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Serum Apolipoprotein AI Synthesis in Rat Hepatocytes and its Secretion as Proform

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Summary: Rat hepatocytes in monolayer or suspension culture synthesize serum lipoprotein AI. It is secreted into the serum-free culture medium. Synthesis and secretion processes were studied in the presence of radiolabelled amino acids. The synthesis product of the hepatocytes and the secretion product from the medium were isolated by immunoprecipitation with a monospecific rabbit antiserum against rat apolipoprotein AI.

The intracellular and secreted products were homogeneous and identical in polyacrylamide gel electrophoresis but had reduced electrophoretic mobility as compared to native apolipoprotein AI. They were submitted to automated Edman degradation. They were present in their proform, the N-terminus of which is extended by a hexapeptide.

In the presence of rat serum the proform is proteolytically transformed into the mature form of apolipoprotein AI.

Synthese des Apolipoproteins AI in Ratten-Hepatozyten und seine Sekretion

Zusammenfassung: Rattenleberzellen in Monolayer-Kulturen oder in Suspension synthetisieren Apolipoprotein AI und sezernieren es in das Medium.

Die Synthese wurde in Gegenwart von radioaktiven Aminosäuren durchgeführt. Mit Hilfe von monospezifischen Antikörpern gegen Ratten-Apolipoprotein AI wurden das Syntheseprodukt aus den Hepatozyten und das Sekretionsprodukt aus dem serumfreien Medium isoliert. Beide radioaktiven Apoproteine wurden dem Edman-Abbau unterworfen. Ihre N-terminalen Sequenzen waren identisch.

Hepatozyten sezernieren das Apolipoprotein AI in seiner Pro-Form in serumfreies Medium. Der N-Terminus des Sekretionsproduktes ist um ein Hexapeptid extendiert.

In Gegenwart von Rattenserum wird die Pro-Form proteolytisch in die reife Form des Apolipoproteins AI umgewandelt.

Abbreviations:
Apo AI, AII = apolipoprotein AI, AII of serum high-density lipoproteins,
HDL = serum high-density lipoprotein,
KIE = kallikrein inhibitor units.

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Key words: Hepatocyte monolayers and suspension, labelling of apolipoprotein AI, immunoprecipitation, secretion of proapolipoprotein AI.

Cell-free translations of mRNA from rat liver[1] and intestinal mucosa[2] as well yielded apolipoprotein AI, a main apolipoprotein of serum high-density lipoproteins. In these studies not only its synthesis in these two cell types was demonstrated, but also that the primary translation product is extended at the N-terminal end by 24 amino-acid residues forming two segments: an 18 amino-acid residues-containing pre-sequence (signal peptide) which is processed cotranslationally by a signal peptidase of the endoplasmic reticulum membrane, the proform of apo AI is released, which contains a hexapeptide extending the N-terminus of mature apo AI.

We reported recently[3] that human liver synthesizes a preproapolipoprotein AI also composed of an 18 residues long strongly hydrophobic signal peptide and a six residues long pro-segment. Human apoprotein AII mRNA yields a cell-free primary translation product of 100 amino acids. The N-terminus of the monomeric form is extended by a 23 amino-acids residue sequence, 17 of which are released by the signal peptidase during cotranslational processing. Again the proform of apo AII can be obtained translocated into the vesicles of the endoplasmic reticulum. Sequence studies showed that again a hexapeptide forms the pro-segment.

The events of the secretion process of apolipoproteins, which lead from the synthesis of the apolipoprotein precursor via the transport through the endoplasmic reticulum and Golgi-apparatus into the surrounding medium (plasma) can be studied most suitably in a whole-cell system. We therefore supplemented our current in vitro system for apolipoprotein synthesis and processing by a hepatocyte suspension system. This approach should not only confirm our in vitro studies but allow insight into the secretion, the structure of the secretion product and finally in the assembly process of the lipoprotein particle.

The studies reported here demonstrate, that rat hepatocytes in suspension exhibit an efficient apo AI synthesis. The product isolated from the postmitochondrial fraction is the proform of apo AI exclusively. The hepatocytes secrete the proform into the serum-free medium. It is identical with the intracellular proform.

Addition of serum leads to the proteolytic processing to mature apolipoprotein AI.

Materials and Methods

Materials

Collagenase, hyaluronidase, pepstatin and leupeptin were purchased from Boehringer Mannheim GmbH (D-6800 Mannheim), protein A-Sepharose CL-4B from Pharmacia Fine Chemicals (D-7800 Freiburg), Trasylol from Bayer (D-5090 Leverkusen) and benzamidine from Sigma Chemical Corp., St. Louis.

The following labelled amino acids were obtained from Amersham-Buchler: L-[2,3,4,5,6-^{3}H]phenylalanine, spec. act. 115 Ci/mmol, L-[3,4-^{3}H]valine, spec. act. 32 Ci/mmol and L-[^{35}S]methionine, spec. act. 800 Ci/mmol.

Rat apo AI was isolated from rat high-density lipoprotein fraction (1.063–1.21 g/m*l*) as described before[4].

Preparation of isolated hepatocytes

Male rats, weighing 200–300 g, were used in the experiments. Isolated hepatocytes were prepared by recycling liver perfusion with collagenase and hyaluronidase[5]. Cells were more than 95% viable as demonstrated by the trypan blue exclusion test[6].

Incubation of isolated hepatocytes

$2-3 \times 10^8$ cells were suspended in 3–5 m*l* Dulbecco's medium free of methionine, phenylalanine or valine respectively, but supplemented with either [^{35}S]methionine (1 mCi), [^{3}H]phenylalanine (1 mCi) or [^{3}H]-valine (1 mCi) respectively, and incubated in culture flasks in a shaking water bath at 37 °C. After 90 min of incubation the total cell suspension was cooled in an ice bath and 2.5 m*l* cold 0.25M sucrose/$(NH_4)_2CO_3$ buffer pH 8.3, containing protease inhibitors, was added. The final concentrations of the inhibitors were 40 μg phenylmethanesulfonyl fluoride/m*l*, 5mM iodoacetamide, 2mM EDTA, 0.1 mg benzamidine/m*l*, 10 μg pepstatin/m*l*, 10 μg leupeptin/m*l* and 100 KIE Trasylol/m*l*.

The cell suspension was cooled at 0 °C for 2 min and centrifuged for 30 s at 300 x *g* in a labofuge to sediment

intact cells. The clear supernatant was removed and centrifuged for 15 min at 15000 x *g*, to eliminate remaining cell debris. The 15000 x *g* supernatant is referred to as culture medium.

The 300 x *g* pellet, containing intact hepatocytes, was resuspended in 2 m*l* cold 0.25M sucrose/$(NH_4)_2CO_3$ buffer and diluted with 4 m*l* phosphate-buffered saline. The cell suspension was homogenized in a motor-driven Potter-Elvehjem homogenizer (loosely fitting, Braun, Melsungen) with 20 strokes. Cellular debris were removed by centrifugation at 1000 x *g* for 10 min.

Immunoprecipitation

The 1000 x *g* supernatant (6 m*l*) was mixed with 1.5 m*l* of a solution containing the following substances (final concentrations in brackets): Na dodecyl sulfate (0.5%), NaCl (100mM), triethanolamine (50mM), EDTA (2mM) and Trasylol (200 KIE/m*l*); the pH was adjusted to 7.4 with HCl.

The culture medium (3 m*l*) (15000 x *g* supernatant) was adjusted to the same sodium dodecyl sulfate concentration with the solution mentioned above. The two fractions were boiled for 5 min and further diluted with 2 m*l* of a 10% Triton X-100/Tris buffer pH 8.3.

Apolipoprotein AI was immunoprecipitated as described before[1] and purified finally by polyacrylamide gradient gel electrophoresis (10–15%) in 0.1% sodium dodecyl sulfate. The radioactive bands were excised with a razor blade and the labelled protein isolated by electroelution. The electrophoresis apparatus consisted of a glass-tube (0.8 cm diameter, 15 cm height), the bottom of which carried a nylon grid to support the polyacrylamide slices for electroelution. A tightly fitting dialysis tubing immersed as a small bag into the lower (anode) buffer compartment. The electrophoresis buffer 0.2M Tris/HCl, pH 8.2, 0.1% sodium dodecyl sulfate was used. Electroelution at 8 mA/tube was completed after 12 h. Whale myoglobin (3 mg) was added as a carrier into the dialysis bag and the protein solution dialysed, lyophilized and precipitated with acetone as described before[3]. 60–70% of the radioactivity was recovered.

Partial sequence determination

The precipitate was dissolved in 0.6 m*l* formic acid and subjected to automated Edman degradation[7] using a Beckman sequencer model 890C, and a 0.1M quadrol program. The thiazolinones of the amino acids were dried in a stream of nitrogen and their radioactivity counted in a Beckman LS 7500 scintillation counter.

Antibodies

Monospecific antibodies against homogeneous rat apolipoprotein AI were raised in rabbits. The IgG-fraction was partially purified by ammonium sulfate precipitation[8].

Pulse experiments

2 x 10^8 cells, suspended in 6 m*l* Dulbecco's medium free of methionine, were pulsed with 0.8 mCi [^{35}S]methionine. After 10, 20, 30 and 60 min 1.5 m*l* of cells suspension was removed and fractionated into whole cells and supernatant medium. AI was immunoprecipitated, the immunocomplex adsorbed to protein A-Sepharose CL-4B. Non-specifically labelled proteins were washed out thoroughly and the immunocomplex dissociated with sample buffer for sodium dodecyl sulfate polyacrylamide gel electrophoresis (10–15% gradient). Proteins were visualized by subsequent fluorography[9].

Results

Rat liver hepatocytes were prepared by liver perfusion with a collagenase solution recycled between the inferior caval vein and the portal vein[5]. This procedure provided single cell suspension. Aliquot portions of 2 to 3 x 10^8 cells were suspended in Dulbecco's medium which was devoid of the amino acids added as radioactive precursors of protein synthesis, methionine, phenylalanine and valine, incubated at 37 °C for the intervals indicated in the legends of the figures. The flow sheet, presented in Fig. 1, outlines the steps of the experiment. Washed cells and the suspension medium were processed separately to the immunoprecipitation step.

After binding of the apo AI-anti apo AI complex to protein A-Sepharose CL-4B all labelled non-specific proteins were removed by thorough washing from this complex, which was then decomposed for sodium dodecyl sulfate polyacrylamide gel electrophoresis and subsequent fluorography.

Fig. 2 demonstrates the autoradiogram of the radiolabelled ([^{35}S]Met) products precipitated with anti-rat apo AI immunoglobulins, isolated from the hepatocytes (Lanes 2, 5, 8 and 11), and the medium (Lanes 3, 6, 9 and 12) after time intervals of 5, 10, 45 and 90 min. Radioactive apoprotein AI does not appear before 10 min in the medium.

It is apparent that the cells are viable over the period of the experiment.

Lanes 1, 4, 7 and 10 indicate the position of mature rat apolipoprotein AI. The immunoprecipitated apo AI, isolated from the hepatocytes, and the secreted products as well exhibit radio-

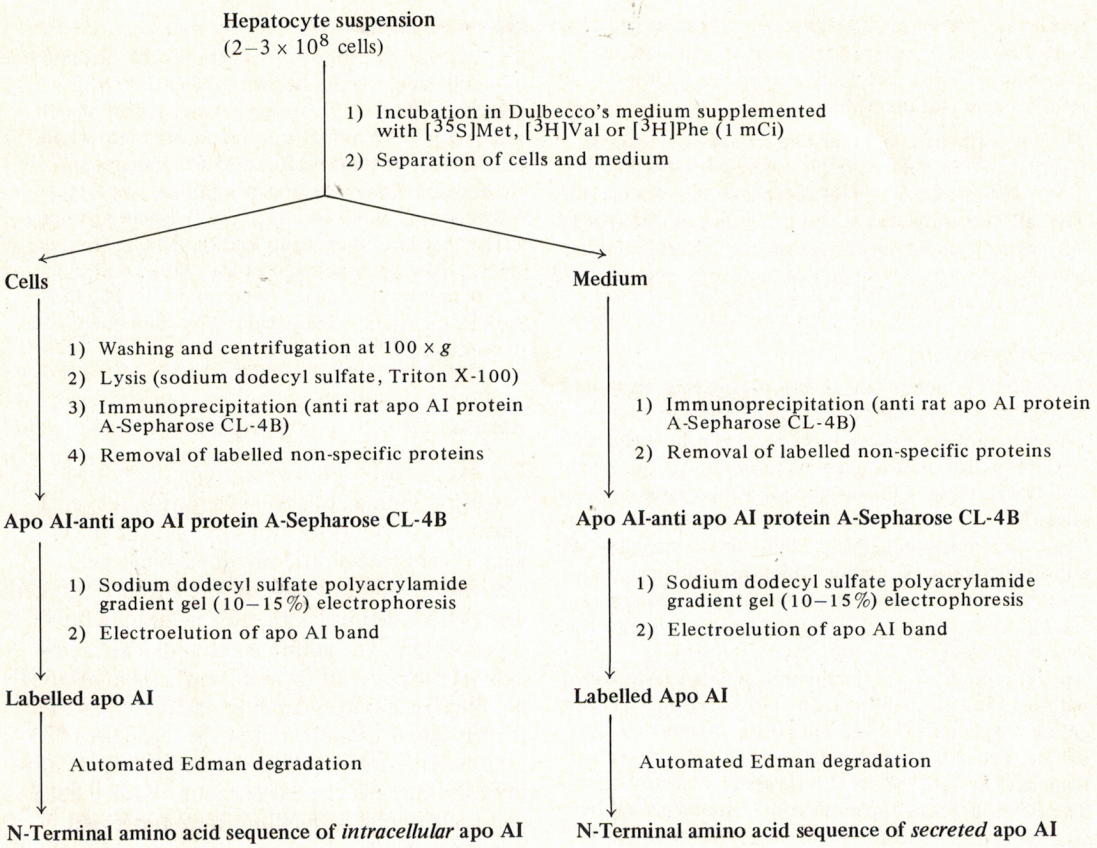

Fig. 1. Flow sheet of steps leading from rat hepatocyte suspension to partial N-terminal sequence determination of intracellularly localized and secreted apolipoprotein AI.

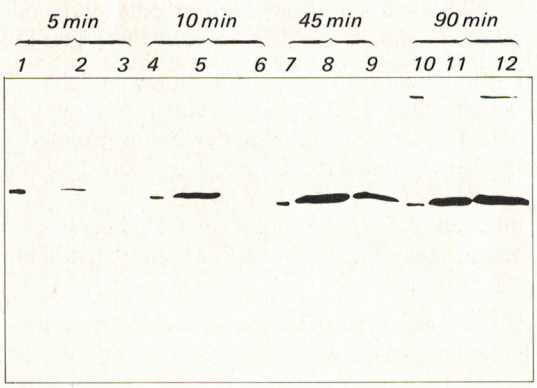

Fig. 2. Autoradiogram of radiolabelled ([35S]Met) immunoprecipitated apolipoprotein AI, isolated from hepatocytes and medium and separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (conditions see Ref.[11]).

2×10^8 cells suspended in 6 ml Dulbecco's medium (Met-free) were pulsed with 1 mCi [35S]methionine. Aliquot portions of 5×10^7 were taken at time intervals indicated in the figure, separated into washed cells and medium and processed for sodium dodecyl sulfate polyacrylamide gel electrophoresis and autoradiography as outlined in the flow sheet (Fig. 1), and under Materials and Methods. Lanes 1, 4, 7 and 10: mature rat apo AI; Lanes 2, 5, 8 and 11: pro apo AI, isolated from hepatocytes (intracellular); Lanes 3, 6, 9 and 12: pro apo AI, isolated from medium (secreted pro apo AI). Immunoprecipitation was performed with rabbit anti rat apo AI-IgG (20 µg/tube) and anti rat apo AIV-IgG (6 µg/tube).

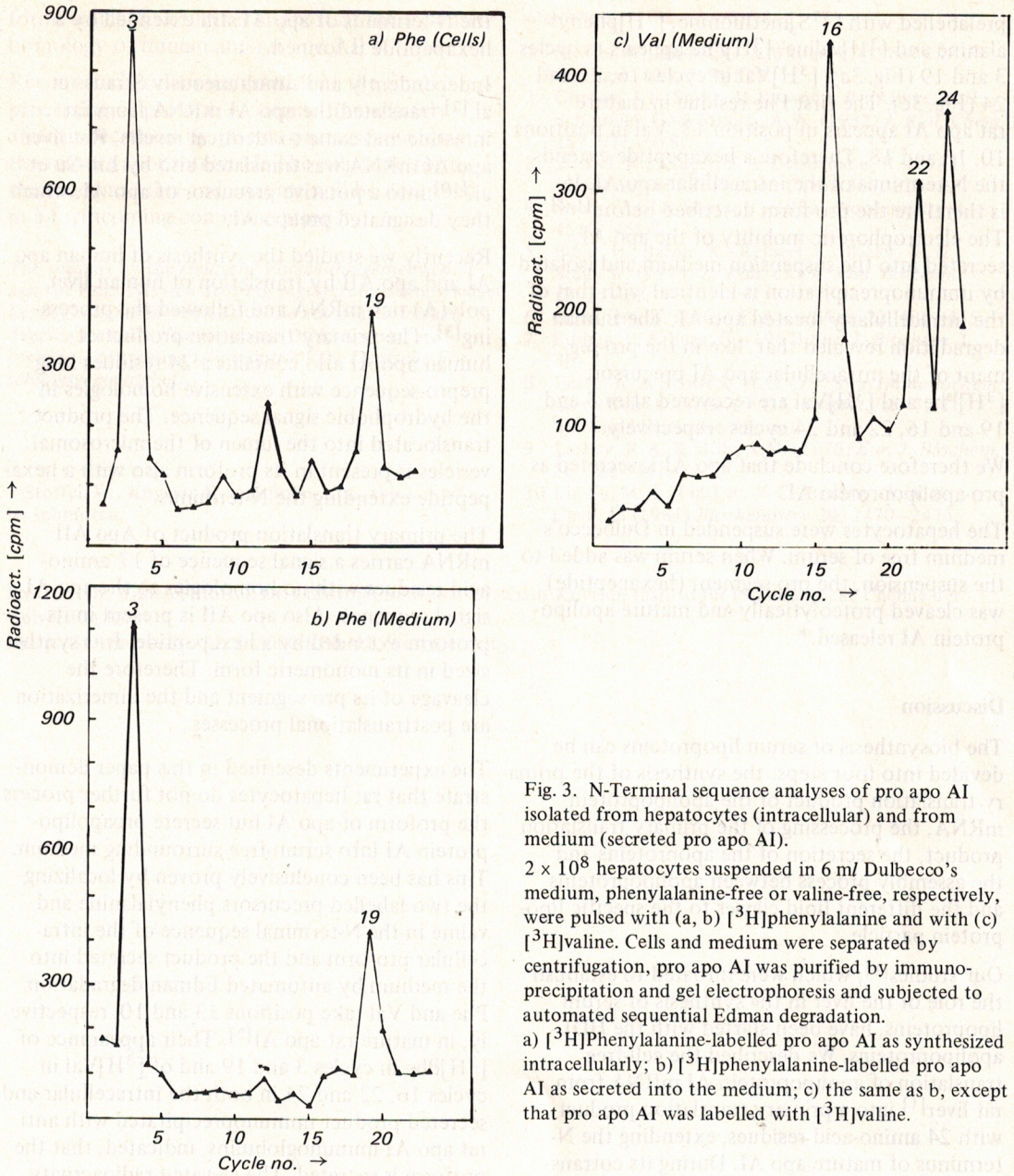

Fig. 3. N-Terminal sequence analyses of pro apo AI isolated from hepatocytes (intracellular) and from medium (secreted pro apo AI).

2×10^8 hepatocytes suspended in 6 ml Dulbecco's medium, phenylalanine-free or valine-free, respectively, were pulsed with (a, b) [^{3}H]phenylalanine and with (c) [^{3}H]valine. Cells and medium were separated by centrifugation, pro apo AI was purified by immunoprecipitation and gel electrophoresis and subjected to automated sequential Edman degradation.

a) [^{3}H]Phenylalanine-labelled pro apo AI as synthesized intracellularly; b) [^{3}H]phenylalanine-labelled pro apo AI as secreted into the medium; c) the same as b, except that pro apo AI was labelled with [^{3}H]valine.

active bands with slightly but distinctly higher molecular mass in sodium dodecyl sulfate gradient polyacrylamide gel electrophoresis (10–15%).

We therefore isolated these bands after labelling with [^{3}H]phenylalanine and [^{3}H]valine for subsequent automated Edman degradation[7] in order to determine the N-terminal sequence of the two products.

Fig. 3 illustrates the results of the Edman degradation of apo AI, isolated from hepatocytes

prelabelled with [^{35}S]methionine, [^{3}H]phenylalanine and [^{3}H]valine. [^{3}H]Phe appears in cycles 3 and 19 (Fig. 3a), [^{3}H]Val in cycles 16, 22 and 24 (Fig. 3c). The first Phe residue in mature rat apo AI appears in position 13, Val in positions 10, 16 and 18. Therefore a hexapeptide extends the N-terminus of the intracellular apo AI. It is therefore the pro-form described before[1,2]. The electrophoretic mobility of the apo AI secreted into the suspension medium and isolated by immunoprecipitation is identical with that of the intracellularly located apo AI. The Edman degradation revealed that, like in the pro-segment of the intracellular apo AI precursor, [^{3}H]Phe and [^{3}H]Val are recovered after 3 and 19 and 16, 22 and 24 cycles respectively.

We therefore conclude that apo AI is secreted as pro-apolipoprotein AI.

The hepatocytes were suspended in Dulbecco's medium free of serum. When serum was added to the suspension, the pro-segment (hexapeptide) was cleaved proteolytically and mature apolipoprotein AI released.*

Discussion

The biosynthesis of serum lipoproteins can be devided into four steps: the synthesis of the primary translation product of the apolipoprotein mRNA, the processing of the primary translation product, the secretion of the apoproteins and the assembly process between apolipoproteins and the different lipid classes to the specific lipoprotein particle.

Our studies[1], which were initiated to establish the role of the liver in the synthesis of serum lipoproteins, have been started with the HDL-apolipoproteins. We described the cell-free translation of apolipoprotein AI mRNA from rat liver[1] into a primary translation product with 24 amino-acid residues, extending the N-terminus of mature apo AI. During its cotranslational translocation through endoplasmic reticulum membranes, the signal sequence with 18 amino-acid residues is lost. A product with the N-terminus of apo AI still extended by a hexapeptide is formed.

Independently and simultaneously Strauss et al.[2] translated the apo AI mRNA from rat intestine and came to identical results. Rat liver apo AI mRNA was translated also by Lin-Su et al.[10] into a putative precursor of apo AI, which they designated preapo AI.

Recently we studied the synthesis of human apo AI and apo AII by translation of human liver poly(A)-rich mRNA and followed the processing[3]. The primary translation product of human apo AI also contains a 24 residues long prepro-sequence with extensive homologies in the hydrophobic signal sequence. The product translocated into the lumen of the microsomal vesicles is present in its proform also with a hexapeptide extending the N-terminus.

The primary translation product of Apo AII mRNA carries a signal sequence of 17 amino-acid residues with no homologies to the apo AI signal sequence. Also apo AII is present in its proform extended by a hexapeptide. It is synthesized in its monomeric form. Therefore the cleavage of its pro-segment and the dimerization are posttranslational processes.

The experiments described in this paper demonstrate that rat hepatocytes do not further process the proform of apo AI but secrete proapolipoprotein AI into serum-free surrounding medium. This has been conclusively proven by localizing the two labelled precursors phenylalanine and valine in the N-terminal sequence of the intracellular proform and the product secreted into the medium by automated Edman degradation. Phe and Val take positions 13 and 10, respectively, in mature rat apo AI[2]. Their appearance of [^{3}H]Phe in cycles 3 and 19 and of [^{3}H]Val in cycles 16, 22 and 24 in both the intracellular and secreted product immunoprecipitated with anti rat apo AI immunoglobulins, indicated, that the proform is secreted. The elevated radioactivity in step 12 of the Edman degradation (Fig. 3a) was only observed in this experiment, but not in a later control. Strauss and coworkers[2], who sequenced rat apo AI over 21 cycles, described an undetermined residue in position 18. On the basis of labelling studies this residue must be valine, corresponding to position 24 in the pro-

* Stoffel, W., Knyrim, K. and Bode, Chr., this j. – to be submitted.

form, which is in further support of the extensive homology of human and rat apo AI[3].

Recent studies demonstrated that the final processing of pro-apo AI occurs immediately after its secretion by plasma proteases yielding the mature apoprotein AI. The substrate form, cleavage products and enzyme will be described in a forthcoming communication.*

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