Immunochemical Isolation and Electrophoretic Characterization of Precursor Prothrombins in H-35 Rat Hepatoma Cells†

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ABSTRACT: Intracellular [^{3}H]leucine-labeled precursor prothrombins, obtained by labeling H-35 rat hepatoma cells for 2 h in the absence of vitamin K, were isolated by immunospecific adsorption to antirat prothrombin coupled to Sepharose. Initially, precursor prothrombins were extracted from cell homogenates with 2% Triton in Tris buffer (pH 9.5) and recovered from the supernatant after high-speed centrifugation (100000*g*). ^{3}H-Labeled precursor prothrombins (supernatant fraction) were purified via affinity antibody chromatography, eluted in urea–sodium dodecyl sulfate ($NaDodSO_4$) buffer, and subsequently characterized by acrylamide gel techniques. As evaluated by isoelectric focusing gels, five distinct [^{3}H]-leucine-labeled precursors of mature prothrombin possessing p*I* values of 7.2, 6.7, 6.2, 5.8, and 5.5 were isolated via these immunochemical procedures. Identification of these proteins as precursors of mature prothrombin was based upon their (a) immunochemical isolation, (b) inability to be adsorbed by insoluble barium salts, (c) common molecular weights of ~75 000 as evaluated by $NaDodSO_4$ gels, and (d) vitamin K dependency; i.e., when cells were labeled in the presence of vitamin, there was a marked reduction (70%) of intracellular p*I* 7.2 and 6.7 species and a moderate reduction (35%) of p*I* 6.2, 5.8, and 5.5 forms with a concomitant appearance of mature prothrombin in the cell culture medium (p*I* value of 5.0, molecular weight 75 000). Quantitative evaluation of the amount of radioactivity incorporated into precursor and mature prothrombins obtained from H-35 hepatoma cells previously labeled with [^{3}H]leucine suggested that vitamin K accelerated the rate of synthesis and/or processing of precursor prothrombins, ultimately resulting in the secretion of mature prothrombin molecules.

The existence of precursor prothrombins in vitamin K[1] deficient and anticoagulant-treated animals has been established by a variety of investigations. Findings in this regard include the presence and absence of γ-carboxyglutamate residues in the N-terminal portion of mature and precursor prothrombin sequences, respectively (Stenflo, 1974; Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974), the conversion of precursor prothrombins to thrombin with *Echis carinatus* venom (Nelsestuen & Suttie, 1972a), and the in vitro vitamin K dependent carboxylation of precursor prothrombins derived from rat liver microsomal preparations (Shah & Suttie, 1974; Girardot et al., 1974; Esmon et al., 1975a,b). More recently, several proteins from microsomes of warfarin-treated rats that possess many of the properties assigned to precursor prothrombins (i.e., molecular weight, amino acid composition, generation of common polypeptides by specific proteases, etc.) have been identified (Nelsestuen & Suttie, 1972b; Stenflo & Ganrot, 1973; Morrissey et al., 1973). Subsequent characterization of these precursors via isoelectric focusing techniques has revealed the presence of two major proteins possessing p*I* values of 7.2 and 5.8 (Esmon et al., 1975a,b; Grant & Suttie, 1976). Whereas the 5.8 isoelectric species has been tentatively identified as the asialo derivative of mature prothrombin (Esmon et al., 1975a,b), the nature of the p*I* 7.2 precursor remains to be elucidated. Although the above studies have provided sufficient evidence for the existence of precursor prothrombins, no information is available regarding their intracellular processing.

Investigations were thus initiated in our laboratory which would permit the rapid and quantitative isolation of small quantities of precursor prothrombins present in cultured H-35 rat hepatoma cells. This culture system was selected for study because (a) sufficient [^{3}H]leucine can be incorporated into the precursor prothrombins undergoing synthesis in H-35 hepatoma cells and (b) these cells respond to exogenous vitamin K in a manner similar to that of vitamin K deficient and/or anticoagulant-treated rats (Munns et al., 1976). In this investigation we wish to describe an immunochemical approach for the rapid isolation of labeled precursor and mature prothrombins obtained from H-35 hepatoma cells previously labeled for 2 h with [^{3}H]leucine. Subsequent characterization of these [^{3}H]leucine-labeled precursors via isoelectric focusing gels has confirmed the existence of the p*I* 7.2 and 5.8 precursors of prothrombin (Grant, 1975; Esmon et al., 1975a,b; Grant & Suttie, 1976), while also revealing the presence of three additional precursors possessing p*I* values of 6.7, 6.2, and 5.5.

Materials and Methods

Materials. Radioactive supplies including [4,5-$^3H_2$]leucine (60 Ci/mmol), [^{125}I]iodine, NCS (tissue solubilizer), and Spectrofluor (concentrated scintillant) were purchased from Amersham/Searle. Ampholyte carrier buffers were obtained from LKB, and CNBr-activated Sepharose 4B, Sephadex G-25, DEAE-Sephadex, and Cellex-D were from Pharmacia. Phylloquinone (vitamin K), as the detergent-solubilized preparation (AquaMephyton), and insulin (Iletin) were obtained from Merck Sharp & Dohme and Eli Lilly, respectively.

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[1] Abbrevations used: vitamin K, phylloquinone; PMSF, phenylmethanesulfonyl fluoride; STI, soybean trypsin inhibitor; $NaDodSO_4$, sodium dodecyl sulfate; PCA, perchloric acid; NaCl–P, phosphate-buffered saline (150 mM NaCl and 10 mM PO_4, pH 7.4); antirat prothrombin–Sepharose, antirat prothrombin antibody coupled to Sepharose; ^{3}H-labeled precursor prothrombins, [4,5-$^3H_2$]leucine-labeled precursor prothrombins; IEF gels, isoelectric focusing gels.

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Phenylmethanesulfonyl fluoride (PMSF), soybean trypsin inhibitor (STI), *E. carinatus* snake venom, and Triton X-100 were purchased from Sigma Chemical Co., and Nonidet P-40 was from Shell Oil Co. All chemicals were of analytical reagent grade.

Cell Culture System. H-35 rat hepatoma cells (Pitot et al., 1964; Munns et al., 1976) were grown in roller cultures at 37 °C in sterilized glass bottles (growth area = 670 cm^2, rpm = 0.75) in an atmosphere of 95% air and 5% CO_2 in the presence of 150 mL of Swim's S-77 media supplemented with 20% horse and 5% fetal calf serum (Gibco). Confluent H-35 cells yielded (10–14) × 10^7 cells/roller culture with a total cell protein, RNA, and DNA content of 4.2 ± 0.3, 0.45 ± 0.04, and 0.17 ± 0.02 mg/10^7 cells, respectively. As evaluated by the *E. carinatus* coagulation assay (Shah & Suttie, 1971), the intracellular prothrombin (precursor) content was 0.5–0.6 μg/10^7 cells. This value represents ~0.012% of the total cell protein.

Labeling Conditions. Confluent cells in roller cultures were maintained for 24 h with serum-free medium containing 1 μg/mL insulin. Radioactive pulses consisted of refeeding cells with 120 mL of leucine-free Swim's S-77 medium containing 1 μg/mL insulin and 5 mCi of [^{3}H]leucine (some cultures also received 0.1 μg/mL vitamin K). Cells were subsequently harvested and washed by low-speed centrifugation in the presence of NaCl–P buffer (150 mM NaCl and 10 mM phosphate, pH 7.4) containing soybean trypsin inhibitor (40 μg/mL) and 1.0 mM PMSF. Media samples (2.0–5.0-mL aliquots) were collected and adjusted to contain 40 μg/mL STI and 1.0 mM PMSF.

Immunochemical Reagents and Procedures. Rat plasma prothrombin was purified as reported by Li & Olson (1967). Preparations demonstrating prothrombin activity (2500–3000 Iowa units/mg of protein) and exhibiting a single protein band in $NaDodSO_4$–acrylamide gels were used as the source of antigen and for radioiodination (Greenwood et al., 1963). Immunization of rabbits was carried out according to Munns et al. (1977) and in most instances yielded 1.0–3.0 mg of antirat prothrombin per mL of antisera as evaluated by quantitative precipitin studies.

Both antibody- and antigen-coupled Sepharose adsorbents were prepared according to the established procedures of Axen et al. (1967) and Porath et al. (1967), while antibody purification was achieved as essentially described by Wallin & Prydz (1975) and Munns et al. (1977). The latter procedure consisted of batchwise adsorption of antirat prothrombin antibodies to prothrombin-coupled Sepharose. Briefly, antiserum (containing STI and PMSF) was incubated in the presence of prothrombin-coupled Sepharose with gentle rotation for 60 min at 25 °C. Unbound serum components were removed by successive washings of the adsorbent with NaCl–P buffer, bicarbonate buffer (0.1 M $NaHCO_3$ and 0.5 M NaCl, pH 7.6), and phosphate buffer (10 mM PO_4, pH 7.0). Following this regimen, a select population of retained antibodies was eluted by incubating the adsorbent in 7 M urea (10 mM PO_4, pH 7.0) for 60 min at 25 °C. Eluted antibodies were dialyzed successively against NaCl–P buffer and bicarbonate buffer (0.2 M $NaHCO_3$ and 0.5 M NaCl, pH 9.0) prior to conjugation to Sepharose.

Immunochemical Isolation of [^{3}H]Leucine-Labeled Precursor Prothrombins. Intracellular precursor prothrombins were initially obtained by homogenizing cell pellets (0.4–0.8 mL of packed cell volume) in the presence of 2.5 mL of 10 mM Tris, pH 9.5, together with protease inhibitors. Cell homogenates were adjusted to contain 2% Triton X-100 and incubated with stirring for 30 min at 4 °C prior to centrifugation at 100000*g* for 60 min. Supernatant fractions (1.0–2.0 mL) were incubated directly with antirat prothrombin antibody coupled Sepharose (i.e., immunoadsorbent, wet weight volume 0.075 mL) for 30 min at 25 °C. Upon completion of this incubation period, the immunoadsorbent was washed successively with NaCl–P buffer (3.0 mL), bicarbonate buffer (3.0 mL), and phosphate buffer (2 × 3.0 mL) by repeated low-speed centrifugation and resuspension. [^{3}H]Leucine-labeled precursor prothrombins (antigens) were eluted by treatment of the immunoadsorbent with 0.25–0.5 mL of urea–$NaDodSO_4$ (7 M urea, 2% $NaDodSO_4$, and 10 mM PO_4, pH 7.2) for 40–60 min at 37 °C. Identical adsorption techniques were performed with aliquots derived from the culture media as well as with [^{125}I]prothrombin standards. The presence of $NaDodSO_4$ in the above eluant was required for the rapid and quantitative elution of antigen. Its presence also enhanced entry of these proteins into the gel systems described below. The exclusion of $NaDodSO_4$, i.e., the use of 7 M urea alone, did not significantly alter the resulting profiles of radioactivity obtained after electrophoresis. It did, however, decrease the yield of protein eluted from the adsorbent.

Electrophoretic Techniques. $NaDodSO_4$–polyacrylamide gel electrophoretic techniques were conducted according to the procedures of Weber & Osborn (1969). Aliquots containing eluted antigens (i.e., in 7 M urea and 2% $NaDodSO_4$) were adjusted to 5% β-mercaptoethanol (v/v), heated for 2 min at 100 °C, and applied directly to precast gels (7.5% acrylamide, 0.5 × 10.0 cm). Electrophoresis was terminated when the tracking dye (bromphenol blue) had migrated to the bottom of the gels.

Isoelectric focusing (IEF) acrylamide gels were prepared as outlined by Piperno et al. (1977). Appropriate quantities of urea, Nonidet, and acrylamide–bis(acrylamide) were dissolved in H_2O (gentle heating) such that their final concentrations were 9.0 M, 2% (w/v), and 4%, respectively. To 20 mL of this solution was added varying amounts of stock ampholytes to construct the desired pH gradients, after which riboflavin and Temed were added. The resulting gel solution was poured into glass tubes (0.5 × 10.0 cm) and subsequently photopolymerized. Both $NaDodSO_4$ and IEF gels were sliced into 1.0- or 2.0-mm sections and incubated for 18 h in tissue solubilizer (NCS, 0.4 mL/mm), and the radioactivity in each slice was determined by employing a toluene-based organic scintillator (Spectrofluor).

Results

Affinity Antibody Chromatography. Immunospecific Retention of Precursor and Mature Prothrombins. For assessment of the ability of antirat prothrombin–Sepharose to retain mature prothrombin, various concentrations of [^{125}I]prothrombin were incubated in the presence of immunoadsorbent. Illustrated in Figure 1 is a representative radioactivity elution profile derived from stepwise washings of the immunoadsorbent after a 30-min incubation in the presence of [^{125}I]prothrombin. Whereas ~10% of the radioactivity was eluted in the first wash fraction (nonretained fraction), 90% was retained by the immunoadsorbent even after numerous washes with a variety of buffers (see legend to Figure 1). This retained radioactivity was quantitatively removed, however, when the adsorbent was treated with 7 M urea containing 2% $NaDodSO_4$. Subsequent characterization of the retained fraction via electrophoresis in $NaDodSO_4$–acrylamide gels revealed a single peak of radioactivity that comigrated with purified prothrombin standard (see Figure 4A). These results indicated that the immunoadsorbent retained ~90% of the [^{125}I]prothrombin that could subsequently be eluted without significant degradation.

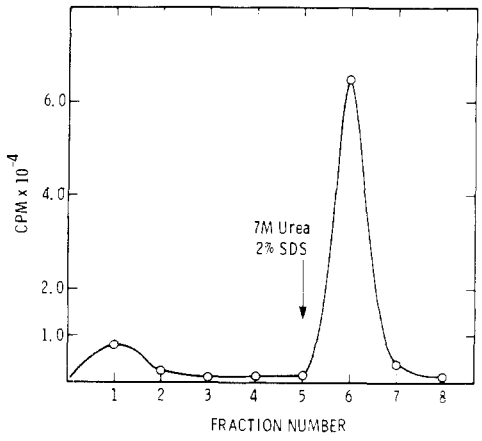

FIGURE 1: Representative radioactivity elution profile of [^{125}I]prothrombin standard (0.5 μg) after a 30-min incubation at 25 °C in the presence of antiprothrombin–Sepharose adsorbent. Fractions 1–5 represent specific wash fractions: fractions 1 and 2 with NaCl–PO_4, pH 7.4; fraction 3 with 0.1 M bicarbonate and 0.5 M NaCl, pH 7.8; fractions 4 and 5 with 10 mM PO_4, pH 7.0. Retained antigen ([^{125}I]prothrombin) was removed by reincubating the adsorbent with 7 M urea containing 2% $NaDodSO_4$ for 30 min at 25 °C. Similar profiles were obtained with quantities of prothrombin ranging from 0.5 to 10.0 μg.

Table I: Immunospecific Adsorption of *E. carinatus* Activatable Protein[a]

adsorbent	*E. carinatus* activatable protein [μg/(2 × 10^7 cells)] in 105000*g* supernatant		
	before $BaSO_4$ and immunospecific adsorption	*after* $BaSO_4$ adsorption	*after* immunospecific adsorption
antiprothrombin-Sepharose			
expt 1	1.05	0.96	ND
expt 2	0.98	1.02	ND
nonimmune IgG-Sepharose			
expt 1	1.10	1.05	1.10
expt 2	1.05	1.00	1.03

[a] Confluent H-35 cells maintained in Swim's medium without serum supplements for 24 h were harvested and homogenized in 10 mM Tris and 2% Triton X-100, pH 9.5 (not containing protease inhibitors). Homogenates were centrifuged at 105000*g*, and aliquots from the supernatant were used directly for $BaSO_4$ adsorption and immunospecific retention studies. *E. carinatus* assays and barium adsorption studies were performed as previously reported (Shah & Suttie, 1971). ND represents no detectable activity.

For assessment of the ability of the immunoadsorbent to retain precursor prothrombins, use was made of previous findings that 105000*g* supernatant fractions of H-35 hepatoma cells possess these precursors, yet little or no mature prothrombin (Munns et al., 1976). As shown in Table I, these supernatant fractions contained ~0.8 μg of precursor prothrombins as evaluated by *E. carinatus* coagulation assays (Shah & Suttie, 1971). Precursors were distinguished from mature prothrombin on the basis that the former was not adsorbed to insoluble barium salts. The ability of antirat prothrombin–Sepharose to retain these precursors was provided by the finding that immunospecific adsorption of supernatant fractions resulted in complete loss of *Echis*-activatable material, i.e., precursor prothrombins. The inability of preimmune rabbit IgG–Sepharose adsorbents to retain *Echis*-activatable material also supported the conclusion that the adsorption of precursor prothrombin was immunospecific.

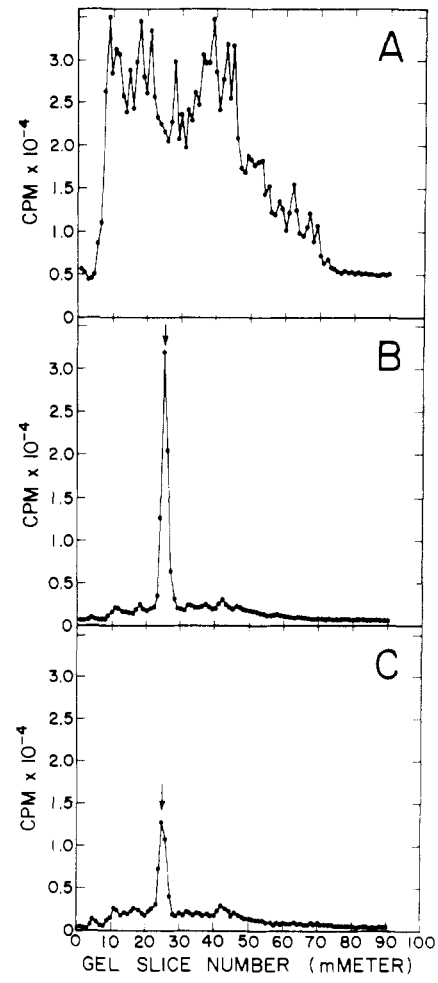

FIGURE 2: Representative radioactivity profiles of *intracellular* [^{3}H]leucine-labeled proteins derived from H-35 cells and electrophoresis in $NaDodSO_4$–acrylamide gels (7.5%). Labeling of cells and preparation of fractions are described under Materials and Methods. (Panel A) Unfractionated 105000*g* supernatant obtained from vitamin K deficient cells (2.0 × 10^5 cells); (panels B and C) immunospecifically adsorbed fractions representing 2.5 × 10^7 cells and labeled in the absence (panel B) and presence (panel C) of vitamin K. Arrows represent the position in the gel where mature prothrombin migrated.

Antirat Prothrombin–Sepharose. Exclusive Retention of [^{3}H]Leucine-Labeled Precursor and Mature Prothrombins. Although the above studies indicated that antirat prothrombin–Sepharose quantitatively retained precursor and mature prothrombins, it remained to be demonstrated whether these were the only proteins immunospecifically adsorbed. For testing of exclusive retention, 105000*g* supernatant (intracellular) and medium (extracellular) samples obtained from cultures previously labeled with [^{3}H]leucine for 2 h were subjected to the immunospecific adsorption procedures described above. Immunospecifically retained, [^{3}H]leucine-labeled protein was eluted in 7 M urea containing 2% $NaDodSO_4$ and electrophoresed in $NaDodSO_4$–acrylamide gels. The resulting radioactivity profiles of these gels together with those of unfractionated preparations are depicted in Figures 2 (intracellular) and 3 (extracellular).

As expected, electrophoresis of an intracellular 105000*g* supernatant aliquot obtained from vitamin K deficient cells (Figure 2A) revealed numerous [^{3}H]leucine-labeled proteins as evidenced by the disperse pattern of radioactivity throughout the gel. Identical patterns were obtained from vitamin K supplemented cells (not shown), thus indicating the ineffectiveness of the vitamin to alter overall protein synthesis.

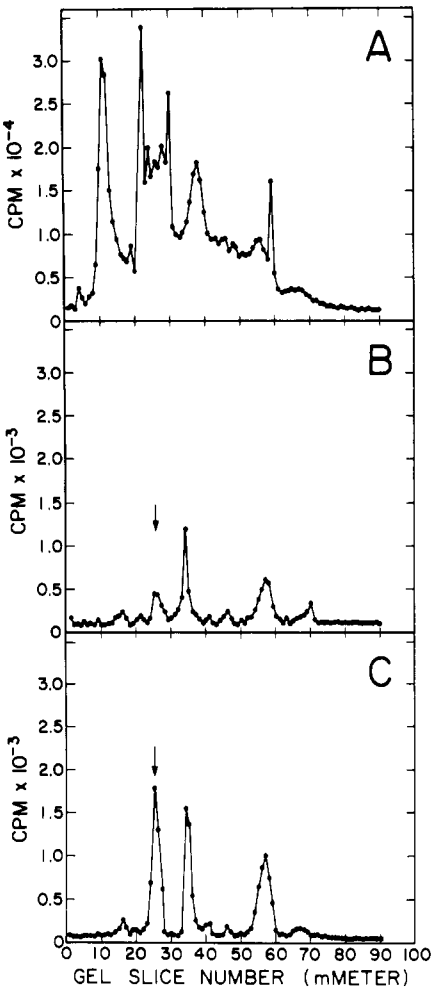

FIGURE 3: Representative radioactivity profiles of *extracellular* [^{3}H]leucine-labeled proteins secreted by H-35 cells into the culture medium and subsequently electrophoresed in NaDodSO$_4$–acrylamide gels (7.5%). Details of these procedures are described under Materials and Methods. (Panel A) Unfractionated medium aliquot from vitamin K deficient cells; (panels B and C) immunospecifically adsorbed medium aliquots derived from vitamin K deficient (panel B) and supplemented (panel C) cells.

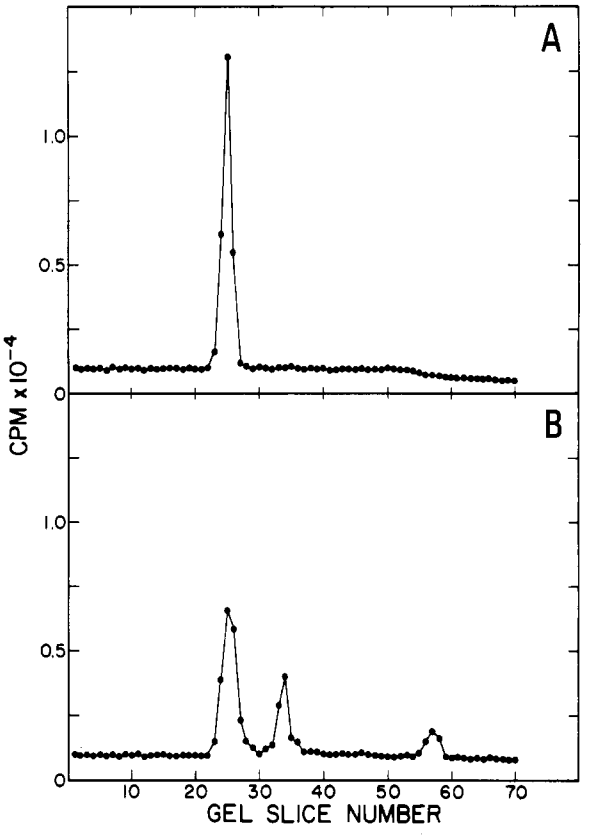

FIGURE 4: Representative NaDodSO$_4$ gel radioactivity profiles of [^{125}I]prothrombin standards (0.2 μg/mL) after incubation in medium of confluent H-35 monolayer cultures for 0 (panel A) and 2 h (panel B). Details of these procedures are as described under Materials and Methods.

However, electrophoresis of immunospecifically retained preparations (parts B and C of Figure 2) revealed the presence of a single peak of radioactive protein that comigrated with mature prothrombin standard. Furthermore, when cells were labeled in the presence of vitamin K, the amount of ^{3}H-labeled protein immunospecifically adsorbed was 50–70% less than that of vitamin K deficient controls.

Electrophoresis of unfractionated medium aliquots also demonstrated the presence of numerous ^{3}H-labeled proteins (Figure 3A). Again, the resulting patterns and amounts of radioactive protein observed with these unfractionated preparations were independent of the vitamin K status of the cells. Analysis of the radioactivity profile from immunospecifically retained medium aliquots (parts B and C of Figure 3) revealed three major protein species, the largest comigrating with the prothrombin standard and two additional proteins observed in gel slices 33–35 and 55–58. Although not extensively characterized, evidence to suggest that these lower molecular weight forms were derived from proteolysis of the 75 000 molecular weight protein was provided by the finding that similar peaks of radioactivity were observed by incubating [^{125}I]prothrombin in the medium for 2 h (compare parts A and B of Figure 4). Molecular weight estimates of these additional proteins indicated values of 60 000 and 35 000 ± 5000 and are in agreement with the molecular weights assigned to various fragments (prethrombin and thrombin) resulting from the proteolysis of prothrombin with activated factor X (Xa) as described by Grant & Suttie (1976) and Morrissey et al. (1973).

A comparison of parts B and C of Figure 2 with parts B and C of Figure 3 also revealed the vitamin K dependency of these immunospecifically retained proteins. Thus, when exogenous vitamin K was present, a marked reduction in the amount of intracellular ^{3}H-labeled protein(s) was observed with a concomitant elevation (twofold) of extracellular ^{3}H-labeled proteins appearing in the culture medium. These vitamin K dependent changes will be discussed in connection with the data presented in Table III.

Isoelectric Forms of Precursor Prothrombins. Although the above data with NaDodSO$_4$–acrylamide gels suggested a molecular weight homogeneity of intracellular precursor prothrombin, previously published data have revealed the presence of multiple isoelectric forms (Esmon et al., 1975a,b; Grant & Suttie, 1976). In view of these findings, immunospecifically retained fractions were characterized via isoelectric focusing acrylamide gel techniques. The results of these investigations are presented in Figure 5 and illustrate the resulting pH gradients and radioactivity profiles of ^{3}H-labeled proteins established during electrophoresis. Panels A and B of Figure 5 reflect the different forms of precursor prothrombins contained in 105000g supernatants of cells maintained in the absence (A) and presence (B) of vitamin K. In both instances, five discrete precursor forms were resolved that possessed pI values of 7.2, 6.7, 6.2, 5.8, and 5.5. Whereas the presence of vitamin resulted in a marked decrease in precursors possessing pI values of 7.2 and 6.7, only moderate decreases

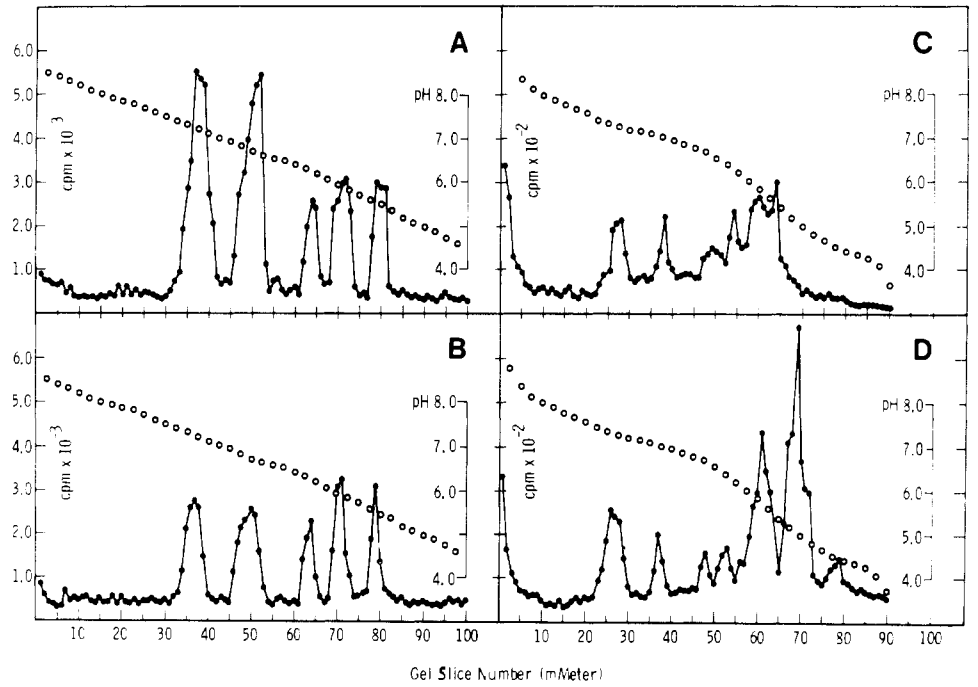

FIGURE 5: Representative radioactivity profiles of [^{3}H]leucine-labeled proteins derived from the H-35 cell culture system and electrophoresis in isoelectric focusing acrylamide gels. Labeling of cells and preparation of various intra- and extracellular fractions are described under Materials and Methods. Panels A and B represent immunospecifically adsorbed 105000*g* supernatant fractions of Triton X-100 extracted whole-cell homogenates. Panel A represents 3.4×10^7 cells labeled in the absence of vitamin while panel B represents 4.3×10^7 cells labeled in the presence of vitamin. Panels C and D represent immunospecifically adsorbed medium aliquots from cell cultures previously labeled in the absence (panel C) and presence (panel D) of vitamin K and correspond to 0.15×10^7 cells. Open circles (o) represent the pH of individual gel slices.

Table II: Distribution of [^{3}H]Leucine-Labeled, Intracellular (105000*g* Supernatant) Precursor Prothrombins. Effects of Vitamin K[a]

precursor prothrombins (p*I*)	cpm incorpd/10^7 cells (% control)	
	vitamin absent	vitamin present
7.2	7647 (100)	2255 (30)
6.7	7058 (100)	2512 (35)
6.2	2341 (100)	1545 (66)
5.8	3088 (100)	2110 (68)
5.5	2573 (100)	1602 (62)

[a] These data were derived from the radioactive profiles illustrated in Figure 5, panels A (vitamin absent) and B (vitamin present).

were observed with forms possessing p*I* values of 6.2, 5.8, and 5.5. The vitamin K dependent decreases in these forms are presented in Table II.

Panels C and D of Figure 5 represent the various forms of [^{3}H]leucine-labeled prothrombins and/or their degradation products secreted into the culture medium in the absence (C) and presence (D) of vitamin. Although these radioactivity profiles are difficult to interpret in view of the extensive proteolysis that occurred in the culture medium (see Figures 4 and 5), it is significant to note the presence of large quantities of mature prothrombin (p*I* value of 5.0) *only* in the media of cell cultures labeled in the presence of vitamin K (see panel D of Figure 5). Exhibited in both of these latter gels was a relatively high level of radioactivity corresponding to p*I* values of and between 5.8 and 5.5.

[^{3}H]Leucine-Labeled Precursor Prothrombins. Effects of Vitamin K. On the basis of the data presented thus far, it was concluded that the antirat prothrombin–Sepharose adsorbent quantitatively and exclusively retained precursor and mature prothrombins as well as various proteolytic fragments derived therefrom. It was therefore of interest to obtain a quantitative assessment of the ability of vitamin K to alter both intra- and extracellular levels of [^{3}H]prothrombins derived from cells labeled with [^{3}H]leucine for 2 h. These data are presented in Table III and revealed that vitamin K had no effect on overall protein synthesis since the levels of [^{3}H]leucine incorporated into total cell homogenate, 105000*g* supernatant, and media protein were equivalent and hence independent of vitamin. However, the presence of vitamin K markedly reduced the intracellular levels of precursor prothrombin (i.e., from 25 000 to 10 000 cpm/10^7 cells, listed as 105000*g* supernatant R_{fx}) while increasing by twofold the levels of [^{3}H]prothrombins in the media (i.e., from 50 000 to 95 000 cpm, listed as medium R_{fx}).

Noteworthy was the additional finding that cells labeled in the presence of vitamin consistently yielded a greater amount of total [^{3}H]prothrombins, i.e., the sum of the immunospecifically retained radioactivity derived from intracellular supernatant and medium fractions. Thus, in the presence of vitamin the radioactive content of total prothrombins amounted to ~105 000 cpm ($0.925 \times 10^4 + 9.54 \times 10^4 = 104\,670$ cpm) whereas in the absence of vitamin 77 000 cpm was isolated ($2.389 \times 10^4 + 5.203 \times 10^4 = 76\,920$). Whether this effect is related to a vitamin K dependent increase in the rate of synthesis of prothrombin (Munns et al., 1976) or an enhanced rate of degradation of immature or precursor prothrombins in the medium (see Figures 4 and 5) as a result of their lack of various posttranslational modifications [see Nelsestuen & Suttie (1971)] remains to be investigated. Evidence in support of the latter assumption is provided by the findings presented in parts B and C of Figure 3. These data suggest that the prothrombin secreted by vitamin K supplemented cells is *not* as extensively degraded as the prothrombin secreted by vitamin-deficient cells (compare parts B and C of Figure 3).

Discussion and Conclusions

Although a number of investigators (Esmon et al., 1975a,b; Grant & Suttie, 1976) have successfully purified intracellular precursors of rat prothrombin by employing conventional techniques (e.g., salt fractionation and ion-exchange chro-

Table III: Immunospecific Adsorption of Intracellular and Extracellular [^{3}H]Leucine-Labeled PCA-Precipitable Protein. Effects of Vitamin K Administration[a]

	(cpm × 10^{-4})/10^7 cells (% of total homogenate protein)				
		105000g supernatant		medium	
expt	cell homogenate	NR_{fx}	R_{fx}	NR_{fx}	R_{fx}
vitamin absent					
expt 1	11 414 (100)	5211 (45.7)	2.4 (0.021)	2226 (19.5)	5.2 (0.046)
expt 2	10 993 (100)	5407 (49.2)	2.6 (0.024)	2120 (19.3)	5.4 (0.049)
vitamin present					
expt 1	11 680 (100)	5660 (48.5)	0.9 (0.008)	2197 (18.8)	9.5 (0.082)
expt 2	11 176 (100)	5987 (53.6)	1.1 (0.010)	2243 (20.1)	9.2 (0.082)

[a] Confluent H-35 hepatoma cells in roller cultures were labeled with [^{3}H]leucine (5 mCi/roller flask) in otherwise leucine-free medium for 2 h in the presence or absence of vitamin K (0.1 μg/mL). Upon completion of the radioactive pulse, 2.5-mL aliquots of medium were collected prior to washing and harvesting cells. Cells were homogenized and the resulting homogenates processed to yield 105000g supernatants. Aliquots of both supernatants and medium were immunospecifically fractionated (antirat prothrombin–Sepharose) to obtain nonretained (NR) and retained (R) fractions. Aliquots of these fractions were precipitated with 2.5% PCA, and after thorough washing the protein pellet was digested in NCS–H_2O (9:1) prior to addition of scintillant and determination of radioactivity. PCA-precipitable protein was also determined for unfractionated cell homogenates, 105000g supernatants, and medium samples. The latter two determinations are now shown, yet they equaled the sum of their respective nonretained and retained fractions ± 5%. Each experimental value represents the average of a minimum of duplicate determinations. All data are expressed on the basis of 10^7 cells with 7.5 mL of culture medium being used per 10^7 cells.

matography), such procedures have resulted in (a) low, nonquantitative yields of precursors and (b) the loss of some precursor species altogether. Furthermore, relatively large amounts of starting material were required for their isolation, i.e., 20–30 rat livers. In view of these findings, other approaches were sought that would result in both the rapid and quantitative isolation of small quantities of precursor prothrombins present in cultured hepatoma cells and liver microsomes. Since previous reports have indicated that antibodies elicited in response to mature prothrombin immunospecifically recognize precursor prothrombins as well (Stenflo, 1972; Wallin & Prydz, 1975; Munns et al., 1976), the feasibility of an immunochemical approach for the isolation of precursor prothrombins was examined.

The results presented herein have established the ability of an antiprothrombin–Sepharose adsorbent to retain [^{3}H]-leucine-labeled precursor and mature prothrombins obtained from H-35 hepatoma cells previously labeled with [^{3}H]leucine. This immunochemical approach was designed to obtain a quantitative recovery of labeled prothrombins as well as their complete solubilization for subsequent electrophoretic separation. On the basis of the properties of dodecyl sulfate as an efficient and nondiscriminating solubilizing agent, immunospecifically retained protein fractions were eluted in the presence of 7 M urea containing 2% $NaDodSO_4$. Such an eluant permits direct characterization of the antigen(s) in both $NaDodSO_4$ and IEF gels. The presence of the nonionic detergent NP-40 in the latter gel system effectively sequesters the $NaDodSO_4$ in the form of micelles, which rapidly migrate into the acid reservoir during electrophoresis. This sequestering effect allows the proteins in the applied samples to migrate according to their native charge (Ames & Nikaido, 1976). The application of this technique permitted us to resolve five discrete precursors of mature prothrombin possessing pI values of 7.2, 6.7, 6.2, 5.8, and 5.5. When cells were labeled in the presence of vitamin K, there appeared a marked reduction in these intracellular precursors with a concomitant appearance of significant quantities of mature prothrombin (pI 5.0) in the culture medium.

In all likelihood each of the precursor prothrombins revealed by isoelectric focusing techniques represents specific post-translational modifications (i.e., N-terminal cleavage, carboxylation, and core and terminal glycosylation). Since the vitamin K dependent processing step results in the γ-carboxylation of glutamate residues located near the NH_4 terminus of prothrombin (Stenflo et al., 1974; Nelsestuen et al., 1974; Magnusson et al., 1974), it was originally anticipated that vitamin-supplemented cells would possess additional, more acidic precursors not present in control preparations. Yet as the radioactivity profiles in Figure 5 demonstrate, only the *amount* and not the *type* of precursors present intracellularly were altered when cells were labeled in the presence of vitamin. Although this apparent inconsistency remains to be resolved, similar observations have been reported by Grant & Suttie (1976) and Stenflo (1972) with precursor and mature prothrombins obtained from bovine plasma. In the latter investigation both precursor and mature prothrombin preparations possessed three identical protein bands after isoelectric focusing.

Although, from the data presented herein, it is impossible to determine which precursor (or precursors) serves as the substrate for the vitamin K dependent carboxylase reaction, the more basic species, i.e., the pI 7.2 and 6.7 precursors, are likely candidates. This assumption is based upon their vitamin-dependent decrease (~70%) relative to the moderate decreases (~30%) observed with the more acidic species (Table II). This interpretation is further supported by the findings of Grant (1975), who isolated a ^{14}C-labeled precursor of prothrombin with a pI value of 6.8 from vitamin K deficient rat liver microsomes previously incubated in the presence of vitamin K and [^{14}C]carbonate. Furthermore, when Grant (1975) incubated an identical microsomal fraction with postmitochondrial supernatant, additional [^{14}C]bicarbonate-labeled proteins were obtained possessing pI values of and between 6.8 and 5.8. Although these results are difficult to interpret with certainty, they suggest that (a) more than one pI form of precursor prothrombin participates in the vitamin K dependent carboxylase reaction and/or that (b) additional modification events associated with prothrombin processing are operable in these in vitro systems. These interpretations are consistent with the data presented in Figure 5, which indicate the presence of multiple forms of precursor prothrombins.

Whereas the exact nature of the modifications associated with the pI 7.2, 6.7, and 6.2 precursors remains to be elucidated, in all likelihood those precursors possessing pI values of 5.8 and 5.5 represent asialo and/or partially sialated forms of prothrombin, respectively. This identification stems from the observation reported by Grant & Suttie (1976) that upon complete removal of sialic acid from mature prothrombin

(neuraminidase treatment) the resulting asialo prothrombin possessed a p*I* of 5.8. Furthermore, since the addition of sialic acid reflects terminal glycosylation, it is not unreasonable to assume that these precursors are in the final stages of prothrombin processing. However, it is intriguing that the intracellular pool of prothrombins is devoid of mature prothrombin (p*I* 5.0) which appears to be secreted into the medium by cells supplemented with vitamin K. The inability to detect mature protein intracellularly suggests that the secretory phase of prothrombin processing is rapid and likely to be associated with a terminal modification event.

That posttranslational modifications in general and sialic acid additions in particular provide stability to mature prothrombin was evident by the degree of degradation associated with various preparations of ^{3}H-labeled prothrombins derived from the medium. Whereas extensive proteolysis appeared to be associated with precursors secreted by vitamin K deficient cells (Figure 3B), only moderate degradation was apparent when mature prothrombin was secreted (Figure 3C) or when [^{125}I]prothrombin standard was incubated directly in the medium (Figure 4B). Coupled with the findings that the bulk of ^{3}H-labeled precursors secreted to the medium appeared to be devoid of sialic acid residues (as evidenced by p*I* values of and between 5.5 and 5.8, see Figure 5), these results are consistent with the data of Nelsestuen & Suttie (1971), who reported significant decreases (10-fold) in the circulating half-life of asialo rat prothrombin.

In conclusion, the application of immunochemical procedures for isolating precursor prothrombins from rat hepatoma cell culture systems appears promising. The employment of antibodies elicited in response to a mature protein antigen that immunospecifically recognize precursors of the antigen should serve as a useful probe for investigating numerous aspects of protein processing. Studies using these procedures are now in progress to characterize precursor prothrombins with respect to (a) the nature of their posttranslational modifications, (b) the subcellular levels at which they occur, (c) the time frame in which they appear, and (d) their potential functional roles as related to processing, secretion, coagulation, and degradation.

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