Immunochemistry, 1975, Vol. 12, pp. 339–343. Pergamon Press. Printed in Great Britain

COMMUNICATION TO THE EDITORS

IMMUNOCHEMICAL RELATIONSHIPS OF THE INTERMEDIATES OF PROTHROMBIN ACTIVATION*

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(*Received* 28 *June* 1974)

Abstract—The mechanism of prothrombin activation previously reported from this laboratory (Heldebrant *et al.*, 1973) has been immunochemically verified by analysis of the precursor, intermediate, and product relationships of the proteins with a micro Ouchterlony precipitin test. The cross reactions between purified bovine prothrombin, intermediates of activation, and thrombin were examined with rabbit and chicken antisera to each of these proteins. In each instance, the derivative was found to cross react with its parent protein, yet was found to be completely distinct from any other protein derived from the same cleavage. The results support a mechanism in which prothrombin (70,000 daltons) is cleaved to yield intermediates 1 (51,000 daltons) and 3 (23,000 daltons); intermediate 1 is then cleaved to yield intermediates 2 (41,000 daltons) and 4 (13,000 daltons); thrombin (39,000 daltons) is finally produced from intermediate 2.

INTRODUCTION

The first theory of blood coagulation, the classic theory of Morawitz, established in 1905 the importance of the activation of prothrombin to thrombin as a necessary preliminary reaction which leads to fibrin polymerization (cf. Owen *et al.*, 1969). However, it has only been within the past few years that developments in biochemical technology have permitted the description of prothrombin activation on a molecular basis. Previous studies of the partial reactions and physical and chemical properties of the activation intermediates have enabled our laboratory to propose a mechanism for prothrombin activation (Heldebrant *et al.*, 1973*a*). Studies of the amino terminal amino acid sequence of prothrombin and of each intermediate have further confirmed the proposed mechanism (Heldebrant *et al.*, 1973*b*).

Prothrombin is a single chain glycoprotein with a mol. wt of 70,000. The activation of prothrombin proceeds by the sequential removal of peptides. Each cleavage results in removal of an inactive peptide from the amino terminal portion of the original protein. In the first activation step, (1) prothrombin $\xrightarrow{\text{Factor Xa or thrombin}}$ intermediate 3 + intermediate 1 prothrombin is cleaved by factor Xa or thrombin to yield intermediate 3 (23,000 daltons) from its amino terminal end, and intermediate 1 (51,000 daltons) from its carboxyl terminal end. In the second activation step, (2) intermediate 1 $\xrightarrow{\text{Factor Xa}}$ intermediate 4 + intermediate 2, intermediate 1 is cleaved by factor Xa only, and not by thrombin, to yield intermediate 4 (13,000 daltons) from its amino terminal end, and intermediate 2 (41,000 daltons) from its carboxyl terminal end. In the final activation step, (3) intermediate 2 $\xrightarrow{\text{Factor Xa}}$ α-thrombin, intermediate 2 is cleaved by factor Xa to generate the active two-chain α-thrombin molecule (39,000 daltons).

If this mechanism is correct, each intermediate must be a unique entity, i.e. there must be no overlapping portions of intermediates derived from the same cleavage. Since immunochemical analysis could most readily resolve the question of the uniqueness of the intermediates, we have tested the validity of this mechanism by analyzing the precipitin reactions between prothrombin, the intermediates, and thrombin, and antisera produced against them. Results from these reactions reported here clearly verify the previously proposed mechanism.

MATERIALS AND METHODS

Antigens

Bovine Factor X and prothrombin were purified from whole blood, assayed, and stored as described by Bajaj and Mann (1973). Thrombin was prepared freshly from Parke–Davis topical thrombin by the procedure of Lundblad as modified by Mann *et al.* (1971). Prothrombin activation intermediates 1, 2, 3 and 4 were produced and purified by the method of Heldebrant *et al.* (1973*a*).

Antisera

For immunization the antigens (prothrombin, intermediates 1, 2, 3, 4) were emulsified in Freund's adjuvant containing killed tubercle bacilli. Final concentration of the antigen was 0·1 mg/ml and of the bacilli was 5 mg/ml. Rabbits and chickens received weekly subcutaneous injections of 0·2 ml of these preparations. Since clot retraction of chicken blood is often very slight we increased the yield of serum from chickens by collecting blood in heparin, centrifuging to obtain the plasma and heating the plasma at 60°C for 1 hr to precipitate fibrinogen.

Immunoprecipitation

We used the micro agar precipitin test of Auernheimer and Atchley (1962), and allowed the immunodiffusion reactions to proceed for 48 hr. For experiments using chicken antisera, the concentration of the NaCl in the agar was increased to 9·0%, as recommended by Goodman *et al.* (1951).

* A preliminary account of this work has been submitted to the 1974 Westinghouse Science Talent Search. This research was supported by a grant from the National Heart and Lung Institute (United States Public Health Service) HL-15381, and by General Research Support Funds (5501 RR 05530-10) of the Mayo Foundation.

† Recipient of a Camille and Henry Dreyfus Foundation Teacher-Scholar Grant; established investigator of the American Heart Association, to whom correspondence should be addressed.

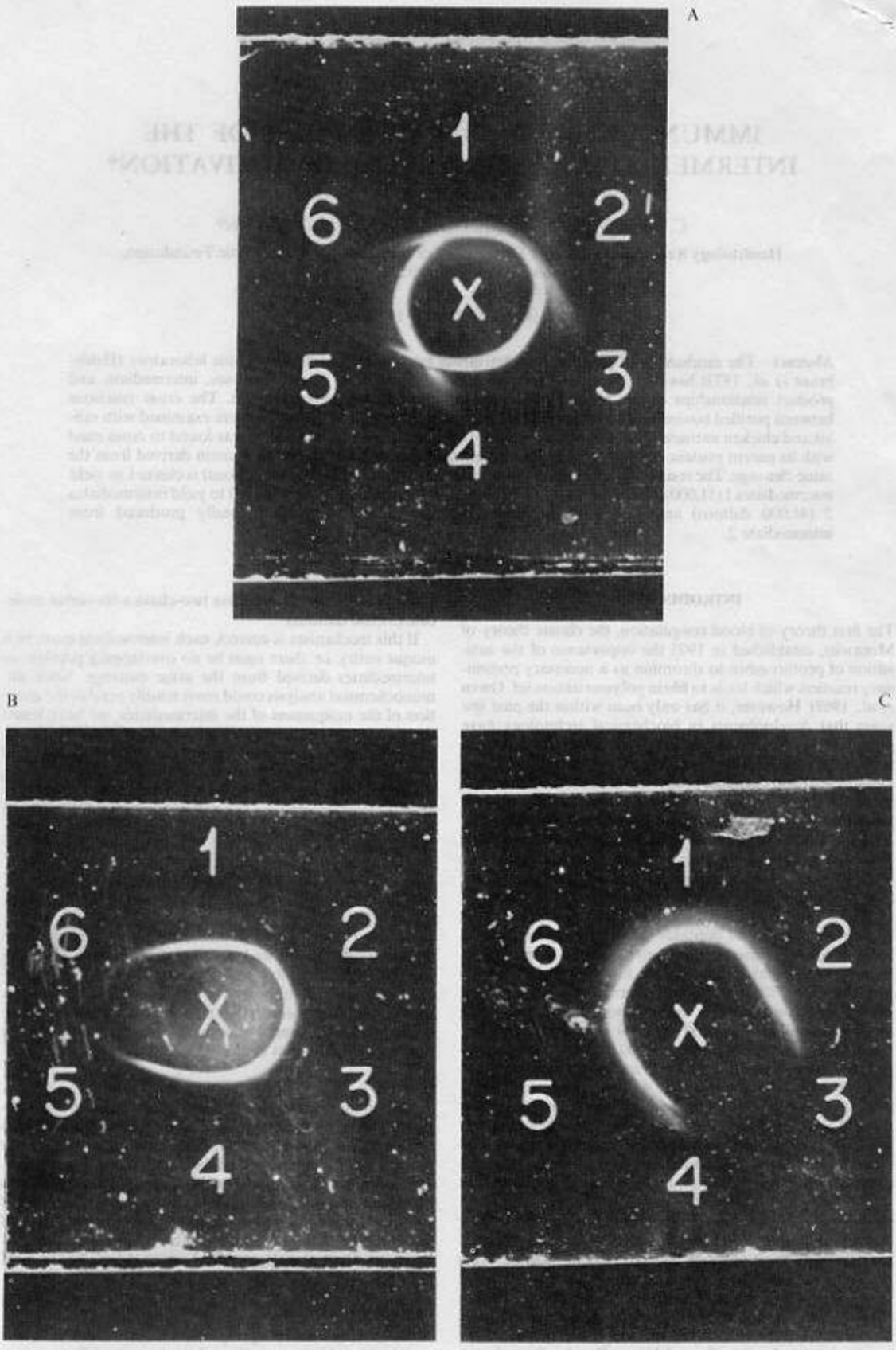

Fig. 1. Cross reaction among prothrombin and the products of cleavage reaction 1—intermediates 1 and 3. Wells 1 and 2, prothrombin; wells 3 and 4, intermediate 1; wells 5 and 6, intermediate 3. Center wells—1A, anti prothrombin; 1B, anti intermediate 1; 1C, anti intermediate 3.

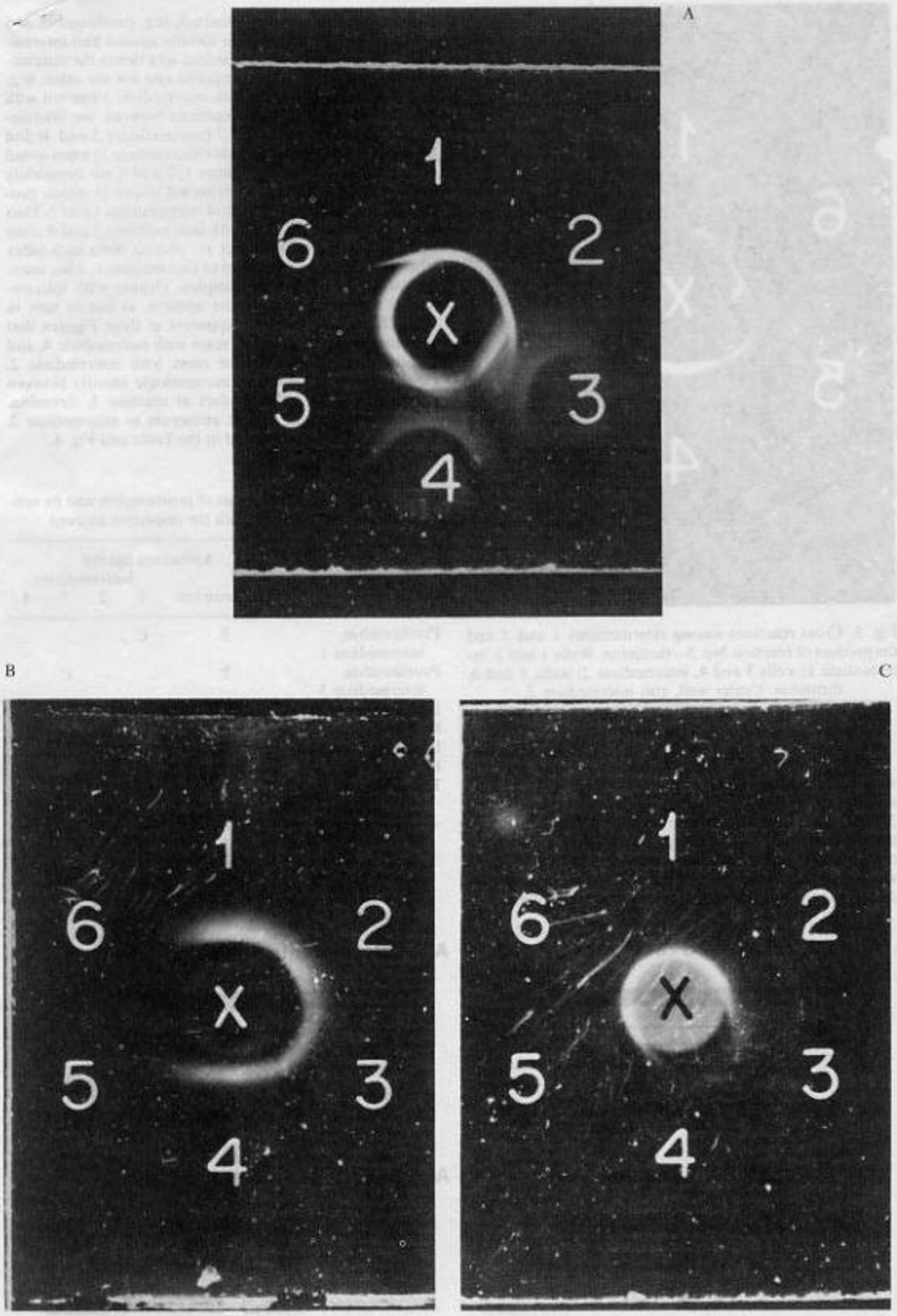

Fig. 2. Cross reactions among intermediate 1 and the products of cleavage reaction 2—intermediates 2 and 4. Wells 1 and 2, intermediate 1; wells 3 and 4, intermediate 2; wells 5 and 6, intermediate 4. Center wells—2A, anti intermediate 1; 2B, anti intermediate 2; 2C, anti intermediate 4.

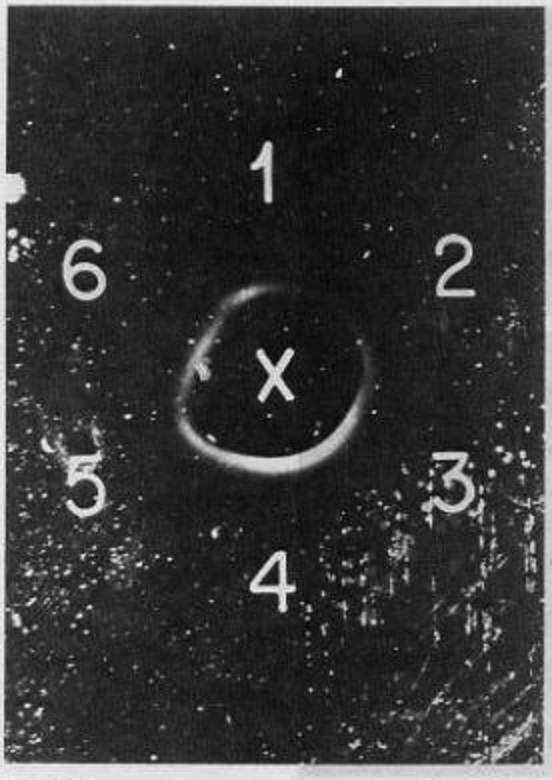

Fig. 3. Cross reactions among intermediates 1 and 2 and the product of reaction No. 3—thrombin. Wells 1 and 2, intermediate 1; wells 3 and 4, intermediate 2; wells 5 and 6, thrombin. Center well, anti intermediate 2.

RESULTS

In Fig. 1A can be seen the cross reactions of prothrombin with the products of reaction 1, (intermediates 1 and 3), against anti-prothrombin serum. Both intermediates 1 and 3 cross react with their parent protein prothrombin, but are distinct from each other. In Figs. 1B and 1C are shown the reactions between prothrombin, intermediates 1 and 3, and antisera to intermediates 1 and 3. Each of these two intermediates shows complete identity with prothrombin when tested against its specific antiserum, (e.g. prothrombin and intermediate 3 show complete identity against anti-intermediate 3). Also, the anti-intermediate sera detect the intermediate against which it was produced and not the other, (e.g. anti-intermediate 3 reacts with intermediate 3 but not with intermediate 1). The cross reactions between the intermediates produced by reaction 2 (intermediates 2 and 4) and their immediate parent protein (intermediate 1) when tested against antisera to intermediates 1, 2 and 4 are completely analogous to the reactions reported above in which prothrombin is the parent protein of intermediates 1 and 3. Thus Fig. 2A demonstrates that both intermediates 2 and 4 cross react with intermediate 1, but are distinct from each other when tested against antiserum to intermediate 1. Also, intermediates 2 and 4 display complete identity with intermediate 1 against their respective antisera, as can be seen in Figs. 2B and 2C. It is also apparent in these Figures that anti-intermediate 2 does not react with intermediate 4, and anti-intermediate 4 does not react with intermediate 2. Finally Fig. 3 demonstrates immunologic identity between intermediate 2 and the product of reaction 3, thrombin, when they are tested against antiserum to intermediate 2. These results are summarized in the Table and Fig. 4.

Table 1. Summary of reactions of prothrombin and its activation intermediates with the respective antisera

Antigens compared	Antiserum against Prothrombin	Intermediates 1	2	3	4
Prothrombin, intermediate 1	P	C			
Prothrombin, intermediate 3	P			C	
Intermediates 1,3	N				
Intermediates 1,2		P	C		
Intermediates 1,4		P			C
Intermediates 2,4		N			
Thrombin, intermediate 2		C	C		

C = complete identity.
P = partial identity.
N = nonidentity.
Blank space indicates not done.

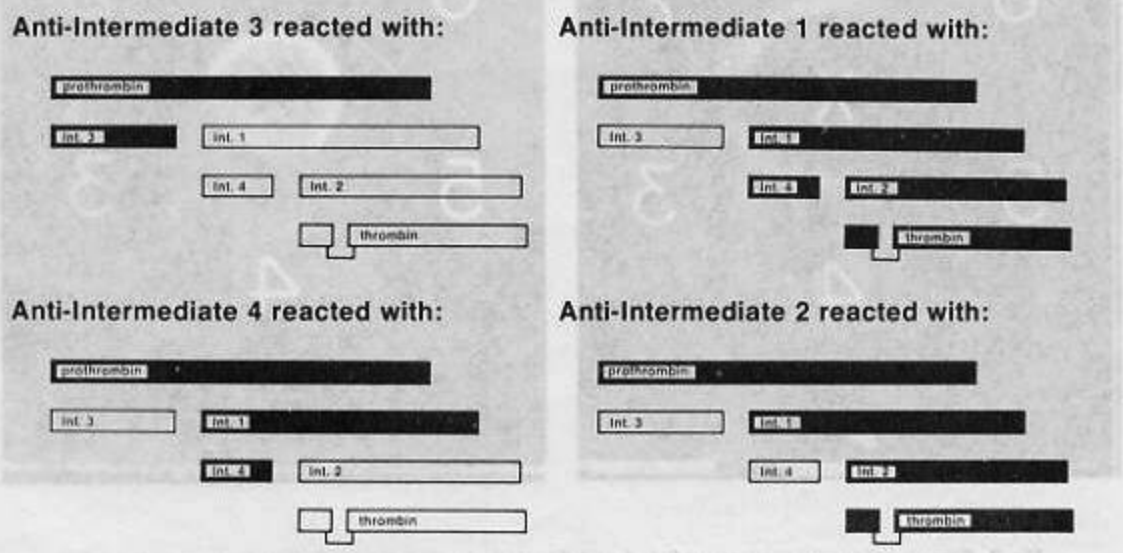

Fig. 4. Schematic diagram of mechanism of prothrombin activation: ■ portion of molecule reacting with indicated antiserum; □ portion of molecule not reacting with indicated antiserum.

DISCUSSION

According to the postulated activation mechanism, intermediates 1 and 3 are two separate fragments from the first cleavage of the original protein, prothrombin. Therefore, since each fragment should possess only a portion of the antigenic determinants of prothrombin, anti-prothrombin serum should detect both prothrombin and the intermediates 1 and 3, while antisera directed against an individual intermediate, 1 or 3, should detect its intermediate, 1 or 3, and prothrombin, but not the other intermediate. Since anti-prothrombin serum contains antibodies against the whole prothrombin molecule, while anti-intermediates 1 and 3 sera have antibodies against only part of the molecule, there should be complete identity between prothrombin and an intermediate against the anti-intermediate serum however only partial identity against the anti-prothrombin serum. A similar argument can be applied to the second step of the activation mechanism. Here the parent protein, intermediate 1, is analogous to the parent protein prothrombin and the derivatives of intermediate 1 (intermediates 2 and 4) are analogous to the first derivatives of prothrombin (intermediates 1 and 3). Reaction 3 probably involves cleavage of a peptide bond within a disulfide loop since there is no significant change in mol. wt nor release of any detectable peptide fragment following the activation of intermediate 2 to thrombin (Heldebrandt *et al.*, 1973*a*). Such a slight change is not detectable by immunoprecipitation with antisera to intermediate 2. A more subtle method such as radioimmunoassay employing anti intermediate 2 after absorption with thrombin might be capable of demonstrating an antigenic difference.

Acknowledgements—We wish to thank Mr. Ralph Butkowski for preparing some of the prothrombin intermediates and Mr. James Peterson for valuable technical assistance.

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