution patterns following gel filtration (Kingdon, 1969; Østerud and Schiffman, 1972) and different electrophoretic mobilities (Schiffman *et al.*, 1964).

In the present experiments, the molecular events associated with the activation of factor IX have been examined employing homogeneous preparations of bovine factor IX and factor $XI_a$. The data indicate that the activation of factor IX involves the splitting of two peptide bonds in the precursor protein with the liberation of a glycoprotein of molecular weight 9000. This gives rise to factor $IX_a$ (mol wt 44,500), an enzyme which is composed of two polypeptide chains held together by a disulfide bond(s).

## Experimental Section

### *Materials*

Bovine factor IX was purified to a homogeneous state as previously described (Fujikawa *et al.*, 1973), except that diisopropyl phosphofluoridate was added to the barium sulfate eluate at a final concentration of $2.5 \times 10^{-4}$ M. Bovine factor XI was purified by $BaSO_4$ adsorption of contaminants, heparin-agarose column chromatography, CM-Sephadex column chromatography, gel filtration, and DEAE-column chromatography. This preparation, which shows one band on SDS[2] gel electrophoresis, was converted to factor $XI_a$ in the presence of pancreatic trypsin (Kato *et al.*, 1974). The details of the isolation and activation of factor XI will be published elsewhere. Human factor IX deficient plasma was provided by Dr. A. R. Thompson, Harborview Hospital, Seattle, and bovine factor X-deficient plasma was prepared according to Bachmann *et al.* (1958). Fibrinogen (98% clottable protein) was kindly provided by Dr. S. Iwanaga, Institute for Protein Research, Osaka, Japan.

Cephalin (rabbit brain extract), mannose, galactose, galactosamine, *p*-dimethylaminobenzaldehyde, acetylace-

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[1] The nomenclature for various coagulation factors is that recommended by an international nomenclature committee (Wright, 1959).

[2] Abbreviation used is: SDS, sodium dodecyl sulfate.

tone, *N*-acetylneuraminic acid, imidazole (grade I), and thiobarbituric acid were obtained from Sigma Chemical Co., St. Louis, Mo. Sephadex G-100, G-50, and G-25 were products of Pharmacia Fine Chemicals, Piscataway, N.J. Benzamidine hydrochloride, diisopropyl phosphofluoridate, and cyclohexanone were purchased from Aldrich Chemical Co., Milwaukee, Wis. 2-Mercaptoethanol, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and acrylamide (recrystallized before use) were purchased from Eastman Kodak Co., Rochester, N.Y. Guanidine hydrochloride (extreme purity) was obtained from Heico, Inc., Delaware Water Gap, Pa. 4-Vinylpyridine (distilled before use) was a product of Baker Chemical Co., Phillipsburg, N.J. All other chemicals were commercial preparations of the highest quality available.

*Methods*

SDS polyacrylamide gel electrophoresis was performed by the method of Weber and Osborn (1969) employing 8.5% gels unless otherwise stated. Samples were run at 8 mA/tube for 6 hr, and the gels were stained for protein with Coomassie Brilliant Blue. The per cent of each protein in the gels was determined by scanning each gel at 650 nm using a Gilford Linear Transport Model 24108 gel scanner and recorder attached to a Beckman DU spectrophotometer.

Zone electrophoresis was carried out in 0.05 M sodium barbital buffer (pH 8.6) on microscope slides (25 × 75 cm) layered with 1% agarose, as described by Williams and Chase (1971). Samples (2 μl containing 2 μg of protein) were placed in a small well and run at room temperature for 4 hr with 5 mA/slide. The slide was stained for protein with Coomassie Brilliant Blue.

The isolation of various fragments of the factor IX activation mixture was carried out as follows. Factor IX (50 mg) was incubated at 37° with 2 mg of factor $XI_a$ in the presence of $5 \times 10^{-3}$ M $CaCl_2$, 0.2 M NaCl, and 0.025 M Tris-HCl buffer (pH 7.2) in a final volume of 10 ml. After 30 min, the reaction was terminated by the addition of 0.75 ml of 0.1 M EDTA followed by lyophilization. The lyophilized sample was then reduced and *S*-pyridylethylated by the method of Friedman *et al.* (1970). Salt and excess reagents were removed by gel filtration on a Sephadex G-50 column (1.8 × 47 cm) in the presence of 9% formic acid. Protein fractions from the column were combined and lyophilized. Alkylated fragments were then separated by gel filtration on a Sephadex G-100 column (2.5 × 90 cm) in the presence of 3 M urea and 9% formic acid. Salt-free materials were obtained by passing the protein sample through a column of Sephadex G-25 (2.2 × 55 cm) with 9% formic acid followed by lyophilization.

Amino acid analyses and preparation of samples were carried out by the methods of Moore and Stein (1963) and Spackman *et al.* (1958) employing a Spinco Model 120 amino acid analyzer. Samples were hydrolyzed for 24, 48, 72, and 96 hr in evacuated tubes in 6 N HCl at 110°. Values for serine and threonine were determined by extrapolation to zero-time hydrolysis. Half-cystine was determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). Valine and isoleucine were calculated from the sample hydrolyzed for 96 hr.

Hexosamine was determined after hydrolyzing samples in 2 N HCl for 24 hr at 110° by the method of Elson and Morgan as described by Gardell (1957) using galactosamine as a standard. Neutral sugar was determined by the phenol–sulfuric acid method of Dubois *et al.* (1956) using a 1:1 mixture of galactose and mannose as a standard. Neuraminic acid was determined by the method of Warren (1959) using *N*-acetylneuraminic acid as a standard.

Amino-terminal analyses were performed with a Beckman sequencer, Model 890A. The operation of this instrument and the methods employed are adaptations (Hermodson *et al.*, 1972) of the technique of Edman and Begg (1967).

Ultracentrifugation was carried out in a Beckman Model E analytical ultracentrifuge as previously described (Fujikawa *et al.*, 1972a, 1973). Sedimentation equilibrium data were derived from the short column, high-speed technique of Yphantis (1964). Experiments were performed at three different concentrations of protein (0.75, 0.5, and 0.25 mg/ml) in the presence of 0.05 M sodium acetate (pH 5.5) and 6 M guanidine hydrochloride. A partial specific volume was determined by amino acid composition and corrected for carbohydrate content as described by Cohn and Edsall (1943) and Longsworth (1953).

Protein concentrations for factors IX and $IX_a$ were routinely determined by optical density at 280 nm. Values of 14.9 and 14.3 were determined for the $\epsilon_{280}(1\%)$ for factor IX and factor $IX_a$, respectively, in the analytical centrifuge employing the methods of Babul and Stellwagon (1969) and Richards *et al.* (1968). A refractive index increment of $1.86 \times 10^{-4}$ ml/mg determined for human plasma glycoproteins (Schmid, 1953) was employed for factor IX and factor $IX_a$. The protein concentration of factor $XI_a$ was also determined by absorbance at 280 nm assuming an $\epsilon_{280}(1\%)$ of 10.0. Carbohydrate analysis of the glycoprotein was determined by dry weight analysis after drying the sample *in vacuo* over phosphorus pentoxide.

For the routine assay of factor $IX_a$ activity, 0.1 ml of diluted sample in Michaelis buffer ($3.6 \times 10^{-2}$ M sodium acetate, $3.6 \times 10^{-2}$ M sodium barbital, and $1.45 \times 10^{-1}$ M sodium chloride (pH 7.4) containing 0.1 mg/ml of bovine serum albumin) was incubated at 37° for 30 sec with 0.1 ml of citrated normal bovine plasma and 0.1 ml of phospholipid suspension (one vial of rabbit brain extract, Sigma, suspended in 100 ml of 0.15 M NaCl). A 0.1-ml solution of $2.5 \times 10^{-2}$ M $CaCl_2$ was then added to the mixture and the clotting time was determined. Activity was calculated from a calibration curve where the log of factor $IX_a$ concentration was plotted against the log of the clotting time. This plot was linear from 40 to 130 sec when fresh bovine plasma was used as a substrate. For the specific assay of factor $IX_a$, 0.1 ml of diluted sample in Michaelis buffer and 0.1 ml of phospholipid solution was incubated with 0.1 ml of human factor IX deficient plasma for 30 sec at 37°. The clotting time was determined after the addition of 0.1 ml of 0.025 M $CaCl_2$. Factor IX was assayed as previously described (Fujikawa *et al.*, 1973). Factor $X_a$ activity was assayed by the method of Bachmann *et al.* (1958) except Russell's viper venom protease was deleted from the assay mixture. Thrombin activity was assayed by incubating 0.1 ml of test sample with 0.1 ml of 1% fibrinogen solution in 0.15 M NaCl.

## Results

*Activation of Bovine Factor IX by Factor $XI_a$.* A time curve for the activation of factor IX by factor $XI_a$ is shown in Figure 1. In these experiments, factor IX was activated at pH 8.0 in the presence of $5 \times 10^{-3}$ M $CaCl_2$. The weight ratio of substrate to enzyme was 50:1. Under these condi-

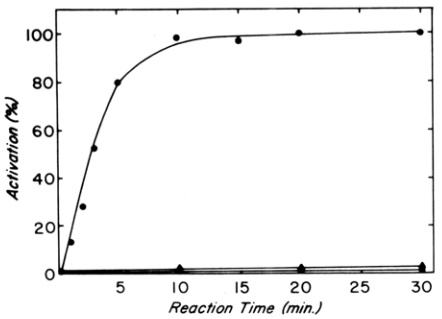


FIGURE 1: Time curve of activation of factor IX with factor $XI_a$. Factor IX (1 mg) was incubated at 37° with 20 $\mu$g of factor $XI_a$ in the presence of 0.05 M Tris-HCl (pH 8.0) and $5 \times 10^{-3}$ M $CaCl_2$ in a final volume of 2.0 ml. At the time desired, 0.2 ml of the reaction mixture was withdrawn to a test tube containing 20 $\mu$l of 0.1 M EDTA. Factor $IX_a$ activity was assayed as described in the Methods after a 1000-fold dilution of sample with Michaelis buffer. The activity of the 30-min reaction mixture was defined as 100% activity, and per cent activity of each reaction was determined from a standard curve. (●) complete reaction; (▲) reaction without $Ca^{2+}$; (■) reaction without factor $XI_a$.

tions, the reaction was essentially complete in 15 min (solid circles). In the absence of $Ca^{2+}$ (solid triangles) or factor $XI_a$ (solid squares), no activation of factor IX occurred.

Factor IX and factor $IX_a$ were next subjected to gel filtration on Sephadex G-100 to compare the two proteins. In these experiments, factor $IX_a$ from a 30-min incubation mixture (as shown in Figure 1) was employed. The protein peak for factor IX (top panel, Figure 2) appeared after 63 ml of effluent passed through the column. The protein peak for factor $IX_a$ (lower panel, Figure 2) appeared after 72 ml of effluent passed through the column. A small shoulder was observed just prior to factor $IX_a$ (lower panel) which was due to the presence of factor IX. This was due to the incomplete conversion of the precursor to the active enzyme.

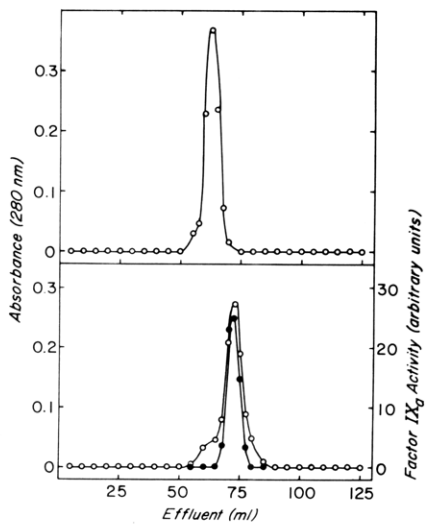


FIGURE 2: Gel filtration patterns of factors IX and $IX_a$. Factor IX (2.9 mg) was activated in a final volume of 1.5 ml with 56 $\mu$g of factor $XI_a$ under the same conditions as described in Figure 1 except that 7 $\mu$g of factor $XI_a$ was added after 25-min incubation and the reaction permitted to proceed for an additional 10 min. The reaction was then terminated by the addition of 0.1 ml of 0.1 M EDTA, and the complete reaction mixture was applied to a Sephadex G-100 column ($1.7 \times 88$ cm) and the protein was eluted with 0.05 M imidazole buffer (pH 6.0) containing 0.2 M NaCl, $5 \times 10^{-4}$ M EDTA, and $2 \times 10^{-3}$ M benzamidine. The top panel is the elution profile for factor IX and the bottom is for factor $IX_a$. (○) Optical density at 280 nm; (●) factor $IX_a$ activity. The absorbance due to protein was calculated by subtracting the basal absorbance due to $2 \times 10^{-3}$ M benzamidine.

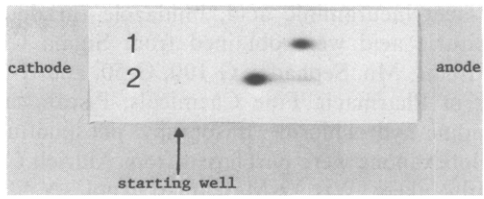


FIGURE 3: Zone electrophoresis of bovine factor IX and factor $IX_a$. Each protein (2 $\mu$g) was applied to the starting wells and electrophoresis was run at 5 mA/slide for 4 hr as described in Methods. Sample 1 is factor IX and sample 2 is factor $IX_a$.

A difference in the electrophoretic properties of factor IX and factor $IX_a$ was also apparent in 0.05 M barbital buffer (pH 8.6) (Figure 3). Under these conditions, factor IX (sample 1) has a faster electrophoretic mobility than factor $IX_a$ (sample 2). The change in gel filtration and electrophoretic properties of these two proteins indicates that the molecular weight of factor IX has been reduced during the activation reaction and this change is due to the release of an acidic fragment(s) from the precursor protein. These results extend those of Kingdon (1969), Østerud and Schiffman (1972), and Schiffman *et al.* (1964) who previously reported a small difference in the gel filtration pattern and electrophoretic mobility of partially purified factors IX and $IX_a$.

Further evidence of a change in molecular weight in factor IX during the activation reaction was shown in experiments employing SDS polyacrylamide gel electrophoresis (Figure 4). In these experiments, aliquots were removed at various times from a factor IX activation mixture (corresponding to that shown in Figure 1) and analyzed by gel electrophoresis. At zero time, a single protein band was observed for factor IX. During the first few minutes of incubation, a new faster moving band appeared and this band corresponds to the band obtained for factor $IX_a$ isolated by gel filtration (gel on the far right, Figure 2). After 20–30 min, nearly all of the factor IX was converted to the band corresponding to factor $IX_a$. These experiments provide further evidence to indicate that a fragment(s) has been split from factor IX, giving rise to factor $IX_a$, a protein of lower molecular weight. Furthermore, these experiments suggest that the fragment(s) split from factor IX during the activa-

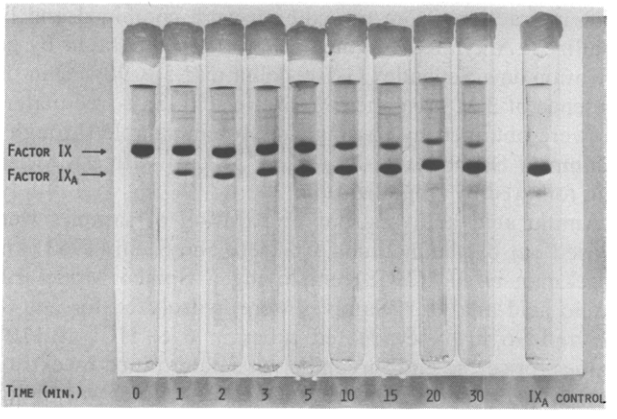


FIGURE 4: SDS polyacrylamide gel electrophoresis of factor IX following activation with factor $XI_a$. Each 25 $\mu$l of sample containing 12.5 $\mu$g of protein was removed from a reaction mixture corresponding to that shown in Figure 1 and incubated at 37° for 4 hr with 2% SDS in the absence of reducing reagent. Electrophoresis was carried out at 8 mA/tube for 6 hr. The gel pattern at the far right of the figure is a control sample of factor $IX_a$ prepared as described in the legend for Figure 2.

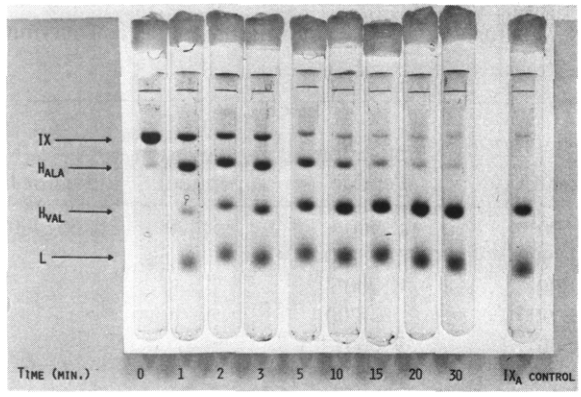


FIGURE 5: SDS polyacrylamide gel electrophoresis of factor IX activation with factor $XI_a$ after reduction of the reactants. The samples and conditions are identical with those described in Figure 4 except the protein was incubated with 2% SDS and 5% 2-mercaptoethanol before electrophoresis.

tion reaction is probably quite small since it was not detected by the staining procedure and probably migrated to the end of the gels under the conditions employed.

Since bovine factor IX is composed of a single polypeptide chain (Fujikawa *et al.*, 1973), it was of interest to examine the activation mixture by gel electrophoresis after reduction with 2-mercaptoethanol to determine whether new polypeptide chains were formed during the activation reaction. The results of these experiments are shown in Figure 5. The zero-time sample showed one band, corresponding to the single chain of factor IX. Within the first minute of incubation, however, three new polypeptide chains appeared. The two faster moving protein bands (labeled $H_{Val}$ and L) corresponded to the two polypeptide chains which are found in factor $IX_a$ (last gel on the right). The protein band migrating just ahead of factor IX (labeled $H_{Ala}$) appeared very rapidly and then disappeared during the next 15–20 min.

The per cent of the total protein present in each of the various bands shown in Figure 5 was then estimated by a scanning densitometer (Figure 6). The open circles correspond to factor IX and show the rapid disappearance of the precursor protein with time. The open squares correspond to the second band in Figure 4 labeled $H_{Ala}$. This band reaches a peak within the first minute of incubation and rapidly decreases as the activation reaction continues. The solid circles correspond to the third band in Figure 5 labeled

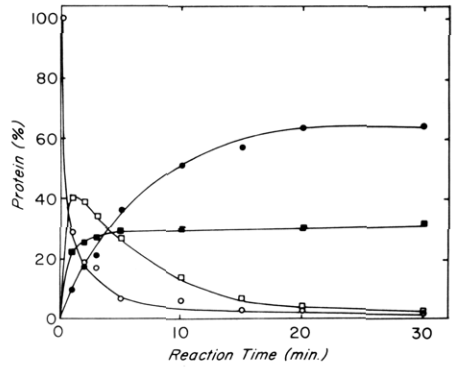


FIGURE 6: Gel scan of the reduced proteins shown in Figure 5 for the activation of factor IX. The gels in Figure 5 were scanned as described in Methods. The per cent of each protein fragment was plotted against reaction time. (○) Factor IX; (□) $H_{Ala}$ band; (●) $H_{Val}$ band; (■) L band.

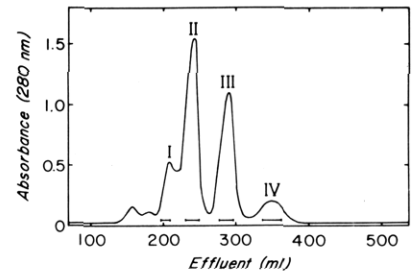


FIGURE 7: Gel filtration pattern for *S*-pyridylethylated fragments from a factor IX activation mixture. Factor IX (50 mg) was activated with factor $XI_a$ and *S*-pyridylethylated derivatives were prepared as described in Methods. *S*-Pyridylethylated fragments were separated by gel filtration on a Sephadex G-100 column (2.5 × 90 cm) with 3 M urea in 9% formic acid at a flow rate of about 6 ml/hr. The yield for each component was as follows: 4 mg of peak I, 17 mg of peak II, 16 mg of peak III, and 4 mg of peak IV on a dry weight basis.

$H_{Val}$. The appearance of this band, which is also present in factor $IX_a$, directly coincides with the formation of factor $IX_a$ activity. The solid squares correspond to the fourth band in Figure 5 labeled L. This polypeptide chain is formed during the first 5 min of the incubation and remains constant throughout the remainder of the activation reaction. The molecular weights for the heavy ($H_{Val}$) and light (L) polypeptide chains shown in Figure 5 were 27,300 and 16,600, respectively, as determined by sedimentation equilibrium (data presented below). The molecular weight for the $H_{Ala}$ heavy chain was 38,000 as estimated by SDS polyacrylamide gel electrophoresis. One interpretation of these data is that factor IX is activated by factor $XI_a$ in a two-step reaction. In the first step, factor IX (mol wt 55,400) is converted to an intermediate containing a light chain (L) with a molecular weight of 16,600 and a heavy chain ($H_{Ala}$) with a molecular weight of approximately 38,000. These two chains are held together by a disulfide bond(s). This reaction is then followed by a second step which involves the cleavage of a fragment from the heavy chain of the intermediate. This leads to the formation of factor $IX_a$ which is composed of a light chain (L) with a molecular weight of 16,600 and a heavy chain ($H_{Val}$) with a molecular weight of 27,300. These two chains are also linked together by a disulfide bond(s).

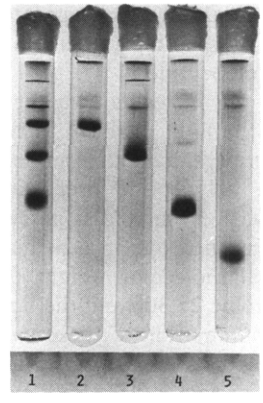


FIGURE 8: SDS polyacrylamide gel electrophoresis of *S*-pyridylethylated fragments isolated by gel filtration. The various *S*-pyridylethylated fragments were isolated as shown in Figure 6 and incubated at 37° for 4 hr with 2% SDS and 5% 2-mercaptoethanol and applied to 10% polyacrylamide gels. Gel 1 is 12.5 μg of the partially activated factor IX taken from a 5-min reaction mixture as shown in Figure 3. Gels 2, 3, and 4 are 12.5 μg each of peaks I, II, and III, respectively. Gel 5 is 40 μg of peak IV.

TABLE I: Molecular Weight of the Heavy and Light Chains of Factor $IX_a$, Factor $IX_a$, and the Activation Peptide, as Determined by Sedimentation Equilibrium.[a]

| | Heavy Chain ($H_{Val}$) | Light Chain | Factor $IX_a$ (heavy + light chains) | Factor $IX_a$ | Activation Peptide | (Factor $IX_a$ + activation peptide) | Factor IX |
|---|---|---|---|---|---|---|---|
| $M_1$ | 27,300[b] ± 600 | 16,600[c] ± 300 | 43,900 | 46,500 ± 900 | 8,900 ± 200 | 55,400 | 55,400[d] |
| $M_1$ | 28,000[e] ± 600 | 18,000[e] ± 300 | | 46,500 ± 900 | 8,900[e] ± 200 | | |
| $M_n$ | 28,800 ± 600 | 18,500 ± 500 | | 47,500 ± 1000 | 9,300 ± 300 | | |
| $M_w$ | 30,300 ± 700 | 19,100 ± 500 | | 48,400 ± 1100 | 9,400 ± 300 | | |
| $M_z$ | 31,300 ± 700 | 19,800 ± 600 | | 49,700 ± 1200 | 10,100 ± 400 | | |
| $\bar{v}$[f] | 0.715 | 0.686 | | 0.706 | 0.652 | | |

[a] Molecular weight averages were calculated as whole cell average molecular weights in experiments employing three different concentrations of protein in 0.05 M NaOAc (pH 5.5), in the presence of 6 M guanidine hydrochloride, as described in Methods. $M_1$ refers to the smallest molecular weight species calculated by the methods described by Teller *et al.* (1969). $M_n$, $M_w$, and $M_z$ refer to the number average molecular weight, the weight average molecular weight, and the Z-average molecular weight, respectively, as defined by Kraemer (1940). [b] Corrected for 7.1 *S*-pyridylethyl residues. [c] Corrected for 14.6 *S*-pyridylethyl residues. [d] From Fujikawa *et al.* (1973). [e] Pyridylethyl preparations were employed. [f] Partial specific volume was determined by amino acid composition and corrected for carbohydrate content (Table III), as described in Methods. No corrections were made for *S*-pyridylethyl residues. The $\bar{v}$ for the activation peptide may be in substantial error since the peptide contains 70% carbohydrate. A 10% error in this value, however, would only change the molecular weight of the activation peptide by ±1150.

To test this proposal, 50 mg of factor IX was activated in the presence of factor $XI_a$ followed by reduction and *S*-pyridylethylation. The chains were separated by gel filtration in the presence of 3 M urea in 9% formic acid (Figure 7). Four major peaks were observed. The proteins in each of these peaks (as shown by the bars) were lyophilized after removal of urea by a Sephadex G-25 column. SDS gel electrophoresis of the protein in each of the four peaks is shown in Figure 8. Gel 1 corresponds to the 5-min incubation sample shown in Figure 5. Gel 2 is peak I from Figure 7 and corresponds to the $H_{Ala}$ heavy chain. Gel 3 is peak II and corresponds to the $H_{Val}$ heavy chain. Gel 4 is peak III and corresponds to the light chain L. Gel 5 is peak IV. Thus, two heavy chains, a light chain, and a new fast moving polypeptide present in peak IV, were separated by gel filtration. The fast moving polypeptide present in peak IV has been called the activation peptide.

Table I shows the molecular weight data for the $H_{Val}$ heavy chain and the light chain of factor $IX_a$, factor $IX_a$, and the activation peptide as determined by sedimentation equilibrium. The polypeptide preparations were *S*-pyridylethylated derivatives isolated as described above and analyzed in the ultracentrifuge in 6 M guanidine hydrochloride. The minimal molecular weight for the $H_{Val}$ heavy chain was found to be 27,300 and the light chain was found to be 16,600. A summation of these two chains is 43,900. This value corresponds well to the molecular weight of 46,500 obtained for factor $IX_a$. The molecular weight for the activation peptide was found to be 8900. A summation of the molecular weights of factor $IX_a$ (46,500) and the activation peptide (8900) is 55,400, and this value is identical with that determined for factor IX (Fujikawa *et al.*, 1973). A summation of the activation peptide in the $H_{Val}$ heavy chain is 36,200. This corresponds to the molecular weight of the $H_{Ala}$ heavy chain as estimated by SDS gel electrophoresis. The molecular weight of the $H_{Ala}$ heavy chain was not determined by sedimentation equilibrium because of the limited availability of this polypeptide.

*Amino-Terminal Sequence.* The amino-terminal sequences for the various polypeptides shown in Figure 8 were carried out on a Beckman sequencer. The light chain was found to contain Tyr-Asn-Ser-Gly- as its amino-terminal sequence. This sequence is the same as that found in the precursor molecule (Fujikawa *et al.*, 1973). Thus, the light chain in factor $IX_a$ arises from the amino-terminal portion of the precursor molecule during the first step of the activation reaction. The $H_{Ala}$ heavy chain and the activation peptide contained the sequence of Ala-Glu-Thr-Ile-Phe-. These data indicate that the $H_{Ala}$ heavy chain is formed from the internal portion of the precursor molecule in the first step of the activation reaction. Furthermore, the activation peptide is split from the amino-terminal end of the $H_{Ala}$ heavy chain in the second step of the activation reaction.

The results of the amino-terminal analysis for the $H_{Val}$ heavy chain are shown in Table II. The first four residues were Val-Val-Gly-Gly-. This sequence is identical with that reported for the B chain of human plasmin and is similar to that for the heavy chain of bovine factor $X_a$ and the B chain of bovine and human thrombin. These studies extend our earlier observations of homology at the amino-terminal end of the precursor form of these plasma proteins (Fujikawa *et al.*, 1974).

*Amino Acid and Carbohydrate Analyses.* The amino acid and carbohydrate compositions for the $H_{Val}$ heavy chain, the light chain, activation peptide, and factors $IX_a$ and IX are shown in Table III. A summation of the amino acids present in the $H_{Val}$ heavy chain and light chain (column 3) provides the amino acid composition of factor $IX_a$ (mol wt 44,000). These values are slightly higher when calculated on the basis of a molecular weight of 46,500 (Table

TABLE II: Amino-Terminal Sequence of the Heavy Chain of Factor $IX_a$ and Other Plasma Proteases.

| Enzyme | Amino-Terminal Sequence | | | |
|---|---|---|---|---|
| Bovine factor $IX_a$[a] | Val | Val | Gly | Gly |
| Bovine factor $X_a$[b] | Ile | Val | Gly | Gly |
| Bovine thrombin[c] | Ile | Val | Glu | Gly |
| Human thrombin[d] | Ile | Val | Glu | Gly |
| Bovine plasmin[e] | Ile | Val | Gly | Gly |
| Human plasmin[f] | Val | Val | Gly | Gly |

[a] $H_{Val}$ heavy chain of factor $IX_a$. [b] Heavy chain of factor $X_a$ (Fujikawa *et al.*, 1972b). [c] B chain of bovine thrombin (Magnusson, 1971). [d] B chain of human thrombin (Thompson *et al.*, 1974). Magnusson and Steele (1965) reported a sequence of Ile-Val-Gly-Gly- for human thrombin. [e] Light chain of bovine plasma (Nagasawa and Suzuki, 1970). [f] B chain of human plasmin (Robbins *et al.*, 1972).

I). A summation of the amino acids present in factor $IX_a$ and the activation peptide (column 4) is provided in column 5, and these data agree reasonably well with the amino acid composition factor IX (column 6).

It is also evident from these data that the activation peptide is a glycopeptide. Indeed, approximately 50% of the carbohydrate present in factor IX was removed during the activation reaction. The recovery of hexose and hexosamine for the various fractions was relatively good. This is evident by comparing a summation of the heavy and light chains and activation peptide (column 5) with factor IX (column 6). The recovery of neuraminic acid, however, was only 30% of the expected amount. Therefore, the number of residues of neuraminic acid in the various chains was increased threefold in the calculations for per cent protein and carbohydrate listed on the bottom of Table III for the heavy and light chains, the activation peptide, and factor $IX_a$. These calculations assume that the loss of neuraminic acid was about the same in each peptide and, thus, these values eventually may require some minor revision.

*Other Properties of Bovine Factor $IX_a$.* Factor $IX_a$ has no effect on factor IX in the presence of calcium ions. In these experiments, a molar ratio of factor IX to $IX_a$ of 100:1 or 10:1 was employed. Under these conditions, no factor IX activation was observed. Furthermore, no change was detected in the SDS gel electrophoresis pattern for factor IX after incubation with factor $IX_a$ in the presence or absence of 2-mercaptoethanol. These experiments indicate that factor $IX_a$ neither activates nor converts factor IX to the intermediate previously described.

Bovine factor $IX_a$ readily clots human factor IX deficient

TABLE III: Amino Acid and Carbohydrate Compositions of Factor IX and Its Components.

| | Heavy Chain ($H_{Val}$) (residues/ 27,300) | Light Chain (residues/ 16,600) | Heavy + Light Chains Factor $IX_a$ (residues/ 44,000) | Activation Peptide (residues/ 8900) | Heavy + Light Chains + Activation Peptide (Factor IX) | Factor IX (residues/ 55,400) |
|---|---|---|---|---|---|---|
| Amino acid | | | | | | |
| Lysine | 17.7 | 12.8 | 30.5 | 0.6 | 31.1 | 27.6 |
| Histidine | 7.7 | 1.0 | 8.7 | 0.2 | 8.9 | 8.1 |
| Arginine | 8.7 | 7.2 | 15.9 | 0.6 | 16.5 | 17.0 |
| Aspartic acid | 16.4 | 16.8 | 33.2 | 3.6 | 36.8 | 36.3 |
| Threonine | 13.4 | 7.2 | 20.9 | 1.6 | 22.5 | 20.1 |
| Serine | 15.2 | 9.4 | 24.6 | 3.1 | 27.7 | 29.0 |
| Glutamic acid | 21.9 | 23.2 | 45.1 | 3.8 | 48.9 | 46.6 |
| Proline | 8.5 | 2.2 | 10.7 | 0.2 | 10.9 | 13.1 |
| Glycine | 23.1 | 9.7 | 32.8 | 0.7 | 33.5 | 29.7 |
| Alanine | 13.6 | 5.3 | 18.9 | 1.0 | 19.9 | 18.8 |
| Half-cystine[a] | 7.1 | 14.6 | 21.7 | 0.3 | 22.0 | 17.2 |
| Valine | 16.7 | 6.9 | 23.6 | 0.7 | 24.3 | 24.9 |
| Methionine | 1.9 | 0.9 | 2.8 | 0.6 | 3.4 | 2.6 |
| Isoleucine | 16.0 | 2.9 | 18.9 | 1.4 | 20.3 | 19.3 |
| Leucine | 13.9 | 5.5 | 19.4 | 4.0 | 23.4 | 18.7 |
| Tyrosine | 12.0 | 2.3 | 15.3 | 0.6 | 15.9 | 16.9[b] |
| Phenylalanine | 7.3 | 6.0 | 13.3 | 1.3 | 14.6 | 15.2 |
| Tryptophan | | | | | | 11.4 |
| Carbohydrate | | | | | | |
| Hexose | 8.7 | 6.7 | 15.4 | 12.2 | 27.6 | 32.6 |
| *N*-Acetylhexosamine | 6.1 | 1.2 | 7.3 | 11.8 | 19.1 | 16.4 |
| *N*-Acetylneuraminic acid | 1.3 | 0.2 | 1.5 | 3.7 | 5.2 | 15.6 |
| Protein (%) | 87 | 92.6 | 89 | 30 | | 74.2 |
| Carbohydrate (%) | 13 | 7.4 | 11 | 70 | | 25.8 |

[a] Determined as *S*-pyridylethylcysteine by the method of Friedman *et al.* (1970). [b] The original determination by the spectrophotometric assay of Bencze and Schmid (1957) gave a value of 9.2 (Fujikawa *et al.*, 1973). The new value of 16.9 was determined by amino acid analysis and shows better agreement with the summation of tyrosine content in the activation peptide and factor $IX_a$.

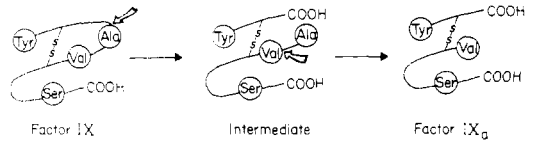


FIGURE 9: The mechanism of activation for bovine factor IX by bovine factor $XI_a$.

plasma in the absence of kaolin. In the assay as described in Methods, 80 $\eta$g of factor $IX_a$/0.4 ml of assay mixture results in a clotting time of 75 sec. With fresh normal bovine plasma in the absence of kaolin, 40 $\eta$g of protein/0.4 ml of assay mixture results in a clotting time of 40 sec. This same amount of factor $IX_a$, however, will clot bovine factor X deficient plasma in 88–90 sec. This is equivalent to a factor $X_a$ contamination of about 0.25%. Factor X deficient plasma prepared by the method of Bachmann *et al.* (1958), however, is not free of factor X. Thus, the apparent factor $X_a$ activity shown in the factor X deficient plasma assay is very likely due to the activation of residual factor X by factor $IX_a$. This interpretation is also consistent with the fact that neither factor IX nor factor XI contains detectable factor X activity.

Factor $IX_a$ was also found to be free of thrombin activity. In these experiments, 50 $\mu$g of factor $IX_a$/0.2 ml was incubated with fibrinogen. No clot formation occurred after 4 hr of incubation at 37°. Factor $IX_a$ proved to be very stable and no loss of activity was detected after several months of storage at −20° in 0.2 M NaCl containing 0.05 M imidazole buffer (pH 6.0).

## Discussion

The present experiments indicate that the activation of bovine factor IX by factor $XI_a$ results in the cleavage of two peptide bonds in the precursor protein. Furthermore, both of these steps are catalyzed by factor $XI_a$. The mechanism of this activation reaction is illustrated in Figure 9. Factor IX is composed of a single polypeptide chain with an amino-terminal tyrosine. In the first step of the activation reaction, factor IX is cleaved into a light chain (mol wt 16,600) with an amino-terminal tyrosine and a heavy chain (mol wt 38,800) with an amino-terminal alanine. This process results in the formation of a two-chain intermediate held together by a disulfide bond(s). This protein lacks enzymatic activity. In the second step of the activation reaction, an activation peptide is split from the amino-terminal end of the heavy chain, producing a smaller heavy chain with an amino-terminal valine. This final cleavage is coincident with the formation of factor $IX_a$ (mol wt 46,500) which now has enzymatic activity. Thus, factor $IX_a$ is composed of a heavy and a light chain held together by a disulfide bond(s). Furthermore, the light chain in factor $IX_a$ arose from the amino-terminal portion of the precursor molecule.

There is no clear lag phase in the formation of factor IX during the activation reaction (Figure 1). This indicates that the first step in the activation reaction is extremely rapid and the second step is rate limiting. This interpretation is further indicated by the rapid appearance of the $H_{Ala}$ heavy chain (Figure 6) and the relatively slow appearance of the $H_{Val}$ heavy chain which coincides with the formation of factor $IX_a$ activity.

Another possible mechanism for the initial activation of factor IX might be that it occurs by the cleavage of a single internal peptide bond by factor $XI_a$. This would give rise to factor $IX_a$ with an amino-terminal tyrosine and valine and the same molecular weight as factor IX. The resulting factor $IX_a$ could then cleave factor IX leading to the formation of the intermediate containing an amino-terminal alanine and tyrosine. This intermediate could then be activated by factor $XI_a$, as shown in Figure 9. This mechanism for the activation of factor IX, however, appears unlikely since no change in factor IX has been detected in the presence of factor $IX_a$ and calcium ions.

The second step in the activation of factor IX, as shown in Figure 9, is very similar to that for the activation of bovine factor X (Fujikawa *et al.*, 1972b). Factor X can be activated by factor $IX_a$ and factor VIII (intrinsic pathway) (Lundblad and Davie, 1964; Biggs and Macfarlane, 1965; Schiffman *et al,* 1966; Hougie *et al.*, 1967; Barton, 1967; Radcliffe and Barton, 1973) or by tissue factor and factor VII (extrinsic pathway) (Flynn and Coon, 1953; Hjort, 1957; Hougie, 1959; Straub and Duckert, 1961; Deutsch *et al.*, 1964; Nemerson and Spaet, 1964; Williams and Norris, 1966; Nemerson and Pitlick, 1970; Østerud *et al.*, 1972; Radcliffe and Barton, 1973; Jesty and Nemerson, 1974). It is also activated by other proteolytic enzymes such as trypsin (Ferguson *et al.*, 1960; Pechet and Alexander, 1960; Papahadjopoulos *et al.*, 1964; Yin, 1964; Radcliffe and Barton, 1973; Bajaj and Mann, 1973) and a protease from Russell's viper venom (Macfarlane 1961; Esnouf and Williams, 1962; Papahadjopoulos *et al.*, 1964; Fujikawa *et al.*, 1972b; Radcliffe and Barton, 1972, 1973; Jesty and Esnouf, 1973). In each of these mechanisms, a small glycopeptide is split from the amino-terminal end of the heavy chain, giving rise to the active enzyme (Fujikawa *et al.*, 1972b, 1974b). Factor X when isolated from bovine plasma already contains a heavy and a light chain (Fujikawa *et al.*, 1972a; Jackson, 1972). This protein corresponds to the factor IX intermediate described above. Mattock and Esnouf (1973), however, have reported that factor X may be present in plasma as a single polypeptide chain. Thus, the activation of a single chain factor X preparation could be analogous to the activation of factor IX involving a two-step reaction.

Factor $IX_a$, like factor $X_a$ and thrombin, is a serine esterase with a reactive serine residue located in the heavy chain.[3] The light chain in these three clotting enzymes may be instrumental in conferring the high degree of substrate specificity which each of these proteases possess. The origin of these three light chains differ, however, in relationship to the precursor molecule. The light chain (A chain) of thrombin arises from an internal portion of prothrombin while the light chains of factor $IX_a$ and factor $X_a$ originate from the amino-terminal portion of the zymogen molecule. The fragment released from the amino-terminal portion of prothrombin (mol wt 22,000–25,000) (Heldebrant and Mann, 1973; Owen *et al.*, 1974) shows a striking similarity in sequence with the light chains of factor $IX_a$ and factor $X_a$ (Fujikawa *et al.*, 1974a). Also, there is a marked similarity in the amino acid composition of these three proteins which have a very high aspartic acid (including asparagine) and glutamic acid (including glutamine) content. The recent experiments of Stenflo *et al.* (1974) indicate that some of these glutamic acid residues are actually $\gamma$-carboxyglutamic acid which play an important role in calcium binding to prothrombin. Furthermore, these residues result from a carboxylation reaction related to vitamin K. Thus, it appears highly likely that some of the glutamic acid residues present

[3] Enfield, D., personal communication.

in the light chain of factor $IX_a$ and factor $X_a$ are also γ-carboxyglutamic acid since these two proteins are also dependent on vitamin K for their biosynthesis.

The activation of factor IX by factor $XI_a$ requires the presence of calcium ions. In the absence of calcium ions, there is a slow conversion of factor IX to the inactive intermediate.[4] Factor $XI_a$, however, is unable to convert the factor IX intermediate to factor $IX_a$ in the absence of calcium ions. Thus, calcium ions greatly accelerate the first step in the activation of factor IX and are an absolute requirement in the second step of the activation reaction. Factor IX prepared in the absence of diisopropyl phosphofluoridate occasionally contains some of the two-chain intermediate shown in Figure 9. Whether this reaction is also catalyzed by the presence of traces of partially activated factor $XI_a$ or some other protease in the plasma is not known.

At the present time, the peptide bonds in factor IX which are split by factor $XI_a$ during the activation reaction have not been completely identified. Factor $XI_a$, however, has esterase activity toward benzoyl arginyl ethyl ester (Kingdon *et al.*, 1964).[5] This suggests that an arginyl–alanine bond may be split in the first step and an arginyl–valine bond may be split in the second step of the activation of factor IX.

Whether factor IX can be activated directly by splitting only one internal peptide bond at the valine residue is not known. As previously mentioned, such a reaction could give rise to factor $IX_a$ without a change in molecular weight. Experiments are now in progress in our laboratory to test whether other proteolytic enzymes or coagulation factors are capable of activating factor IX and whether this can be accomplished in a single step reaction.

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References

Babul, J., and Stellwagen, E. (1969), *Anal. Biochem. 28,* 216.

Bachmann, F., Duckert, F., and Koller, F. (1958), *Thromb. Diath. Haemorrh. 2,* 24.

Bajaj, S. P., and Mann, K. G. (1973), *J. Biol. Chem. 248,* 7729.

Barton, P. G. (1967), *Nature (London) 215,* 1508.

Bencze, W. L., and Schmid, K. (1957), *Anal. Chem. 29,* 1193.

Biggs, R., and Macfarlane, R. G. (1965), *Thromb. Diath. Haemorrh., Suppl. 17,* 23.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N.Y., Reinhold, p 370.

Davie, E. W., and Kirby, E. P. (1973), *Curr. Top. Cell. Regul. 7,* 51.

Deutsch, E., Irsigler, K., and Lomoschitz, H. (1964), *Thromb. Diath. Haemorrh. 12,* 12.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28,* 350.

Edman, P., and Begg, G. (1967), *Eur. J. Biochem. 1,* 80.

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

Ferguson, J. H., Wilson, E. G., Iatridis, S. G., Rierson, H. A., and Johnston, B. R. (1960), *J. Clin. Invest. 39,* 1942.

Flynn, J. E., and Coon, R. W. (1953), *Amer. J. Physiol. 175,* 289.

Friedman, M., Krull, L. H., and Cavins, J. F. (1970), *J. Biol. Chem. 245,* 3865.

Fujikawa, K., Coan, M. H., Enfield, D. L., Titani, K., Ericsson, L. H., and Davie, E. W. (1974a), *Proc. Nat. Acad. Sci. U. S. 71,* 427.

Fujikawa, K., Coan, M. H., Legaz, M. E., and Davie, E. W. (1974b), *Biochemistry* (in press).

Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972a), *Biochemistry 11,* 4882.

Fujikawa, K., Legaz, M. E., and Davie, E. W. (1972b), *Biochemistry 11,* 4892.

Fujikawa, K., Thompson, A. R., Legaz, M. E., Meyer, R. G., and Davie, E. W. (1973), *Biochemistry 12,* 4938.

Gardell, S. (1957), *Methods Biochem. Anal. 6,* 289.

Heldebrant, C. M., and Mann, K. G. (1973), *J. Biol. Chem. 248,* 3642.

Hermodson, M. A., Ericsson, L. H., Titani, K., Neurath, H., and Walsh, K. A. (1972), *Biochemistry 11,* 4493.

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

Hougie, C. (1959), *Proc. Soc. Exp. Biol. Med. 101,* 132.

Hougie, C., Denson, K. W. E., and Biggs, R. (1967), *Thromb. Diath. Haemorrh. 18,* 211.

Jackson, C. M. (1972), *Biochemistry 11,* 4873.

Jesty, J., and Esnouf, M. P. (1973), *Biochem. J. 131,* 791.

Jesty, J., and Nemerson, Y. (1974), *J. Biol. Chem. 249,* 509.

Kato, H., Fujikawa, K., and Legaz, M. E. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1505.

Kingdon, H. S. (1969), *J. Biomed. Mater. Res. 3,* 25.

Kingdon, H. S., Davie, E. W., and Ratnoff, O. D. (1964), *Biochemistry 3,* 166.

Kraemer, E. O. (1940), *in* The Ultracentrifuge, Svedberg, T., and Petersen, K. O., Ed., London, Oxford University Press, p 345.

Longsworth, L. G. (1953), *J. Amer. Chem. Soc. 75,* 5705.

Lundblad, R. L., and Davie, E. W. (1964), *Biochemistry 3,* 1720.

Macfarlane, R. G. (1961), *Brit. J. Haematol. 7,* 496.

Magnusson, S. (1971), *Enzymes, 3rd Ed. 3,* 277.

Magnusson, S., and Steele, B. (1965), *Ark. Kemi 24,* 359.

Mattock, P., and Esnouf, M. P. (1973), *Nature (London), New Biol. 242,* 90.

Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6,* 819.

Nagasawa, S., and Suzuki, T. (1970), *Biochem. Biophys. Res. Commun. 41,* 562.

Nemerson, Y., and Pitlick, F. A. (1970), *Biochemistry 9,* 5100.

Nemerson, Y., and Spaet, T. H. (1964), *Blood 23,* 657.

Nossel, H. L. (1964), The Contact Phase of Blood Coagulation, Davis, Philadelphia.

Østerud, B., Berre, A., Otnaess, A.-B., Bjorklid, E., and Prydz, H. (1972), *Biochemistry 11,* 2853.

Østerud, B. and Schiffman, S. (1972), *Thromb. Diath. Haemorrh. 28,* 317.

Owen, W. G., Esmon, C. T., and Jackson, C. M. (1974), *J. Biol. Chem. 249,* 594.

[4] Fujikawa, K., and Davie, E. W., unpublished data.

[5] Kato, H., and Davie, E. W., unpublished data.

Papahadjopoulos, D., Yin, E. T., and Hanahan, D. J. (1964), *Biochemistry 3,* 1931.
Pechet, L., and Alexander, B. (1960), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 19,* 64.
Radcliffe, R. D., and Barton, P. G. (1972), *J. Biol. Chem. 247,* 7735.
Radcliffe, R. D., and Barton, P. G. (1973), *J. Biol. Chem. 248,* 6788.
Ratnoff, O. D., and Davie, E. W. (1962), *Biochemistry 1,* 677.
Richards, E. G., Teller, D. C., and Schachman, H. K. (1968), *Biochemistry 7,* 1054.
Robbins, K. C., Bernabe, P., Arzadon, L., and Summaria, L. (1972), *J. Biol. Chem. 247,* 6757.
Schiffman, S., Rapaport, S. I., and Chong, M. M. Y. (1966), *Proc. Soc. Exp. Biol. Med. 123,* 736.
Schiffman, S., Rapaport, S. I., and Patch, M. J. (1963), *Blood 22,* 733.
Schiffman, S., Rapaport, S. I., and Patch, M. J. (1964), *J. Clin. Res. 12,* 110.
Schmid, K. (1953), *J. Amer. Chem. Soc. 75,* 60.
Soulier, J.-P., Prou-Wartelle, O., and Menache, D. (1958), *Rev. Fr. Etud. Clin. Biol. 3,* 263.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Stenflo, J., Fernlund, P., Egan, W., and Roepstorff, P. (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 2730.
Straub, W., and Duckert, F. (1961), *Thromb. Diath. Haemorrh. 5,* 402.
Teller, D. C., Horbett, T. A., Richards, E. G., and Schachman, H. K. (1969), *Ann. N. Y. Acad. Sci. 164,* 66.
Thompson, A. R., Ericsson, L. H., and Enfield, D. L. (1974), American Heart Association Abstracts, National Conference on Thrombosis and Hemostasis, Dallas, Tex.
Waaler, B. A. (1959), *Scand. J. Clin. Lab. Invest., Suppl. 11, No. 37,* 1.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Williams, C. A., and Chase, M. W. (1971), *Methods Immunol. Immunochem. 3,* 103.
Williams, W. J., and Norris, D. G. (1966), *J. Biol. Chem. 241,* 1847.
Wright, I. (1959), *J. Amer. Med. Ass. 170,* 325.
Yin, E. T. (1964), *Thromb. Diath. Haemorrh. 12,* 307.
Yphantis, D. A. (1964), *Biochemistry 3,* 297.

# Imidazole Catalysis of Amino Proton Exchange in 2′,3′-Cyclic Adenosine Monophosphate. A General Exchange Mechanism†

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ABSTRACT: Imidazole and 2-methylimidazole increase the proton magnetic resonance (pmr) line widths of the amino protons of 2′,3′-cAMP. This amine-induced broadening is a function of pH and is described by an exchange mechanism in which the initial exchange event is protonation of the ring nitrogen (N-1) of adenine. Protonation of N-1 lowers the acid dissociation $pK_a$ of the purine amino from $pK_a$ = 18–19 to $pK_a$ = 10.6–11.7. The range for the latter value represents agreement in the $pK_a$ value determined separately from the same mechanism for three different proton acceptors at different temperatures, *i.e.,* the conjugate bases of imidazole, 2-methylimidazole, and the nucleotide N-1 itself. The experimental $pK_a$'s of the proton acceptors and of the purine N-1 are unique values required for data fitting. From this a novel method is implied for the measurement of (N-1) ligand dissociation constants by the measurement of $-NH_2$ pmr line widths.

The slow intrinsic exchange of amino protons of purines and pyrimidines is more characteristic of the proton exchange of amides, rather than that of aliphatic and aromatic amines (McConnell and Seawell, 1972, 1973; Suchy *et al.,* 1972). This is not surprising, since amide-like character would be associated with amino groups linked to carbon atoms adjacent to electronegative atoms (nitrogen) in the conjugated ring systems of nucleic acid bases. The fact that these amino groups are not titratable in aqueous solution is further testimony of their amide-like character (Christensen *et al.,* 1970). Diffusion controlled (fast) exchange would be expected only for groups that are titratable in water to give "normal" $pK_a$ values (Eigen, 1964).

Although exchange could be initiated by direct protonation of the amino nitrogen, there is evidence that a more complex mechanism is involved. Amines such as imidazole catalyze hydrogen exchange in double helical DNA (McConnell and von Hippel, 1971). Phosphate and imidazole catalyze $-NH_2$ exchange in mononucleotides (McConnell and Seawell, 1972, 1973). As discussed previously (McConnell and Seawell, 1972), direct protonation of the amino nitrogen by $H_3O^+$ as the predominant rate-limiting event would exclude the ability to observe catalysis by phosphate and amines. Proton transfer theory leads to the prediction that such catalysis would be seen at moderate catalyst concentration only if proton transfer from $H_3O^+$ to the nucleotide were diffusion controlled (Eigen, 1964). This

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