Characterization of the Major Apolipoproteins Secreted by Two Human Hepatoma Cell Lines†

V. I. Zannis,* J. L. Breslow, T. R. SanGiacomo, D. P. Aden, and B. B. Knowles

ABSTRACT: Two newly described human hepatoma derived cell lines, Hep G2 and Hep 3B [Knowles, B. B., Howe, C. C., & Aden, D. P. (1980) *Science (Washington, D.C.) 209*, 497–499], synthesize and secrete into the culture medium most of the major plasma apoproteins (apoA-I, apoA-II, apoB, apoC-II, apoC-III, and apoE). The synthesized apoproteins were identified by direct two-dimensional gel analysis of the culture medium or by two-dimensional analysis following purification of the apoproteins by ultracentrifugation or immunoprecipitation. We found that the apoA-I synthesized by both of the hepatoma cell lines consists of two isoproteins designated 2 and 3 which are more basic than the major plasma apoA-I isoproteins designated 4 and 5. The apoE synthesized by both cell lines is composed mainly of an array of isoproteins with increasingly higher molecular weights and lower isoelectric points as compared to those of the major apoE isoproteins found in plasma. These precursors of apoE are converted to the major apoE isoproteins upon treatment with *Clostridium perfringens* neuraminidase and represent sialo apoE isoproteins. ApoA-II, apoC-II, apoC-III-1, and apoC-III-2 correspond to the protein forms present in plasma. The human hepatoma cell lines (Hep G2 and Hep 3B) provide a unique model for studies of the regulation of human apoprotein and lipoprotein synthesis and catabolism.

Apoprotein synthesis takes place in the liver and small intestine (Mahley et al., 1970; Windmueller et al., 1973; Noel & Rubinstein, 1974; Marsh, 1976; Hamilton et al., 1976; Felker et al., 1977; Glickman & Green, 1977; Wu & Windmueller, 1978, 1979; Rachmilewitz et al., 1978; Imaizumi et al., 1978; Green et al., 1978; Schonfeld et al., 1978; Hamilton, 1978; Noel et al., 1979; Windmueller & Wu, 1981). Experiments with rats have shown that the liver can synthesize all the major apoproteins (Windmueller et al., 1973; Marsh, 1976; Felker et al., 1977; Imaizumi et al., 1978; Noel et al., 1979; Wu & Windmueller, 1979; Windmueller & Wu, 1981). The apoproteins synthesized by the liver are found in very low density lipoproteins (VLDL) and high density lipoproteins HDL (Windmueller et al., 1973; Hamilton et al., 1976; Felker et al., 1977; Hamilton, 1978; Noel et al., 1979). The HDL produced by rat livers perfused with medium containing an inhibitor of the enzyme lecithin cholesterol acyltransferase consists of disk-shaped HDL particles which have high apoE to apoA-I ratios (nascent HDL) (Hamilton et al., 1976; Felker et al., 1977; Hamilton, 1978). Rat small intestine has been shown to synthesize large amounts of apoA-I, apoA-IV, and apoB but only small amounts of apoC peptides and litttle or no apoE (Glickman & Green, 1977; Wu & Windmueller, 1978, 1979; Imaizumi et al., 1978; Green et al., 1978; Schonfeld et al., 1978; Windmueller & Wu, 1981). The apoproteins synthesized by the intestine are distributed in chylomicrons, VLDL, and HDL (Windmueller et al., 1973; Wu & Windmueller, 1978; Green et al., 1978). Apoprotein (and lipoprotein) synthesis has also been demonstrated by liver cells in monolayer cultures (Tarlow et al., 1977; Davis et al., 1979; Kempen, 1980; Dashti et al., 1980) and by organ cultures of intestine and liver (Zannis et al., 1980, 1981b; Zannis & Breslow, 1980a). In addition to synthesizing lipoproteins, liver cells are also involved in lipoprotein catabolism (Redgrave, 1970; Bergman et al., 1971; Grundy & Mok, 1976; Floren & Nilsson, 1977, 1978; Cooper & Yu, 1978; Sherrill & Dietschy, 1978; Gardner & Mayes, 1978; Sigurdsson et al., 1978, 1979; Van Berkel & Van Tol, 1978; Chao et al., 1979; Soltys & Portman, 1979; Ose et al., 1979; Carrella & Cooper, 1979). Recent findings indicate that apoprotein E (which is synthesized almost exclusively by the liver) may also be the recognition site for the hepatic uptake and catabolism of chylomicron remnants and other apoE-containing lipoprotein particles (Sherrill et al., 1980; Windler et al., 1980a–c; Quarfordt et al., 1980; Shelburne et al., 1980).

In this paper, we report that two previously described human hepatoma derived cell lines Hep 3B and Hep G2 (Knowles et al., 1980) are capable of synthesizing the major human apoproteins. The apoA-I and apoE are synthesized as precursors which are different from the isoprotein forms present in plasma. These apoproteins have the same characteristics as those synthesized by the human liver or intestine in organ cultures (Zannis et al., 1980, 1981b; Zannis & Breslow, 1980a). The use of these apoprotein-producing hepatoma cell lines will facilitate in vitro studies of lipoprotein and apoprotein metabolism.

Experimental Procedures

Materials. Bovine serum albumin, ovalbumin, lysozyme, trypsin inhibitor, *o*-phenanthroline, phenylmethanesulfonyl fluoride, neuraminidase (*Clostridium perfringens*), Tris, glycine, and agarose were purchased from Sigma Chemical Co. Acid and alkaline phosphatase were purchased from Worthington and phospholipase C from Calbiochem. Ampholines, pH 2.5–4 and 5–8, were obtained from LKB. Nonidet P-40 was purchased from Particle Data Laboratories, Ltd. Sodium dodecyl sulfate ($NaDodSO_4$), manufactured by British Drughouse Chemical, Ltd., was purchased through Gallard-Schleisinger. Acrylamide, bis(acrylamide), *N,N,N′,N′*-tetramethylenediamine, ammonium persulfate, Coomassie brilliant blue, bromophenol blue, and biolites (ampholines, pH 4–6) were obtained from Bio-Rad. Urea, ultrapure grade, was a

† From the Children's Hospital Medical Center and Harvard Medical School, Boston, Massachusetts 02115 (V.I.Z., J.L.B., and T.R.S.), and the Wistar Institute of Anatomy and Biology, Philadelphia, Pennsylvania 19104 (D.P.A. and B.B.K.). *Received May 22, 1981.* This work was supported by grants from the National Institutes of Health (HL15895, CA18470, and CA25875) and the Americal Cancer Society (IM215). Partial salary support for Dr. Zannis was provided by the Medical Foundation, Boston, MA. J.L.B. is an Established Investigator of the American Heart Association. D.P.A. is a recipient of an RCDA (CA00510).

0006-2960/81/0420-7089$01.25/0

product of Schwarz/Mann. [^{35}S]Methionine (300 Ci/mmol) was obtained from New England Nuclear. X-ray film, Cronex-4, was purchased from Du Pont. Human anti-HDL was purchased from Miles Laboratories. Sheep anti-apoA-I was a gift from Dr. Peter Herbert. All other materials were of the purest grade commercially available.

Cell Cultures. The cell lines Hep G2 and Hep 3B were grown in 100 mm diameter Petri dishes with Williams E or Eagles's minimum essential medium supplemented with 10% fetal bovine serum. So that synthesized proteins could be labeled, confluent monolayers were washed with Earle's balanced salt solution (EBSS) and incubated for 7 h with 5 mL of specially prepared methionine-free Williams E or Eagle's minimum essential medium containing 1 mCi of [^{35}S]-methionine/Petri dish.

Preparation of Cell Culture Samples for Two-Dimensional Analysis. After incubation, the medium was collected and centrifuged at 30000*g* for 25 min in a Beckman 75 rotor, and the supernatant was retained. A small aliquot of the supernatant was dialyzed, lypophilized, and used for two-dimensional analysis without immunoprecipitation. The remaining supernatant was used for immunoprecipitation and density-gradient ultracentrifugation (Redgrave et al., 1975). The antibodies used for immunoprecipitation were anti-apo A-I and anti-HDL. The anti-HDL antibody immunoprecipitates apoA-I, apoE, and small amounts of apoA-II and the apoC peptides (Zannis et al., 1980). The anti-apo A-I antibody immunoprecipitates apoA-I, but in the presence of carrier HDL, this antibody also precipitates small amounts of apoE, apoA-II, and the apoC's. The anti-apoA-I antibody does not immunoprecipitate VLDL or purified apoE. Maximum immunoprecipitation for both of these antibodies was achieved by adding to the culture medium a predetermined amount of carrier plasma HDL and incubating at 4 °C for 4 days as previously described (Zannis et al., 1980, 1981b; Zannis & Breslow, 1980a). The immunoprecipitate was collected by centrifugation for 3 min in a Beckman microfuge and washed twice with 1.0 mL of cold EBSS and once with distilled water. The immunoprecipitate was then dissolved in lysis buffer (O'Farrell, 1975) and analyzed on the two-dimensional gel system.

Enzymatic Treatments of Newly Synthesized Apoproteins. A small portion of the culture medium and portions of the immunoprecipitates were dissolved in 0.5 mL of either 0.1 M sodium acetate (pH 5), 0.1 M Tris-HCl (pH 8), or 0.1 M Tris-HCl (pH 7.3) and 0.05 M $CaCl_2$. These fractions were then treated as follows: (a) fractions in sodium acetate buffer were incubated with either 2 units of neuraminidase (*C. perfringens*) at 37 °C for 2 h or 250 μg of acid phosphatase at 25 °C for 1 h, (b) fractions in 0.1 M Tris-HCl (pH 8) were incubated with 2 units of alkaline phosphatase at 25 ° C for 1 h, and (c) fractions in 0.1 M Tris-HCl (pH 7.3) and 0.05 M $CaCl_2$ were incubated with 40 μg of phospholipase C at 37 °C for 2 h. Control experiments were also performed where the above fractions were supplemented with a combination of protease inhibitors consisting of 20 μg/mL trypsin inhibitor, 3×10^{-4} M phenylmethanesulfonyl chloride, 3×10^{-4} M *o*-phenanthroline, and 1×10^{-2} M EDTA. After incubation, the fractions were heated at 55 °C for 10 min. The treated samples were dialyzed against water, lyophilized, dissolved in O'Farrell's (1975) lysis buffer, and analyzed by two-dimensional polyacrylamide gel electrophoresis.

Density-Gradient Ultracentrifugation. Density-gradient ultracentrifugation was performed according to the method of Redgrave et al. (1975). Four milliliters of culture medium was adjusted to a density of 1.21 g/mL with potassium bromide, placed in a cellulose nitrate tube, and overlayed sequentially with 3 mL each of $d = 1.063$ g/mL and $d = 1.019$ g/mL potassium bromide solutions followed by 2.9 mL of normal saline. The tubes were then centrifuged in a Beckman SW41 rotor at 38 000 rpm for 44 h. After centrifugation, ten 1-mL fractions were collected from the top of the tube, and an aliquot of 0.3 mL from each fraction was dialyzed, lyophilized, and analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis.

One-Dimensional $NaDodSO_4$–Polyacrylamide Slab Gel Electrophoresis. Electrophoresis was performed on a vertical gel apparatus as previously described (Zannis et al., 1980). The separating gel [11.7% acrylamide and 0.32% bis(acrylamide)] and the stacking gel [4.4% acrylamide and 0.12% bis(acrylamide)] were prepared according to the method of Davis (1964).

Two-Dimensional Polyacrylamide Gel Electrophoresis. The method used was similar to that described by O'Farrell (1975) with the following modifications. The isoelectric focusing tubes were 15 cm in length with an inner diameter of 3 mm. The ampholines used were 1.4% (pH 5–8), 0.45% (pH 4–6), and 1.16% (pH 2.5–4). The samples were loaded on the first dimension without prerunning, and focusing was carried out for a total of 9500 V-h with the equilibration time between the two dimensions limited to 15 min.

For the second dimension, the focused cylindrical gels were placed on slab gels identical with those described above for $NaDodSO_4$ slab gel electrophoresis. After electrophoresis, the gels were fixed in 50% methanol–10% acetic acid for 1 h and stained in a solution of 0.25% Coomassie brilliant blue, 50% methanol, and 10% acetic acid for 1 h. Destaining was accomplished by alternating exposure of the gel for 1-*h* periods to either 200 mL of fixing solution or 400 mL of water until the proper stain background was achieved. Two such exchanges were usually sufficient.

Results

Identification by Two-Dimensional Gel Electrophoresis of the Apoproteins Synthesized and Secreted into the Culture Medium by the Hepatoma Cell Lines. Aliquots of [^{35}S]-methionine-labeled culture medium from Hep 3B and Hep G2 cells were dialyzed and lyophilized. This material was mixed with 20 μg of a pool of human VLDL (consisting of a mixture of apoE subclasses βII, βIII, and βIV) and 20 μg of human HDL and subjected to two-dimensional gel analysis and autoradiography. The protein stained gel obtained from the analysis of Hep 3B culture medium shows the positions of the carrier VLDL and HDL apoproteins (Figure 1A). The autoradiogram of the gel reveals the positions of the newly synthesized [^{35}S]methionine-labeled apoproteins (Figure 1B). A comparison of the plasma with the hepatoma apoproteins can be obtained by superimposing the autoradiogram on the corresponding protein stained gel (Figure 1C). The autoradiogram of a similar analysis of Hep G2 cell culture medium is shown in Figure 1D. Figure 1 reveals that Hep 3B and Hep G2 cells synthesize apoA-I, apoA-II, apoB, apoC-II, apoC-III-1, -2, and -3, apoE (subclass βIII), and a lower molecular weight peptide, designated X. The nature of peptide X and any possible relationship to apoA-II or the apoC's are under investigation. ApoE is synthesized abundantly by both Hep 3B and Hep G2 cells. Hep G2 synthesizes greater amounts of apoA-I and lesser amounts of apoA-II andd apoC peptides compared to Hep 3B cells. The apoA-I synthesized by the Hep 3B and Hep G2 cell lines is composed exclusively of isoproteins 2 and 3, whereas the major plasma apoA-I iso-

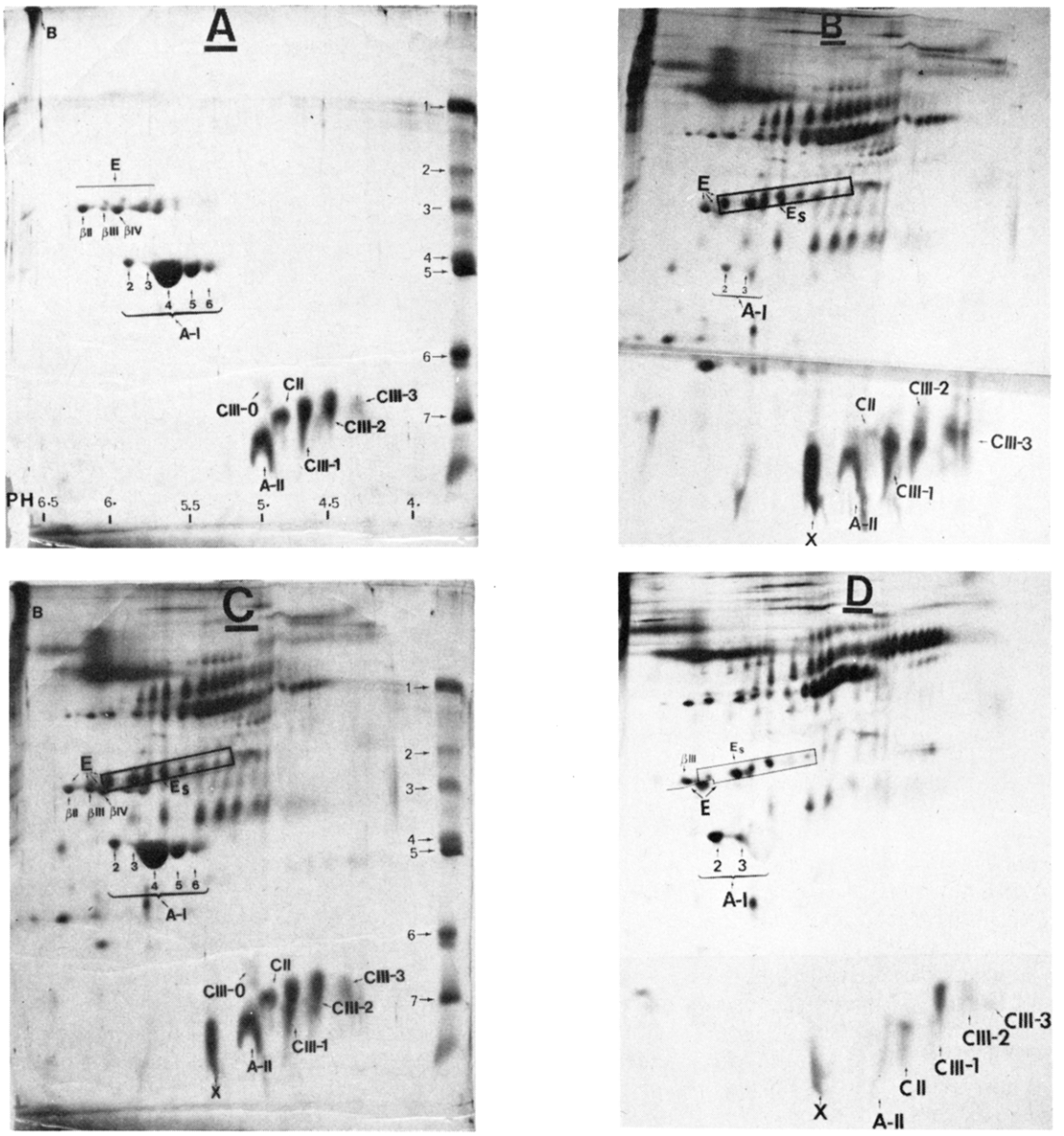

FIGURE 1: Analysis of the culture medium from the hepatoma cell lines incubated in the presence of [^{35}S]methionine by two-dimensional gel electrophoresis. An aliquot of 0.15 mL of the Hep 3B culture medium was mixed with 20 μg of a pool of human VLDL (consisting of a mixture of apoE subclasses βII, βIII, and βIV) and 20 μg of human HDL and subjected to two-dimensional gel analysis. Panel A shows the protein stained gel and reveals the position of the plasma HDL and VLDL apoproteins. The indicated positions of the apoproteins have been established by using either purified apoproteins or apoproteins immunoprecipitated with monospecific antibodies. Panel B shows the autoradiogram of the gel shown in panel A and reveals the position of the [^{35}S]methionine-labeled proteins synthesized and secreted by the Hep 3B cells into the culture medium. The upper part of the film was exposed for 6 days, and the lower part of the film (in the vicinity of apoA-II and the apoC's) was exposed for 40 days. Panel C shows the autoradiogram in panel B superimposed on the protein stained gel in panel A. The positions of the newly synthesized apoA-I (isoproteins 2 and 3) and of the apoE, mainly sialo apoE (E_s) isoproteins, are indicated. Panel D is an autoradiogram derived from a similar two-dimensional analysis of the culture medium obtained from Hep G2 cells. The upper part of the film was exposed for 6 days, and the lower part of the film (in the vicinity of apoA-II and apoC's) was exposed for 40 days. The numbers 1–7 at the right side of panels A and C indicate protein molecular weight markers as follows: (1) bovine serum albumin, 68 000; (2) ovalbumin, 43 000; (3) aldolase, 40 000; (4) bovine purine nucleoside phosphorylase, 30 000; (5) human apoA-I, 28 000; (6) trypsin inhibitor, 19 000; (7) egg white lysozyme, 14 300. In this and all subsequent photographs and autoradiograms, the cathode is on the left and the anode is on the right.

proteins are 4 and 5. The apoE synthesized by both cell lines is composed mainly of an array of isoproteins with increasingly higher molecular weights and lower isoelectric points as compared to those of the major apoE isoproteins found in plasma. Similar analysis of the cell culture medium of another previously described cell line PLC/PRF/5 (Macnab et al., 1976) showed that this line synthesized mainly apoE and only traces of apoA-I (data not shown).

Separation of Hepatoma Lipoproteins by Density-Gradient Ultracentrifugation and Characterization of the Apoproteins by One- and Two-Dimensional Polyacrylamide Gel Electrophoresis. One-dimensional $NaDodSO_4$–polyacrylamide gel electrophoresis of the fractions obtained by density-gradient ultracentrifugation reveals the distribution of the apoproteins in the various lipoprotein fractions. Figure 2 is an autoradiogram of such an analysis and shows the newly synthesized apoA-I, apoE, and apoB in lipoprotein particles of d = 1.03–1.17 g/mL. The same figure also reveals size heterogeneity of apoE which is the result of sialo apoE isoproteins.

Two-dimensional analysis of lipoprotein particles of d = 1.03–1.17 g/mL (Figure 3) demonstrates sialo apoE and precursor apoA-I isoproteins that were seen when the culture medium was directly analyzed (Figure 1). A similar result was obtained with the Hep G2 cell line.

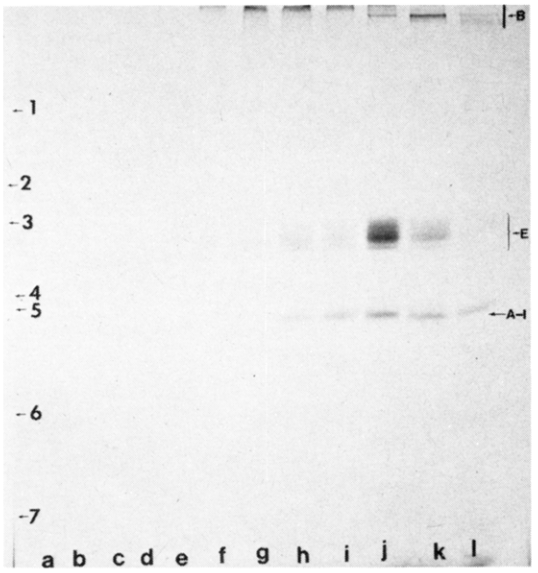

FIGURE 2: Analysis of the culture medium obtained from the hepatoma cell lines by density-gradient ultracentrifugation, $NaDodSO_4$–polyacrylamide gel electrophoresis, and autoradiography. The autoradiogram obtained from the analysis of the culture medium of Hep 3B cells grown in [^{35}S]methionine is shown. Lane a of the gel contained the same molecular weight markers as in Figure 1. Lane b contained purified apoE as indicated. The densities (grams per milliliter) of the lipoprotein fractions contained in lanes c–l are as follows: c = 1.002; d = 1.008; e = 1.011; f = 1.019; g = 1.031; h = 1.042; i = 1.068; j = 1.096; k = 1.125; l = 1.17. Similar results are obtained by analysis of the culture medium of the Hep G2 cell line.

Identification by Immunoprecipitation of Apoproteins A-I and E Synthesized by the Hepatoma Cell Lines. The protein identified as apoA-I can be immunoprecipitated by using monospecific anti-apoA-I as well as anti-HDL antibodies. Both antibodies cause concomitant immunoprecipitation of apoE and small amounts of apoA-II, the apoC's, and protein X. The ratio of apoA-I to apoE observed in the culture medium is roughly maintained after immunoprecipitation with anti-HDL or anti-apoA-I antibodies. Figure 4 shows immunoprecipitation of the culture medium of Hep G2 (panels A–C and E) and Hep 3B (panel D) cells with anti-HDL (panels A–D) or anti-apoA-I (panel E) antibodies. The isoprotein patterns for apoA-I and apoE after immunoprecipitation are identical with those seen upon direct analysis of the culture medium (Figure 1) and after ultracentrifugation (Figure 3).

Newly Synthesized ApoE Exists Primarily as Sialo ApoE. The newly synthesized apoE isoproteins, which are more acidic and of higher molecular weight than the plasma apoE isoproteins, are converted to apoE isoproteins of M_r 38 000 after treatment with *C. perfringens* neuraminidase as shown by direct analysis of the culture medium (Figure 5A–C). Thus, the acidic and higher molecular weight apoE components represent sialo apoE isoproteins. The conversion of the acidic and higher molecular weight apoE isoproteins to M_r 38 000 still takes place in the presence of a combination of protease inhibitors (Morihara, 1974). This strongly suggests that the disappearance of the sialo isoproteins is not the result of preferential destruction of the higher molecular weight acidic isoproteins by protease impurities that may be present in the neuraminidase preparation. Treatment of the culture medium with acid phosphatase, alkaline phosphatase, and phospholipase C does not effect any of the apoproteins synthesized by the cells. The conversion of sialo to asialo apoE isoproteins was also observed when the neuraminidase treatment was applied to immunoprecipitates of the culture medium (Figure 5D). In the experiments shown in Figure 5, the neuraminidase treatment resulted in increased intensity of the major apoE isoprotein of M_r 38 000 and the appearance of a new, more acidic isoprotein differing by approximately 0.5 negative charge unit from the major isoprotein. It is not known whether this new apoE isoprotein is of physiological significance or results from aging of the samples. We have previously shown a smearing of the plasma apoE isoprotein patterns and a shift to more acidic isoelectric points after prolonged storage (Zannis & Breslow, 1981; Zannis et al., 1981).

Absence of Detectable Intracellular Pools of Apoproteins in the Hep 3B and Hep G2 Cell Lines. In contrast to the analysis of the culture medium, two-dimensional gel electrophoretic analysis of [^{35}S]methionine-labeled cell extracts of both Hep 3B and Hep G2 cell lines revealed no detectable apoproteins even after immunoprecipitation with anti-apoA-I or anti-HDL antibodies. This suggests that apoproteins are secreted soon after synthesis without the accumulation of a large intracellular pool.

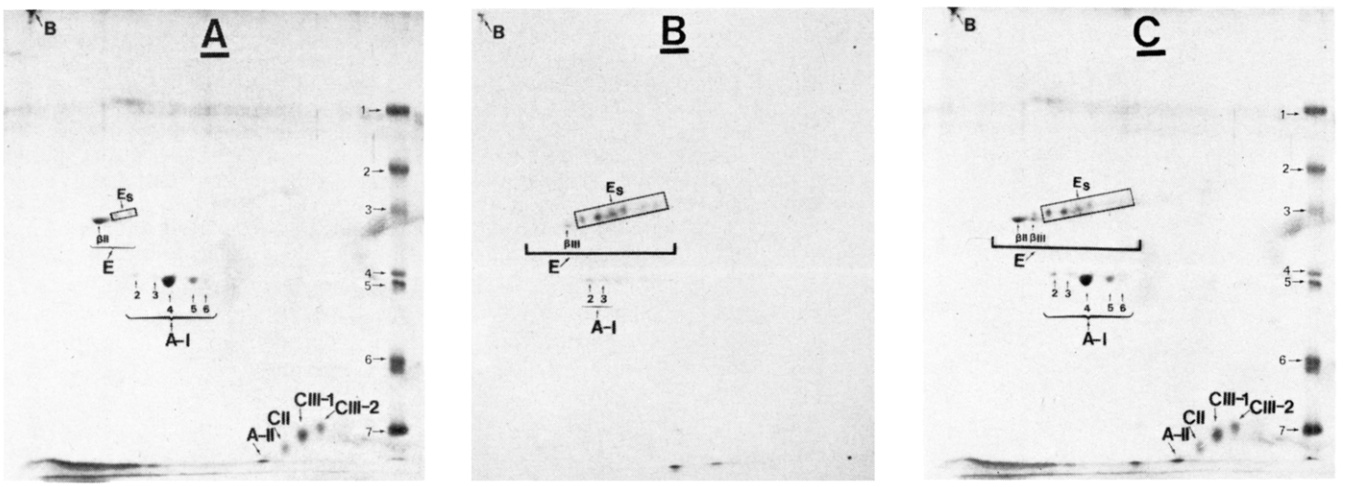

FIGURE 3: Two-dimensional gel electrophoresis and autoradiography of [^{35}S]methionine-labeled apoproteins separated by density-gradient ultracentrifugation. Fractions 5–10 of the density-gradient separation (which correspond to lanes g–l of Figure 2) were pooled, mixed with 10 μg of human VLDL (apoE subclass βII) and 20 μg of human HDL, and analyzed on a two-dimensional gel system. Panel A shows the protein stained gel. Panel B shows the autoradiogram of the gel shown in panel A. Panel C shows the autoradiogram of panel B superimposed on the gel of panel A. The positions of plasma and newly synthesized Hep 3B apoproteins are indicated. The numbers 1–7 at the right side of panels A and C indicate protein molecular weight markers as explained in Figure 1.

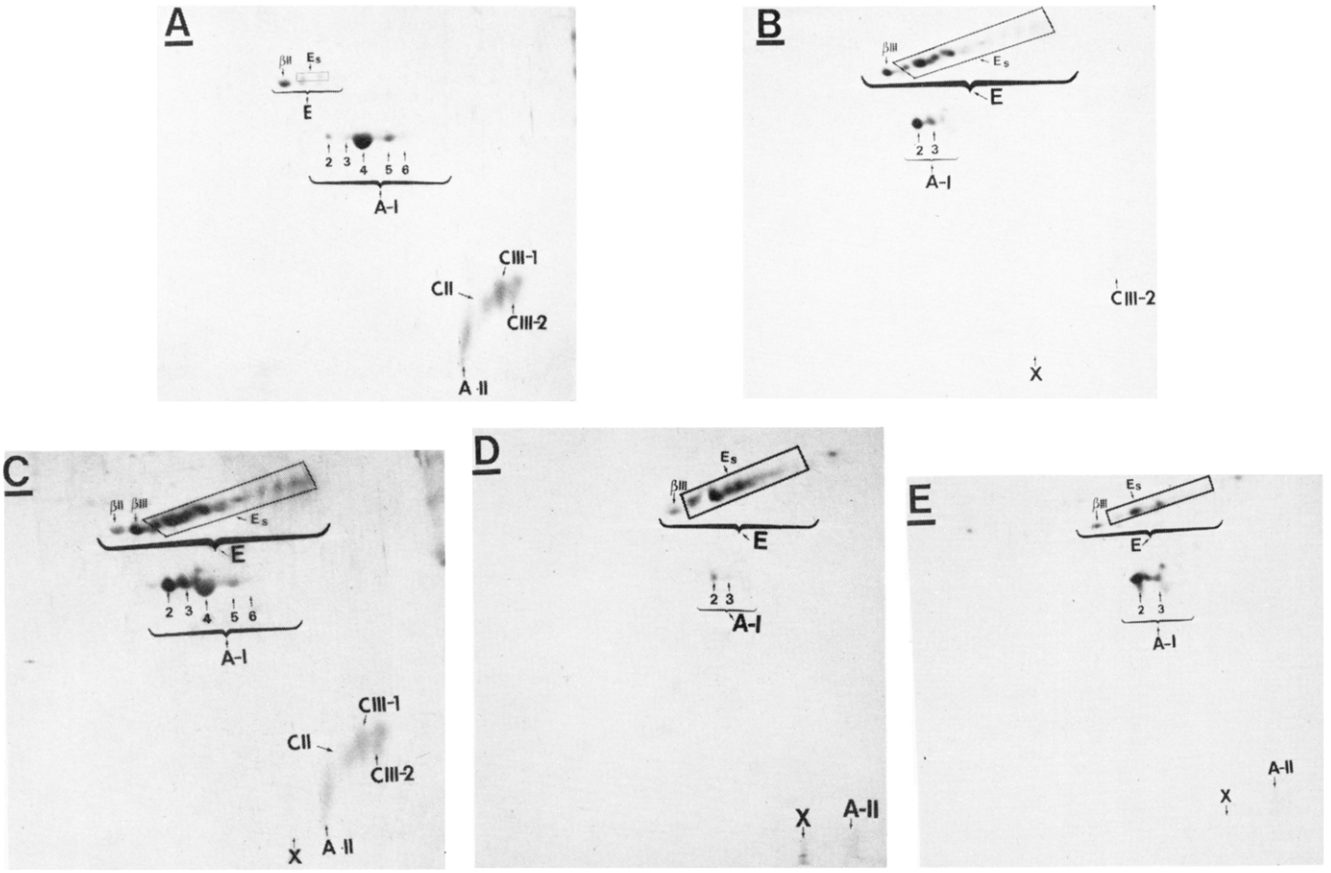

FIGURE 4: Two-dimensional gel electrophoresis of [^{35}S]methionine-labeled apoproteins immunoprecipitated from the culture medium of hepatoma cells with anti-HDL (panels A–to D) or anti-apoA-I (panel E) antibodies. An aliquot of the culture medium of Hep G2 cells was mixed with 15 μg of human HDL and immunoprecipitated as explained under Experimental Procedures. The immunoprecipitate was mixed with 10 μg of human VLDL (apoE subclass βII) and subjected to two-dimensional gel analysis. Panel A shows the protein stained gel. The positions of plasma apoA-I, apoE, apoA-II, and apoC's are indicated. Panel B shows the autoradiogram of the gel in panel A. The position of the immunoprecipitable ^{35}S-labeled apoproteins is indicated. Panel C shows the autoradiogram in panel B superimposed on the protein stained gel in panel A. Panel D shows an autoradiogram obtained after a similar analysis of the culture medium from Hep 3B cells. Panel E shows autoradiograms obtained after a similar analysis of the culture medium from Hep G2 cells, following immunoprecipitation with anti-apoA-I antibodies. In all panels, only the area of the gel (or autoradiogram) in the vicinity of the proteins of interest are shown. The sets of photographs shown in panels A–C were taken at the same magnification.

Discussion

Liver cells perform both lipoprotein synthesis and catabolism. These cells synthesize VLDL, HDL, and all the known apoproteins (Mahley et al., 1970; Windmueller et al., 1973; Neol & Rubinstein, 1974; Marsh, 1976; Hamilton et al., 1976; Felker et al.,, 1977; Imaizumi et al., 1978; Hamilton, 1978; Wu & Windmueller, 1979; Windmueller & Wu, 1981) and catabolize chylomicron remnants and other lipoprotein particles of $d > 1.019$ g/mL (Redgrave, 1970; Bergam et al., 1971; Grundy & Mok, 1976; Floren & Nilsson, 1977, 1978; Cooper & Yu, 1978; Sherrill & Dietschy, 1978; Gardner & Mayes, 1978; Sigurdsson et al., 1978, 1979; Van Berkel & VanTol, 1978; Chao et al., 1979; Soltys & Portman, 1979; Ose et al., 1979; Carrella & Cooper, 1979; Sherrill et al., 1980; Windler et al., 1980a–c; Quarfordt et al., 1980; Shelburne et al., 1980). In spite of significant advances in our understanding of hepatic apoprotein and lipoprotein biosynthesis and catabolism, the detailed molecular mechanisms underlying these processes, especially in cells of human origin, remain unknown. The demonstration that the Hep 3B and Hep G2 cell lines derived from human hepatomas retain their apoprotein synthesizing capability makes these cell lines a model in which to study the detailed molecular mechanisms underlying the regulation of lipoprotein and apoprotein metabolism.

It is of great interest that apoA-I and apoE synthesized by the hepatoma cells are in the form of precursor isoproteins and thus have the same features as the corresponding apoproteins synthesized by the human liver or intestine in organ culture (Zannis & Breslow, 1980a; Zannis et al., 1980, 1981b). The apoA-I synthesized by the hepatoma cells is composed exclusively of isoproteins 2 and 3, whereas the major plasma apoA-I isoproteins are 4 and 5. This implies that isoproteins 2 and 3 are converted (probably in plasma) to isoproteins 4 and 5 by a posttranslational charge modification. The physiological significance of the conversion of isoproteins 2 and 3 to the major plasma isoproteins 4 and 5 is not clear. Recently, it was shown that isoproteins of apoA-I which correspond (on the basis of their relative abundance and their isoelectric point) to isoproteins 4–6 have similar amino acid composition and are the same in their ability to activate lecithin cholesterol acyltransferase (LCAT) (Nestruck et al., 1980). The isoprotein forms of apoA-I synthesized by the hepatoma cell lines could be isolated and their ability to activate LCAT studied. In addition, these isoproteins could be used as substrates to isolate activities in human plasma which convert isoproteins 2 and 3 to the major plasma isoproteins 4 and 5. We have recently shown that the apoA-I synthesized by the human intestine in organ culture has protein components both of higher and of lower molecular weights than the major apoA-I isoprotein synthesized and that these components cross-react with anti-apoA-I and anti-HDL (Zannis et al., 1980). Some higher molecular weight cross-reacting com-

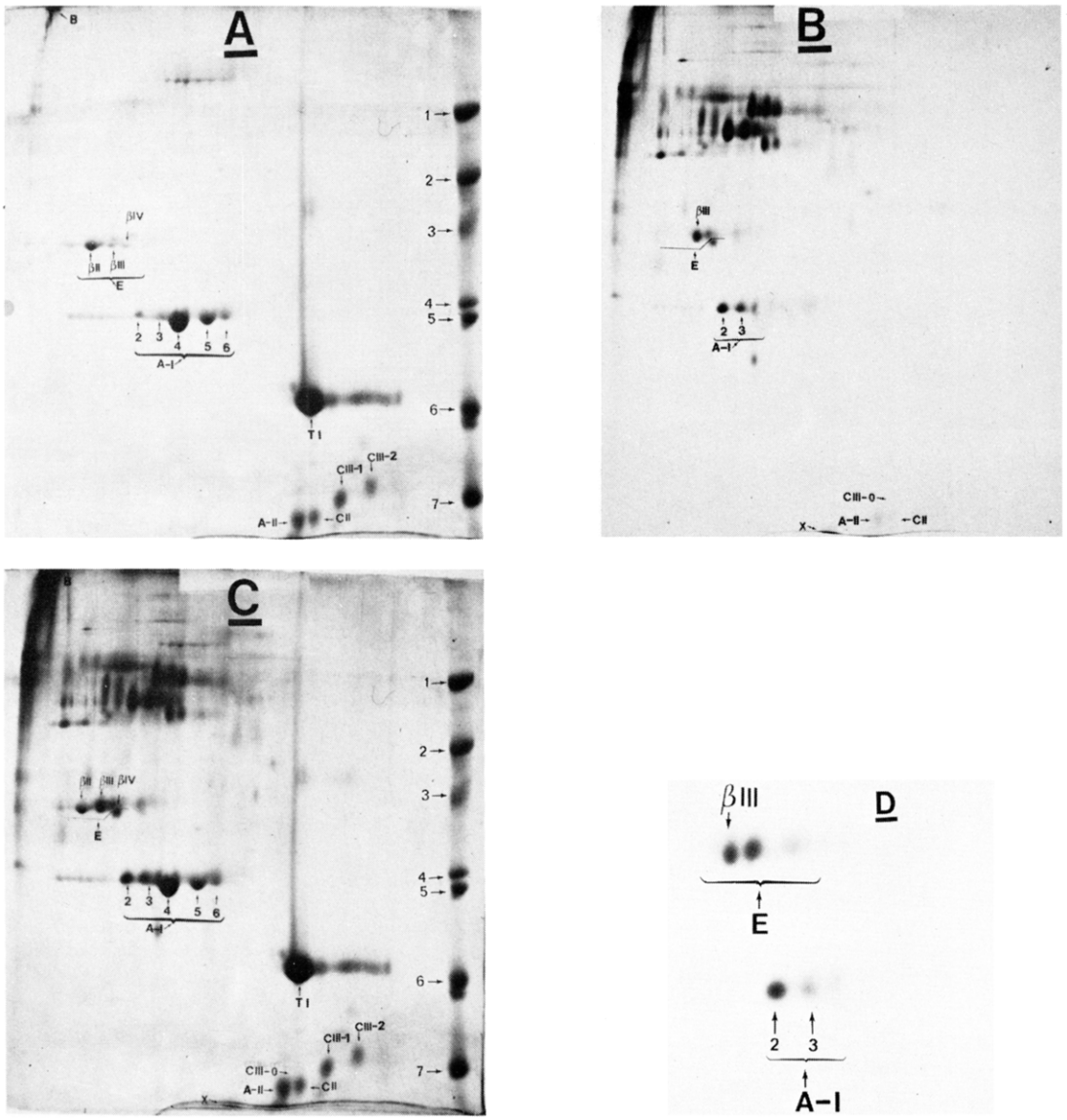

FIGURE 5: Two-dimensional gel electrophoresis of the hepatoma apoproteins following treatment with *C. perfringens* neuraminidase. Panels A–C show the analysis of the culture medium. Panel D shows the analysis of the apoproteins immunoprecipitated with anti-HDL antibodies. An aliquot of 0.10 mL of the Hep 3B culture medium was treated with *C. perfringens* neuraminidase as described under Experimental Procedures. After treatment, the medium was mixed with 10 μg of human VLDL (apoE subclass βII) and 20 μg of HDL and subjected to two-dimensional gel electrophoretic analysis. Panel A shows the protein stained gel and reveals the position of the plasma HDL and VLDL apoproteins. TI is the trypsin inhibitor which was added to the sample for reasons explained under Experimental Procedures. Panel B shows the autoradiogram of the gel shown in panel A and reveals the positions of the [^{35}S]methionine-labeled Hep 3B proteins. Numbers 1–7 at the right side of panels A and C indicate protein molecular weight markers described in Figure 1. Panel C shows the autoradiogram in panel B superimposed on the gel of panel A. Similar results were obtained by neuraminidase treatment and two-dimensional analysis of the Hep G2 culture medium. A comparison with Figure 1 reveals that the acidic and higher molecular weight apoE isoproteins are converted to M_r 38 000 isoproteins. In addition, apoC-III, 1–3 isoprotiens, are converted to apo C-III-0. Panel D shows autoradiograms obtained after Hep G2 culture medium was immunoprecipitated with anti-HDL antibody, treated with neuraminidase, and subjected to two-dimensional gel electrophoretic analysis. The analysis of the corresponding samples without neuraminidase treatment is shown in Figure 4, panel B.

ponents are observed on the acidic site of Hep G2 apoA-I purified by immunoprecipitation with anti-apoA-I (Figure 4E). The physiological significance of these components is not known.

ApoE synthesized by the hepatoma cell lines consists mainly of an array of isoproteins with increasingly higher molecular weights and lower isoelectric points as compared to the major apoE isoproteins found in plasma (Zannis & Breslow, 1980b, 1981; Zannis et al., 1981a). These acidic and higher molecular weight apoE isoproteins are converted to M_r 38 000 apoE isoprotein(s) upon treatment with *C. perfringens* neuraminidase and represent sialo apoE isoproteins. Since we have previously shown that only 10–20% of plasma apoE's are sialo apoE isoproteins (Zannis & Breslow, 1981), we postulate that apoE is synthesized first as sialo apoE and subsequently desialated in plasma to asialo apoE. Recent experiments have indicated that apoE may be the recognition site for the uptake and catabolism of chylomicron remnants and other apoE-containing lipoprotein particles (Sherrill et al., 1980; Windler et al., 1980a–c; Quarfordt et al., 1980, Shelburne et al., 1980). It has also been reported that acidic apoE isoproteins apoE-I and apoE-II (separated by one-dimensional techniques) are poorly catabolized by the perfused rat liver (Havel et al., 1980). We have recently shown by two-dimensional gel electrophoresis that these acidic apoE isoproteins are enriched in sialo apoE components (Zannis & Breslow, 1981). Our observations together with these other findings suggest that apoE is synthesized in its sialo apoE form and it is in this form that it

leaves its site of synthesis, the liver. ApoE must then be desialated in plasma before it can be effectively recognized and taken up by the liver. The hepatoma cell lines may allow us to isolate the sialo apoE isoproteins and directly test this hypothesis. In addition, the availability of [^{35}S]methionine-labeled sialo apoE might help in the isolation of activities from human plasma which convert the sialo apoE isoproteins to their asialo form.

The hepatoma cell lines Hep 3B and Hep G2 synthesize and secrete into the culture medium apoB, apoA-II, apoC-II, apoC-III-1, and apoC-III-2 (but not apoC-III-0) which have the same molecular weights and isoelectric points as their corresponding plasma counterparts. ApoB synthesis by these cells has been previously suggested by the Ouchterlony immunodiffusion technique (Knowles et al., 1980). Two additional peptides are synthesized in large amounts by the Hep 3B and Hep G2 cells. One of them, designated X, with an apparent isoelectric point of 5.28, is not present in detectable quantities in plasma. We are currently investigating the possible relationship of this peptide with apoA-II or the apoC's. The other peptide designated as apoC-III-3, corresponds to a minor apoC-III isoprotein previously described (Marcel et al., 1979). ApoC-I is a relatively basic protein and even if synthesized by the hepatoma cells cannot be analyzed with this gel system.

The hepatoma cell lines should allow in the future even more detailed studies of the regulation of human apoprotein and lipoprotein synthesis. In addition, further studies of the physiological roles of the apoA-I and apoE precursor isoproteins will be possible. Finally, if the hepatoma cell lines have maintained the capability of the parent liver cells to bind and catabolize lipoproteins, they will provide a unique system for in vitro studies of the regulation of hepatic lipoprotein degradation.

References

Bergman, E. N., Havel, R. J., Wolfe, B. M., & Bohmer, T. (1971) *J. Clin. Invest. 50*, 1831–1839.

Carrella, M., & Cooper, A. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 338–342.

Chao, Y. S., Windler, E. E., Chen, G. C., & Havel, R. J. (1979) *J. Biol. Chem. 254*, 11360–11366.

Cooper, A. D., & Yu, P. Y. S. (1978) *J. Lipid Res. 19*, 635–642.

Dashti, N., McConathy, W. J., & Ontko, J. A. (1980) *Biochim. Biophys. Acta 618*, 347–358.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Davis, R. A., Engelhorn, S. C., Pangburn, S. H., Weinstein, D. B., & Steinberg, D. (1979) *J. Biol. Chem. 254*, 2010–2016.

Felker, T. E., Fainaru, M., Hamilton, R. L., & Havel, R. J. (1977) *J. Lipid Res. 18*, 465–473.

Floren, C. H., & Nilsson, A. (1977) *Biochem. J. 168*, 483–494.

Floren, C. H., & Nilsson, A. (1978) *Biochem. J. 174*, 827–838.

Gardner, R. S., & Mayes, P. A. (1978) *Biochem. J. 170*, 47–55.

Glickman, R. M., & Green, P. H. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2569–2575.

Green, P. H. R., Tall, A. R., & Glickman, R. M. (1978) *J. Clin. Invest. 61*, 528–534.

Grundy, S. M., & Mok, H. Y. I. (1976) *Metab. Clin. Exp. 25*, 1225–1239

Hamilton, R. L. (1978) in *Disturbances in Lipid and Lipoprotein Metabolism* (Dietschy, J., Gotto, A. M., Jr., & Ontko, J. A., Eds.) pp 155–171, Clinical Physiology Series, American Physiology Society, Bethesda, MD.

Hamilton, R. L., Williams, M. C., Fielding, C. J., & Havel, R. J. (1976) *J. Clin. Invest. 58*, 667–680.

Havel, R. J., Chao, Y., Windler, E. E., Kotite, L., & Guo, L. S. S. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 4349–4353.

Imaizumi, K., Havel, R. J., Fainaru, M., & Vigne, J. L. (1978) *J. Lipid Res. 19*, 1038–1046.

Kempen, H. J. M. (1980) *J. Lipid Res. 21*, 671–680.

Knowles, B. B., Howe, C. C., & Aden, D. P. (1980) *Science (Washington, D.C.) 209*, 497–499.

Macnab, G. M., Alexander, J. J., Lecatsas, G., Bey, E. M., & Urbanowicz, J. M. (1976) *Br. J. Cancer 34*, 509–515.

Mahley, R. W., Bersot, T. P., Lequire, V. S., Levy, R. I., Windmueller, H. G., & Brown, W. V. (1970) *Science (Washington, D.C.) 168*, 380–382.

Marcel, Y. L., Bergseth M., & Nestruck, A. C. (1979) *Biochim. Biophys. Acta 573*, 175–183.

Marsh, J. B. (1976) *J. Lipid Res. 17*, 85–90.

Morihara, K. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 179–243.

Nestruck, A. C., Suzue, G., & Marcel, Y. L. (1980) *Biochim. Biophys. Acta 617*, 110–121.

Noel, S. P., & Rubinstein, D. (1974) *J. Lipid Res. 15*, 301–308.

Noel, S. P., Wong, L., Dolphin, P. J., Dory, L., & Rubinstein, D. (1979) *J. Clin. Invest. 64*, 674–683.

O'Farrel, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.

Ose, L., Ose, T., Norum, K. R., & Berg, T. (1979) *Biochim. Biophys. Acta 574*, 521–536.

Quarfordt, S., Hanks, J., Jones, R. S., & Shelburne, F. (1980) *J. Biol. Chem. 255*, 2934–2937.

Rachmilewitz, D., Albers, J. J., Saunders, D. R., & Fainaru, M. (1978) *Gastroenterology 75*, 677–682.

Redgrave, T. G. (1970) *J. Clin. Invest. 49*, 465–471.

Redgrave, T. G., Roberts, D. C. K., & West, C. F. (1975) *Anal. Biochem. 65*, 42–49.

Schonfeld, G., Bell, E., & Alpers, D. H. (1978) *J. Clin. Invest. 61*, 1539–1550.

Shelburne, F., Hanks, J., Meyers, W., & Quarfordt, S. (1980) *J. Clin. Invest. 65*, 652–658.

Sherrill, B. C., & Dietschy, J. M. (1978) *J. Biol. Chem. 253*, 1859–1867.

Sherrill, B. C., Innerarity, T. L., & Mahley, R. W. (1980) *J. Biol. Chem. 255*, 1804–1807.

Sigurdsson, G., Noel, S. P., & Havel, R. J. (1978) *J. Lipid Res. 19*, 628–634.

Sigurdsson, G., Noel, S. P., & Havel, R. J. (1979) *J. Lipid Res. 20*, 316–324.

Soltys, P. A., & Portman, O. W. (1979) *Biochim. Biophys. Acta 574*, 505–520.

Tarlow, D. M., Watkins, P. A., Reed, R. E., Miller, R. S., Zwergel, E. E., & Lane, D. (1977) *J. Cell Biol. 73*, 332–353.

Van Berkel, T. J. C., & Van Tol, A. (1978) *Biochim. Biophys. Acta 530*, 299–304.

Windler, E., Chao, Y., & Havel, R. J. (1980a) *J. Biol. Chem. 255*, 5475–5480.

Windler, E., Chao, Y., & Havel, R. J. (1980b) *J. Biol. Chem. 255*, 8303–8307.

Windler, E. E. T., Kovanen, P. T., Chao, Y., Brown, M. S., Havel, R. J., & Goldstein, J. L. (1980c) *J. Biol. Chem. 255*, 10464-10471.
Windmueller, H. G., & Wu, A. L. (1981) *J. Biol. Chem. 256*, 3012-3016.
Windmueller, H. G., Herbert, P. N., & Levy, R. I. (1973) *J. Lipid Res. 14*, 215-223.
Wu, A. L., & Windmueller, H. G. (1978) *J. Biol. Chem. 253*, 2525-2528.
Wu, A. L., & Windmueller, H. G. (1979) *J. Biol. Chem. 254*, 7316-7322.
Zannis, V. I., & Breslow, J. L. (1980a) in *Electrophoresis 1979* (Radola, B. J., Ed.) pp 437-473, Walter de Gruyter, Berlin and New York.
Zannis, V. I., & Breslow, J. L. (1980b) *J. Biol. Chem. 255*, 1759-1762.
Zannis, V. I., & Breslow, J. L. (1981) *Biochemistry 20*, 1033-1041.
Zannis, V. I., Breslow, J. L., & Katz, A. J. (1980) *J. Biol. Chem. 255*, 8612-8617.
Zannis, V. I., Just, P. W., & Breslow, J. L. (1981a) *Am. J. Hum. Genet. 33*, 11-24.
Zannis, V. I., Kunit, D. M., & Breslow, J. L. (1981b) *J. Biol. Chem.* (in press).

Simple and Rapid Method To Determine the Binding of Blood Clotting Factor X to Phospholipid Vesicles†

Gerbrand van Dieijen,* Guido Tans,‡ Jan van Rijn,§ Robert F. A. Zwaal, and Jan Rosing

ABSTRACT: Negatively charged phospholipid vesicles inhibit the activation of factor X by the factor X activating protein from *Vipera russelli* (RVV-X). It is shown that this inhibition is caused by the fact that RVV-X is not able to activate factor X molecules bound to phospholipid bilayers. By use of the kinetic parameters for the activation of factor X by RVV-X, this finding can be applied to determine free and membrane-bound factor X from which binding parameters can be obtained. Binding of factor X to negatively charged phospholipid bilayers has been found to be rapid and reversible. The parameters that describe factor X binding to phospholipid membranes containing various mole percentages of the negatively charged phospholipids phosphatidylserine (PS) and phosphatidylglycerol (PG) in phosphatidylcholine (PC) were measured at a Ca^{2+} concentration of 10 mM. The dissociation constants obtained from Scatchard plots decrease when the mole percentage of negatively charged phospholipid is increased from 0 to 50 mol %. Factor X has a higher affinity for membranes containing PS than for those containing PG. The number of binding sites increases proportionally with increasing mole percentage of negatively charged phospholipid molecules between 0 and 25 mol % PS. The stoichiometry calculated for PS-containing vesicles is 16-17 PS residues per bound factor X molecule. Since factor X contains 14 γ-carboxyglumatic acid residues, it is likely that one PS molecule is required to link one γ-carboxyglumatic acid residue of factor X via Ca^{2+} to the phospholipid membrane. The number of binding sites on phospholipid vesicle surfaces containing more than 50 mol % PS decreases dramatically, presumably due to Ca^{2+}-induced aggregation of vesicles. Prothrombin, which like factor X is a vitamin K dependent protein containing γ-carboxyglumatic acid residues, competes with factor X for binding sites on the phospholipid membrane.

Phospholipid-protein interactions play an important role in the blood coagulation cascade [for a review, see Zwaal (1978)]. Prothrombin activation and the activation of factor X via the intrinsic and extrinsic pathway take place on a phospholipid surface. Phospholipid provides the surface upon which a complex is formed of the enzyme, the substrate, and an activator protein (factor V for prothrombin activation, factor VIII for the intrinsic factor X activation, and tissue factor apoprotein for the extrinsic factor X activation). Both the enzymes (factors Xa, IXa, and VIIa) and the substrates (prothrombin and factor X) that participate in these complexes belong to the vitamin K dependent proteins of blood coagulation. These proteins contain γ-carboxyglumatic acid residues that are essential for the binding to negatively charged phospholipid surfaces in the presence of calcium ions. To describe the reactions taking place on the phospholipid surface, it is essential to have quantitative information about the binding of these proteins to phospholipid bilayers.

A number of methods have been used to determine the binding parameters of clotting factors to phospholipid bilayers. An extensive study of the binding of vitamin K dependent clotting factors to phospholipid vesicles of different composition was carried out by utilizing the technique of 90° light scattering (Nelsestuen & Broderius, 1977; Nelsestuen et al., 1978; Resnick & Nelsestuen, 1980). Bloom et al. (1979) used the same technique to determine the binding of factor X, factor Va and prothrombin to vesicles of a mixture of Folch fraction III [which is rich in phosphatidylserine (PS)][1] and PC (25/75 w/w). Since phospholipid vesicles with a mole percentage of PS higher than 30-40% tend to aggregate and form nonvesicular structures in the presence of calcium ions (Papahadjopoulos et al., 1975; Day et al., 1977), light scattering methods

† From the Department of Biochemistry, Biomedical Centre, Rijksuniversiteit Limburg, Maastricht, The Netherlands. *Received April 29, 1981.*
‡ Present address: Department of Immunopathology, Scripps Clinic and Research Foundation, La Jolla, CA 92037.
§ Supported by the Netherlands Organization for the Advancement of Pure Scientific Research (Z.W.O.).

[1] Abbreviations used: PS, phosphatidylserine; PG, phosphatidylglycerol; PC, phosphatidylcholine; RVV-X, factor X activator purified from Russel's viper venom; S2222, *N*-benzoyl-L-isoleucyl-L-glutamylglycyl-L-arginine-*p*-nitroanilide hydrochloride.

0006-2960/81/0420-7096$01.25/0