Some Biochemical and Electrophoretic Studies on Purified Prothrombin, Factor VII (Proconvertin) and Factor X (Stuart)

By Garson H. Tishkoff, Liberto Pechet and Benjamin Alexander

STUDIES of the chemical nature of prothrombin have indicated a glycoprotein structure. Data obtained on purified bovine prothrombin have shown the reducing sugar content to be approximately 5 per cent, based on a reference standard of galactose and mannose. Laki et al. reported a value of 6.5 per cent in terms of glucose, and 1.6 to 1.7 per cent hexosamine. Alexander and colleagues observed a value of 6.5 per cent reducing sugar in terms of glucose in a highly purified human prothrombin preparation. A considerable fraction of the carbohydrate is said to be freed during the activation of prothrombin by citrate, becoming soluble in trichloracetic acid, and Miller and Seegers have isolated a polysaccharide of glucose from this moiety. While there thus can be little doubt as to the glycoprotein nature of prothrombin, the carbohydrate composition described above may be considerably influenced by sugar-containing contaminants.

In order to obtain further insight into the structure and function of the carbohydrate of bovine prothrombin, its carbohydrate composition was re-examined in purified preparations from some of which other known clotting factors were effectively separated. Moreover, in addition to reducing sugar and hexosamine, the acid carbohydrate, neuraminic acid (sialic acid) was assayed and found to comprise the largest constituent of the total carbohydrate in bovine prothrombin preparations. More important, it was demonstrated that this carbohydrate is not essential to the prothrombin molecule for its ability to yield thrombin.

Biochemical studies were also carried out on factors VII and X. These substances are frequently concentrated to a large extent in the prothrombin-containing glycoprotein fractions obtained by older fractionation technics and have been difficult to obtain prothrombin-free. More recently this has been achieved by means of starch gel electrophoresis. The isolated factors thus obtained were then studied for their protein and carbohydrate composition.

Additional insight into the chemical nature of prothrombin, factor VII and

*Various forms of neuraminic acid have been described, but no attempt was made here to specify the precise chemical structure as occurs in the prothrombin preparation. The analytic methods employed measure all types of neuraminic acid.
factor X was obtained by splitting neuraminic acid from the glycoprotein-rich preparation with neuraminidase, and then subjecting the mixture to electrophoresis to observe changes in electrophoretic mobility correlated with biologic activity.

**METHODS**

**Separation of Bovine Prothrombin and Factors VII and X from Plasma**

The method employed for obtaining purified prothrombin has been described in detail. Briefly, the procedure consists of adsorption on barium sulfate, elution with five per cent sodium citrate and fractionation with ammonium sulfate. The fractions thus obtained contain prothrombin, factors VII, IX and X and an appreciable quantity of bound carbohydrate. Their specific prothrombin activity was usually approximately 1000 U. (see below) per milligram protein. Studies were performed at a protein concentration of approximately 10 mg. per milliliter.

Electrophoresis on starch gel.—The technic of Smithies was employed with slight modification with respect to the buffer. After investigation of various buffers, a tris-sequestrene-boric acid buffer was found to be particularly effective in the separation of prothrombin from other clotting factors. The buffer was prepared by dissolving 60.5 Gm. tris-(hydroxymethyl) aminomethane (Fisher Scientific Co.), 6 Gm. of (ethylenedinitrilo) tetracetic acid (Eastman Kodak Co.) and 4.6 Gm. of boric acid in a liter of distilled water. This buffer had a pH of 8.8 and was generally used in both cells and starch block. In some later experiments, this buffer was limited to the starch, but was replaced in the cells with borate buffer alone (pH 8.0), as described originally by Smithies.

Each preparation was electrophoresed on a double starch gel block in order to obtain an amount of material sufficient for all our studies. Runs were made at a potential of 200 volts across the buffered starch for 18 hours at 12 C.

After electrophoresis the starch gel block was cut longitudinally. One-half was stained with amidoschwarz 10B dye (Farbenfabriken, Bayer) to locate the protein bands, and the other half was used to express the protein fractions from segmental sections. Another procedure which proved very effective for the detection of protein bands was to impress the entire starch block with a strip of Whatman no. 1 filter paper, which was subsequently stained with bromphenol blue. Although the stained bands were not as prominent on the paper as when the amidoschwarz was applied directly to the starch gel, this procedure had the advantage of both keeping the starch block intact for subsequent studies and also providing opportunity of measuring relative staining density with a Spinco densitometer.

Expression of the proteins from the starch was carried out as follows: The block was cut into 1 cm. segments, the individual segments were frozen for 3 hours at -20 C, then thawed, placed on a sintered glass funnel, and a vacuum applied as described by Kunkel. The volume of expressate thus obtained from each segment was approximately 0.7 ml. Corresponding segments from the two starch trays were combined for the extraction procedure.

In order to remove the buffer, the expressates were individually dialysed against 0.15 M sodium chloride for 16 hours at 4 C. prior to biochemical and clotting studies.

**Assay of Clotting Factors**

Prothrombin.—The two-stage procedure of Ware and Seegers was employed with slight modification. BaSO₄-adsorbed bovine plasma (diluted 1:150 with physiologic saline) was used as a source of factor V, and bovine serum (diluted 1:50) supplied factors VII and X. Conventional acetone-extracted human brain provided the thromboplastin. The standard fibrinogen consisted of a 1.5 per cent solution of bovine fraction I (Armour Co.) in physiologic saline. The reaction mixture was adjusted to pH 7.5 with 0.02 M Veronal buffer. By this procedure bovine plasma assayed at approximately 250 U. of prothrombin per milliliter.

Factor VII.—Factor VII activity was measured by the ability of the dialysed expressates
to correct the poor one-stage prothrombin activity (Quick prothrombin time) of our original patient with factor VII (SPCA) deficiency when admixed with a fixed amount of her plasma. To assure optimal factor V activity in the total mixture, BaSO₄-adsorbed bovine plasma was incorporated as indicated below. A standard curve was prepared from fresh normal bovine plasma diluted in various proportions with saline which was then admixed with the factor VII-deficient plasma. Individual expressates were assayed in a mixture comprising equal volumes of expressate, factor VII-deficient human plasma and BaSO₄ adsorbed bovine plasma. The value, expressed in per cent of the clot-corrective effect of normal plasma, was interpolated from the standard curve described above.

Factor X (Stuart).—Factor X was assayed by the method of Bachmann, Duckert and Koller. A standard curve was prepared with various dilutions of bovine plasma in physiologic saline.

Chemical Analyses

Protein was computed from total nondialysable nitrogen as determined by a semimicro-Kjeldahl method, employing a conversion factor of 6.25. For amounts of protein too small to measure by this procedure, protein was determined with the Folin-Ciocaltau reagent, modified recently by including alkaline copper. Conversion of the intensity of the chromogen thus obtained into protein values was made from the Kjeldahl protein:n:chromogen ratio obtained on a larger sample of glycoprotein-rich prothrombin fraction prior to electrophoretic separation and purification.

Hexose was determined by the orcinol method of Weimer and Moslin with a galactose-mannose standard. Hexosamine was determined by the Elson-Morgan procedure as described by Rimington. Neuraminic acid was assayed by the method of Winzler but with the use of a modified Ehrlich's reagent instead of diphenylamine to develop the chromogen. Neuraminic acid was also determined on some preparations by the procedure of Werner and Odin, with the use of a Bial's reagent. This method measures neuraminic acid in the intact glycoproteins, in contrast to the Wizner procedure which requires prior hydrolysis to free the neuraminic acid. N-acetyl neuraminic acid was employed as a reference standard.

In order to achieve enzymatic splitting of neuraminic acid from the glycoproteins, three partially purified neuraminidase preparations were employed which were obtained from pneumococcus, Clostridium perfringens and Vibrio cholerae, respectively. Following incubation of the prothrombin fraction with the enzyme, the mixture was subjected to trichloracetic acid precipitation at room temperature in a final concentration of 5 per cent. The precipitates were removed by filtration, and the quantity of enzyme liberated neuraminic acid (i.e., trichloracetic acid-soluble and presumably free) was estimated by use of Ehrlich's reagent directly on a suitable aliquot of the filtrate. A control prothrombin fraction in which saline was substituted for enzyme was employed for assay of total neuraminic acid by the Winzler procedure as follows: after the addition of trichloracetic acid, the total mixture was heated in a boiling water bath for 15 minutes in order to free the total neuraminic acid by acid hydrolysis. The precipitated material was removed by filtration, and neuraminic acid was assayed on a suitable aliquot of the filtrate. The percentage of neuraminic acid liberated by the enzyme was thus computed from the ratio of "free" to total neuraminic acid content.

Results

The nature of the carbohydrate moiety of some prothrombin preparations.—The carbohydrate composition of several prothrombin preparations are given

*We are indebted to Dr. H. Eylar of the Department of Biological Chemistry, Harvard Medical School, for pneumococcal neuraminidase; to Dr. E. Popeneo of the Brookhaven National Laboratories for Clostridium perfringens neuraminidase; and to Dr. M. Madloff of the Department of Infectious Diseases, New England Center Hospital, Boston, for Vibrio cholerae neuraminidase.
in table 1. Total carbohydrate comprised 10.4 to 14.7 per cent of the total protein. Except for one preparation (#49), neuraminic acid made up the largest fraction, ranging from 3.2 to 7.2 per cent. Hexose amounted to 3.6 to 6.0 per cent, and the hexosamine content ranged from 1.5 to 4.1 per cent. There was no correlation between the total carbohydrate, or the individual sugars, with the specific prothrombin activity.

The effect of neuraminidase on the prothrombin activity is shown in table 2. Sixty-one and 74 per cent of the neuraminic acid were split from the protein by pneumococcal and clostridium enzyme, respectively, after three hours' incubation, whereas the prothrombin activity decreased only by 6 and 21 per cent, respectively. After five hours' incubation, the clostridium enzyme liberated 100 per cent of the neuraminic acid, yet 71 per cent of the original prothrombin activity remained. In a similar experiment (table 3) employing *Vibrio cholerae* neuraminidase at a slightly more alkaline pH (5.6 vs. 5.25), after four hours' incubation, 82 per cent of the neuraminic acid was liberated while 69 per cent of the control prothrombin activity was still demonstrable. Although some loss of prothrombin activity occurred in all the enzyme studies,

### Table 1.—Carbohydrate Composition of Various Bovine Prothrombin Preparations

<table>
<thead>
<tr>
<th>Preparation no.</th>
<th>Prothrombin (U./mg. protein*)</th>
<th>Neuraminic acid (%)</th>
<th>Hexose (%)</th>
<th>Hexosamine (%)</th>
<th>Total carbohydrate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>42</td>
<td>936</td>
<td>3.2</td>
<td>3.8</td>
<td>3.4</td>
<td>12.0</td>
</tr>
<tr>
<td>44</td>
<td>1350</td>
<td>4.3</td>
<td>4.1</td>
<td>3.6</td>
<td>13.3</td>
</tr>
<tr>
<td>47</td>
<td>1090</td>
<td>5.6</td>
<td>6.0</td>
<td>1.5</td>
<td>10.7</td>
</tr>
<tr>
<td>49</td>
<td>272</td>
<td>3.2</td>
<td>3.9</td>
<td>4.1</td>
<td>13.3</td>
</tr>
<tr>
<td>50</td>
<td>811</td>
<td>5.3</td>
<td>3.6</td>
<td>2.8</td>
<td>10.4</td>
</tr>
<tr>
<td>52</td>
<td>872</td>
<td>7.2</td>
<td>4.0</td>
<td>3.5</td>
<td>14.7</td>
</tr>
<tr>
<td>55</td>
<td>188</td>
<td>4.0</td>
<td>3.6</td>
<td>2.8</td>
<td>10.4</td>
</tr>
<tr>
<td>57</td>
<td>1070</td>
<td>5.6</td>
<td>3.6</td>
<td>4.1</td>
<td>13.4</td>
</tr>
<tr>
<td>105</td>
<td>1174</td>
<td>4.6</td>
<td>4.1</td>
<td>4.1</td>
<td>13.4</td>
</tr>
</tbody>
</table>

*Computed from Kjeldahl nitrogen, using a factor of 6.25.  
†Assayed by method of Werner and Odin. All other neuraminic acid values obtained by Winzler's procedure as described in text.

### Table 2.—Effect of Neuraminidase on Prothrombin

<table>
<thead>
<tr>
<th>Neuraminidase</th>
<th>Enzyme (U./ml.*)</th>
<th>Prothrombin (U./ml)</th>
<th>Neuraminic acid split (%)</th>
<th>Prothrombin (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>—</td>
<td>3570</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>Pneumococcus</td>
<td>80*</td>
<td>3350</td>
<td>61</td>
<td>94</td>
</tr>
<tr>
<td><em>Cl. Perfringens</em></td>
<td>16.5*</td>
<td>2820</td>
<td>74</td>
<td>79</td>
</tr>
<tr>
<td>Control</td>
<td>—</td>
<td>2658</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td><em>Cl. Perfringens</em></td>
<td>16.5*</td>
<td>1875</td>
<td>100</td>
<td>71</td>
</tr>
</tbody>
</table>

Incubation mixture (37 C.): 0.5 ml. enzyme (or saline); 0.5 ml. of 0.2 M acetate buffer, pH 5.25; 0.5 ml. prothrombin. One aliquot treated with pneumococcus neuraminidase, the other with *Clostridium perfringens* neuraminidase. The prothrombin had a specific activity of 1070 U. per milligram protein as measured by Kjeldahl N.

*Units of enzyme are not comparable, but are arbitrary values submitted by the investigators who kindly provided us with the enzymes.
Table 3.—Effect of Neuraminidase* on Biologic Activities of Prothrombin, Factor VII and Factor X

<table>
<thead>
<tr>
<th></th>
<th>Prothrombin activity (U/ml)</th>
<th>Factor VII (%)</th>
<th>Factor X (%)</th>
<th>Neuraminic acid split (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before enzyme exposure</td>
<td>2800</td>
<td>2000</td>
<td>180</td>
<td>0</td>
</tr>
<tr>
<td>After (4 hr.) enzyme exposure</td>
<td>1600</td>
<td>2300</td>
<td>240</td>
<td>82</td>
</tr>
</tbody>
</table>

*Vibrio cholerae, same conditions as in table 2, except with a different prothrombin preparation and at pH 5.6.
†During 4 hours the prothrombin activity of an aliquot not exposed to the enzyme but otherwise maintained under the same conditions had dropped from 2800 to 2340 U. per milliliter.

it was considerably less than the per cent neuraminic acid liberated. No losses in the activities of factors VII and X were observed; if anything there was a slight increase. It should also be noted that no thrombin evolved during treatment with the enzyme, in accordance with earlier observations.24

Starch gel electrophoresis of prothrombin preparations correlated with chemical studies.—The results of a typical electrophoresis of a bovine prothrombin preparation appear in figure 1. Individual segments were concurrently assayed for prothrombin, factor VII and factor X, protein content and neuraminic acid.

It is evident that the various clotting factors migrate at different rates, factor X moving most rapidly toward the anode. Preparations of factor X could thus
Table 4.—Effect of Vibrio Cholerae Neuraminidase on the Specific Activity of Prothrombin

<table>
<thead>
<tr>
<th>Incubation time (hours)</th>
<th>Prothrombin with enzyme (U./mL)</th>
<th>Per cent neuraminic acid split</th>
<th>Specific activity* of prothrombin (U./mg. protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>4800</td>
<td>—</td>
<td>1310</td>
</tr>
<tr>
<td>3</td>
<td>4800</td>
<td>52</td>
<td>1210</td>
</tr>
<tr>
<td>6</td>
<td>3930</td>
<td>64</td>
<td>775</td>
</tr>
</tbody>
</table>

Incubation mixture (37 C.): 0.5 ml. enzyme (or saline), 0.5 ml. prothrombin, 0.5 ml. 0.2 M acetate buffer pH 5.6. The specific activity of the prothrombin preparation before electrophoresis and without addition of enzyme was 900 U. per milligram protein. It did not change significantly during 6 hours' incubation under these conditions. After electrophoresis, the same material had a specific activity of 1310 U. per milligram protein.

*These values represent the specific activity of prothrombin after neuraminidase exposure, electrophoresis and expression from starch segment. It should be noted that they are not to be correlated with those in the first column since the interval elapsing before the determination exceeds 24 hours, during which substantial loss in activity not directly referable to enzymatic carbohydrate splitting may have occurred. Since the enzyme migrated much more rapidly, its presence as a nonspecific protein contaminant could not have affected the specific activity values of the prothrombin.

be obtained completely devoid of factor VII and prothrombin (fig. 1, segment 14). Similarly, factor VII migrated between prothrombin and factor X, and could be obtained free of both prothrombin and factor X (segment 12), although some overlapping was almost always experienced. Prothrombin had the slowest anodic migration, with the peak activity occurring close to the factor VII activity. However, prothrombin completely devoid of factors VII and X could be obtained readily (fig. 1, segments 8 and 9). In other experiments, (one alluded to in the footnote of table 4,) such separation was associated with significant increase in specific activity of the prothrombin.

A close correlation was evident between protein, neuraminic acid and the peak of the prothrombin activity. There was no correlation between peak of factor VII and factor X activity with the quantity of protein and neuraminic acid. Segments 12 to 15, containing factors VII and X activities, were relatively low in protein,* although they exhibited significant quantities of neuraminic acid.

Preparations of prothrombin with significant factor VII and factor X activities were treated with neuraminidase, following which an aliquot was assayed for prothrombin, factors VII and X and neuraminic acid. Another aliquot of neuraminidase-exposed prothrombin was subjected to electrophoresis. A suitable control of the untreated prothrombin dissolved in buffer of the same pH was electrophoresed simultaneously.

Following six hours' incubation (fig. 2), 64 per cent of the neuraminic acid became trichloracetic acid-soluble, and at the same time a definite alteration in electrophoretic mobility of the proteins and the various clotting activities was seen. Two major protein peaks were noted in the neuraminidase-treated prothrombin preparation, in contrast to the nontreated prothrombin counterpart in which a single major protein band was observed. The pro-

*As measured by the Folin-Ciocaltau-alkaline copper chromogen.18
Fig. 2.—Effect of exposure of purified prothrombin preparation to neuraminidase on electrophoretic mobility of proteins and activities of prothrombin, factor VII and factor X. The distribution of the same preparation prior to neuraminidase treatment was, as in our prior experiments including that shown in figure 1, different, as detailed in the text. Incubation was carried out with *Vibrio cholerae* neuraminidase for 6 hours at pH 5.6 in 0.2 M acetate buffer. An electrophoretic run of the neuraminidase enzyme alone resulted in its appearance in segments 10 and 11. It should be noted that 64 per cent of the total neuraminic acid was split during incubation, yet 86 per cent of the prothrombic activity remained demonstrable.

thrombin activity, coinciding with the faster moving protein band, fell in the same segment as that of the control run (fig. 2, segment 9).

After neuraminidase treatment, factor X again was noted to migrate most rapidly, and significant activity was noted in segment 13. In contrast to the control run, in which—in agreement with earlier experiments (see above)—factor VII appeared as a single band moving slightly more rapidly than prothrombin, *after neuraminidase treatment* factor VII migrated at two different velocities. One area of activity coincided exactly with the prothrombin activity, and the other appeared in the segment containing factor X activity.

The effect of neuraminidase on the specific prothrombin activities (units per milligram protein) of the same preparation presented in figure 2 are given in table 4. After neuraminidase treatment for three hours, the specific activity of prothrombin following electrophoresis was approximately that of the control prothrombin, i.e., nonenzyme exposed yet electrophoresed. After six hours, the specific activity was appreciably less.

**DISCUSSION**

Our present studies confirm previous observations that complete separation of prothrombin, factor VII and factor X can be achieved by conventional fractionation technics followed by starch gel electrophoresis. Although some overlapping occurs, it is clear that these factors are discrete entities. It is
noteworthy that the preponderant protein-staining band as well as the tyrosine-
alkaline copper chromogen and the neuraminic acid correlate best with the
prothrombin activity, although additional faint bands have also been demon-
strated by the protein staining methods. Apparently the substances represent-
ed by the latter migrate more rapidly than prothrombin.

Of unusual interest was the observation that relatively little protein, demon-
strable either by staining or by the tyrosine-copper reagent, was associated
with factor X activity, although neuraminic acid was present in significant
quantities (fig. 1). To explain this, several possibilities must be considered.
Either factor X may be nonprotein in nature (yet nondialyzable) or its biologic
activity is associated with trace protein of high neuraminic acid content. It is
also possible that the hydroxyl group of the tyrosine residues may be “masked”
by internal structural configuration. Present information is inadequate for
resolution of these possibilities.

It is profitable to contrast the factor VII-rich expressates with those rich in
factor X. Factor VII migrates ahead of, yet in close proximity to prothrombin,
here again leading to considerable overlap. Nevertheless, expressates with
factor VII activity which are devoid of both prothrombin and factor X (seg-
ment 12, fig. 1) can be obtained which contain relatively more protein than in
present in segments associated with factor X activity but devoid of the other
two clotting factors.

Notably lacking are observations on factor IX (plasma thromboplastin com-
ponent), which we know to have been originally present in our starting ma-
terial. Studies on its distribution and composition are in progress.

The data concerning certain carbohydrate constituents (hexose and hexo-
samine) are consistent with previously reported analyses,6 and accordingly
leave little doubt as to the glycoprotein nature of prothrombin. Nevertheless,
it cannot yet be stated with certainty to what degree the sugars make up
the prothrombin molecule itself, or how much they represent nonspecific glyco-
protein contaminants in the fraction. The lack of correlation between the
specific prothrombin activity of the preparation and their content of indi-
vidual sugars or total carbohydrates points this up. For example, prepara-
tions #49 and #55 (table 1) with very low specific activity had a total carbohydrate
closely comparable to that of preparation #44 with much higher prothrombin
activity. Moreover, purified factor VII fractions derived from serum and devoid
of prothrombin contain substantial carbohydrate (hexose)6 as well as hexo-
samine and neuraminic acid.25 Since we know that the prothrombin fractions
in table 1 contain some factor VII and factor X, it is likely that at least part
of the carbohydrate is derived from these factors. On the other hand, pro-
thrombin devoid of factors VII and X contains carbohydrate, at least neuraminic
acid, as was demonstrated in purified prothrombin obtained by starch gel
electrophoresis (fig. 1). It would therefore appear that the lack of correlation
between specific prothrombin activity and carbohydrate composition of the
several prothrombin preparations is referable partly to contaminants and
partly to substantial denaturation and loss in biologic activity of the prothrom-
bin without parallel loss of biochemical properties determining its behavior
in the fractionation procedure.

In extension of earlier work, new information has been obtained indicating
that neuraminic acid also should be included among the carbohydrates. Indeed, this substance apparently comprises the largest fraction of the total sugar, and of a magnitude comparable to that reported for other serum glycoproteins. With this additional component included, our present values for total carbohydrate constitute a larger fraction of prothrombin than those reported earlier by others as well as by ourselves. In this connection it is of interest that, on the basis of physicochemical data, Laki26 suspected the presence in prothrombin of an unknown acid moiety possibly carbohydrate in nature. It is likely that this postulated entity is neuraminic acid, which is known to endow proteins with acidic properties which are reduced if the acid is split off.27

The disclosure of considerable neuraminic acid in our prothrombin fractions raised the question as to whether it is essential for thrombin formation from prothrombin. Studies with neuraminidases from various bacteria have confirmed earlier observations that substantial neuraminic acid cleavage can occur with far less proportionate reduction in prothrombin activity. This would indicate that this particular carbohydrate as a component of prothrombin is not essential for thrombin formation, or that the neuraminic acid-containing moiety is a contaminant in the prothrombin fractions. In view of what might be anticipated as to the specific activity of pure prothrombin on the basis of analytic values reported for highly "purified" yet admittedly still impure material,6 the neuraminic acid-rich contaminant would have to be of unusually high neuraminic acid content to account for the value found in our preparations. Moreover, from our electrophoretic study the postulated impurity would have to have a mobility remarkably similar to prothrombin, as well as similar other physicochemical properties which would make it accompany prothrombin during plasma fractionation. This alternate hypothesis of a neuraminic acid-containing contaminant as accounting for all the neuraminic acid we consider less likely. It is more probable that much of the acid constitutes part of the prothrombin molecule as it exists in nature.

In addition, significant quantities of neuraminic acid were found in segments which were rich in factor VII yet relatively free of both prothrombin and factor X. Substantial amounts of neuraminic acid were also found in fractions rich in factor X but devoid of both prothrombin and factor VII. It thus appears that all three of these clotting factors are glycoprotein, at least insofar as containing neuraminic acid in common.

That the acid can be cleaved from prothrombin and factors VII and X without proportionate loss of biologic activity has ample precedent in other proteins, i.e., papain,29 ribonuclease,30 trypsinogen,31 in which significant molecular fragmentation can be inflicted without consequent loss of biologic activity. This observation further suggests that the neuraminic acid-containing fragment is situated at the terminal ends of the molecules of these clotting entities.

In striking contrast to the relative innocuousness of neuraminidase treatment are the observations in our laboratory of the effects of many proteolytic enzymes on prothrombin.24 Earlier studies have shown that trypsin can convert prothrombin directly to thrombin, while at the same time considerable prothrombin (as well as thrombin) is destroyed. Other proteolytic enzymes, which are incapable of yielding thrombin, induce profound losses in prothrom-
bic activity. These observations would thus focus attention on the protein moiety of prothrombin rather than the carbohydrate as the center of biologic activity. It is also likely that thrombin does not contain neuraminic acid.

The electrophoretic studies following treatment with neuraminidase indicate that certain physicochemical alterations are thus induced in the glycoproteins, associated with liberation of trichloracetic acid-soluble neuraminic acid. This results in the appearance of an electrophoretically slow moving protein in a concentration approximately the same as the adjacent faster moving entity containing prothrombin activity. Since neuraminic acid endows many glycoproteins with marked acidic properties, which can be markedly reduced following scission of the acid, its cleavage and removal per se would be expected to result in reduction of the net negative charge of the protein moiety, thus leading to the appearance of a protein with slower mobility since it would be more positively charged. Neuraminic acid assays have not been made as yet on expressates containing this slower moving material: obviously such data would clarify this point.

A more challenging question is: What is the source of this material? It should be noted that the slower moving material could not be converted to thrombin. Did it arise from prothrombin as a consequence of neuraminic acid cleavage which thus rendered it more susceptible to denaturation resulting in biologic inertness? Or did it result from physicochemical alteration of a nonprothrombin-contaminating glycoprotein, or did it come from prothrombin which, during neuraminidase scission, was at the same time being fragmented or otherwise denatured by nonspecific proteinases known to be present as contaminants of the neuraminidase preparations? We have already observed that the neuraminidase liberates a large amount of neuraminic acid without proportionate loss in prothrombic activity, and the destructive effect of many proteolytic enzymes on prothrombin has been alluded to above. It is likely that the electrophoretic changes resulting from neuraminidase treatment reflect both neuraminic acid cleavage and some protein denaturation.

Neuraminidase treatment appeared to have no effect on the mobility of either the prothrombin activity or factor X, when compared with non-neuraminidase-treated material electrophoresed concurrently. A definite alteration in the migration of factor VII, however, was obtained. Following neuraminidase treatment, factor VII activity migrated as two distinct activities. One coincided exactly with the prothrombin activity, and the other with factor X. The explanation for this alteration also remains obscure. With regard to prothrombin, this poses a paradox in the light of the concept that neuraminic acid cleavage should result in a more basic residue which is more positively charged and thus of slower mobility. Conceivably, incomplete neuraminic acid cleavage may have left sufficient acid residue in some of the prothrombin to maintain its prior mobility.

**Summary**

1. Complete separation of prothrombin, factors VII and X has been achieved by conventional fractionation technic followed by starch gel electrophoresis.

2. The glycoprotein nature of bovine prothrombin has been confirmed.
3. In addition to hexose and hexosamine, substantial quantities of a hitherto unrecognized constituent, neuraminic acid, have been demonstrated. When included with the other carbohydrates, the total sugars comprise a substantial fraction of prothrombin.

4. In electrophoretic mobility the preponderance of proteins and total neuraminic acid correlate closely with the biologic prothrombin activity.

5. Scission of neuraminic acid by neuraminidase does not result in destruction of the prothrombin, which can subsequently still be converted to thrombin.

6. Significant quantities of neuraminic acid are also found in preparations rich in factors VII and X but devoid of prothrombin. These fractions are relatively low in protein. Here, too, cleavage of neuraminic acid does not destroy biologic activity.

7. Treatment with neuraminidase results in some changes in electrophoretic mobility of some of the protein, resulting in the appearance of a slower moving protein fraction devoid of biologic activity. The mobility of factor VII was slightly altered, but that of factor X remained unchanged.

**Summario in Interlingua**

1. Le complete separation de prothrombina, factor VII, e factor X esseva efectuate per technicas conventional de fractionation post electrophorese per gel a amylo.

2. Le natura glycoproteinic de prothrombula bovin esseva confirmate.

3. Esseva demonstrate—a parte hexosa e hexosamina—quantitates notabile de un previemente non recognoscite constitutente, i.e. acido neuraminic. Le sucros total—inclus le altere hydratos de carbon—representa un fraction mucho respectabile del prothrombina.

4. Le preponderantia de proteinas e total acido neuraminic, judicate super le base del motilitate electrophoretic, es strictemente correlationate con le activitate biologic de prothrombina.

5. Le scission de acido neuraminic per neuraminidase non resulta in le destruction del prothrombina. Isto continua esser convertibile in thrombina.

6. Quantitates significative de acido neuraminic es etiam trovate in preparatos que es ric in factor VII e factor X sed que se disproviste de prothrombina. Iste fractiones es relativamente povere in proteina. Etiam in iste situation, le scission de acido neuraminic non destrue le activitate biologic.

7. Le tractamento con neuraminidase resulta in certe alterationes del motilitate de un parte del protela. Il appare un fraction proteinic de relentate motilitate, e isto es sin activitate biologic. Le motilitate de factor VII esseva levemente alterate. Illo de factor X remaneva sin alteration.

**REFERENCES**


distribution during the activation of purified prothrombin in sodium citrate solution. Arch.Biochem.& Biophys. 45:312, 1953.


Some Biochemical and Electrophoretic Studies on Purified Prothrombin, Factor VII (Proconvertin) and Factor X (Stuart)

GARSON H. TISHKOFF, LIBERTO PECHET and BENJAMIN ALEXANDER