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Hexa- and Pentapeptide Extension of Proalbumin: Feedback Inhibition of Albumin Synthesis by Its Propeptide in Isolated Hepatocytes and in the Cell-Free System†

K. Weigand,* M. Schmid, A. Villringer, Ch. Birr, and P. C. Heinrich

ABSTRACT: Addition of the chemically synthesized proalbumin hexapeptide in a concentration of 110 μM to the medium of isolated rat hepatocytes decreased net albumin synthesis by 12%. The synthesis of other secretory proteins was not altered. A weaker inhibitory effect on albumin synthesis was found for a tetrapeptide, a possible degradation product of the proalbumin hexapeptide. For the uptake of the hexa- and tetrapeptide into the cells, bovine serum albumin is required. In a reticulocyte and in a wheat germ cell-free system a propeptide concentration of 600 μM inhibited albumin synthesis by 50%, whereas total protein synthesis was inhibited by 19% only, and the synthesis of α_1-antitrypsin was not inhibited. These results suggest that the synthesis of preproalbumin is regulated by a feedback mechanism with its propeptide as inhibitor.

Proalbumin is the immediate intracellular precursor of albumin, differing from serum albumin by a hexapeptide extension at the N-terminal end. The amino acid sequence of the rat (Quinn et al., 1975; Russel & Geller, 1975) and bovine (Patterson & Geller, 1977) albumin propeptide has been shown to be NH_2-Arg-Gly-Val-Phe-Arg-Arg, and probably the propeptide of human albumin has the same structure (Brennan & Carrell, 1978). This peptide is split off in the Golgi apparatus (Edwards et al., 1976; Ikehara et al., 1976) by an enzyme with the characteristics of cathepsin B (Judah & Quinn, 1978; Quinn & Judah, 1978). Multiple possible functions of the basic hexapeptide extension have been discussed (Peters & Reed, 1980). It may be involved in the formation of the tertiary structure of the albumin molecule, it may facilitate transport through or export out of the cell,

† From the Department of Clinical Pharmacology, University of Bern, Ch-3010 Bern, Switzerland (K.W. and M.S.), the Biochemical Institute, University of Freiburg, D-7800 Freiburg, FRG (A.V. and P.C.H.), and the Max Planck Institute for Medical Research, D-6900 Heidelberg, FRG (Ch.B.). *Received December 28, 1981; revised manuscript received August 17, 1982.* This work was supported by the Deutsche Forschungsgemeinschaft (We 462/4), the Forschergruppe Lebererkrankungen (De 113/26-3), and the Swiss National Foundation for Scientific Research.

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or it may mask binding sites for substances like fatty acids. Since none of these hypotheses has been adequately supported to date (Peters & Reed, 1980), no specific function of the proalbumin molecule is as yet recognized. It has also been suggested that rather than the proalbumin molecule itself, the propeptide may have a biological function and be involved in the regulation of albumin synthesis (Judah & Quinn, 1976; Schreiber et al., 1976).

The free propeptide has not been isolated from liver so far. However, we were able to synthesize it chemically, as reported in the first paper of this series (Birr et al., 1981). In order to test the hypothesis that the released propeptide is involved in the regulation of albumin synthesis, we studied the effect of the chemically produced hexapeptide on the synthesis of albumin in rat hepatocyte suspensions and in cell-free systems. Since a possible degradation product of the hexapeptide has the sequence Arg-Gly-Val-Phe-, we also investigated the effect of this tetrapeptide on albumin synthesis.

Materials and Methods

Animals and Chemicals. Male Sprague-Dawley rats with a body weight of 150–250 g were used. Animals were fed the Altromin standard diet and water ad libitum and kept under automatic lighting at 24 °C with a humidity of 47%. Protein A–Sepharose CL-4B and *N*-succinimidyl 3-(2-pyridyldithio)propionate were purchased from Pharmacia (Uppsala, Sweden), and sucrose (ribonuclease free) was from Schwarz/Mann (Orangeburg). Hepes [*N*-(2-hydroxyethyl)-piperazine-*N'*-2-ethanesulfonic acid] was purchased from Fluka AG (Chemische Fabrik, Buchs SG, Switzerland), and all other salts were from E. Merck (Darmstadt, FRG). All chemicals were of analytical grade. Collagenase (grade CLS III purity 103 units/mg) was obtained from Worthington Biochemical Corp. (Freehold, NJ) and alkaline phosphatase (calf intestine, 441 units/mg) from Biogenzia Lemania SA (Lausanne, Switzerland). Penicillin–streptomycin solution (10000 units/mL) was from Gibco Bio-Cult (Glasgow, Scotland) and monospecific antiserum against rat serum albumin from Nordic Immunology (Tilburg-Berchem, London). Bovine serum albumin (RHD 20) was obtained from Behring-Werke AG (Marburg, FRG), extensively dialyzed against water, and lyophilized. [1-^{14}C]Leucine (59 mCi/mmol), L-[^{3}H]leucine (60 Ci/mmol), and L-[^{35}S]methionine (>1000 Ci/mol) were purchased from Radiochemical Centre (Amersham, U.K.) and New England Nuclear (Boston, MA).

Chemical Synthesis of Peptides. The hexapeptide was synthesized beginning from the C-terminal end by stepwise N-terminal peptide chain elongation starting from *N-tert*-butyloxycarbonyl-N^{γ}-nitroarginyl-N^{γ}-nitroarginine 4-nitrobenzyl ester; $[\alpha]^{20}_{365}$ −12° (*c* 1, dimethylformamide). The other amino acids were incorporated by excess mixed anhydrides of Ddz amino acids (Ddz, α,α-dimethyl-3,5-dimethoxybenzyloxycarbonyl), yielding the fully protected hexapeptide in crystalline quality. After removal of the protective groups by acid treatment and hydrogenation, the peptide was purified by Dowex ion-exchange and Sephadex chromatography. Purity was confirmed by thin-layer chromatography, HPLC,[1] and amino acid analysis [for details of the synthesis of the hexa- and tetrapeptide see Birr et al. (1981)].

Detection of the Hexapeptide in Rat Liver Homogenate by HPLC. One gram of rat liver was homogenized in 5 mL of water, treated 2 min with ultrasonic, frozen, thawed, and centrifuged for 2 min at 12000 rpm. Subsequently the supernatant was lyophilized. Immediately before use the lyophilysate was redissolved in 3 mL of H_2O/CH_3CN (1:1) and centrifuged again; 50 μL of the supernatant was applied by an automatic injector on a LiChrosorb RP-18 column (4 × 250 mm). The analysis was performed in a Spectra Physics SP 8000 B/SP 8400/Sp 8010 chromatograph. The samples were eluted with a gradient system of 0.1% (v/v) trifluoroacetic acid in H_2O (A) and acetonitrile (B). The gradient was performed in the following way: for 10 min isocratic 98% A plus 2% B, for 5 min isocratic 80% A plus 20% B, for 20 min gradient up to 60% A plus 40% B, within 1 min to 100% B, for 2 min isocratic 100% B, and finally within 1 min to 98% A plus 2% B. The flow rate was 1 mL/min, and the absorbance was monitored at 210 nm and at 260 nm. For comparison 20 nmol of hexapeptide was applied to the same system.

Isolation and Incubation of Rat Hepatocytes. Cells were isolated by recirculating in situ perfusion (Berry & Friend, 1969) modified as described previously (Weigand & Otto, 1974; Weigand et al., 1977) with calcium-free Krebs–Ringer–carbonate buffer containing 0.1% collagenase. After perfusion for 20 min at 36 °C with a flow rate of about 30 mL/min the liver was excised, and the suspended cells were filtered through nylon meshes with 250- and 100-μm pore diameter, washed twice, and centrifuged for 2 min at 10*g*. Viability was estimated by staining with 0.4% Trypan blue in BSA-free medium. About 2 × 10^6 cells/mL were incubated in Krebs–Ringer–carbonate buffer with 30 mM Hepes, pH 7.4 at 37 °C, for 7 h. The buffer contained 15 units/mL penicillin–streptomycin and amino acids in serum concentrations (Scharff & Wool, 1964). Several samples of 10 mL, obtained from the same liver, were shaken simultaneously with 100 rpm in a rotary water bath shaker (New Brunswick Scientific Inc., Edison) in 100-mL Erlenmeyer flasks in an atmosphere of 95% oxygen and 5% carbon dioxide. One of the samples always served as control to which amino acids in the same composition and in equimolar amounts as the corresponding peptide were added. The peptides were added in the presence or absence of 1% BSA either at the beginning as a single bolus (1.1 × 10^{-4} mol/L) or in a sequence every 30 min (3.4 × 10^{-5} mol/L). Aliquots of 0.5 mL were taken in hourly intervals, and incubation was stopped by rapid cooling and subsequent centrifugation for 2 min at 100*g*. The supernatant was centrifuged again for 10 min at 12000*g*. All glass materials were treated with dichlorodimethylsilane to avoid attachment of the cells.

Isolation of RNA. Rat liver polysomes were isolated by sucrose density centrifugation according to Palacios et al. (1972). Poly(A+) RNA was extracted with phenol–chloroform–isoamyl alcohol (Zieve & Penman, 1976) and subsequently chromatographed on poly(U)–Sepharose (Schmelzer & Heinrich, 1980).

Cell-Free Protein Synthesis. L-[^{35}S]Methionine or L-[^{3}H]leucine was incorporated into protein, using a cell-free lysate from wheat germ as described by Roberts & Paterson (1973) or the reticulocyte lysate as described by Kühn et al. (1980). The reaction mixture was incubated with hexapeptide concentrations ranging from 10^{-6} to 10^{-3} mol/L for 90 min at 25 °C in the case of the wheat germ system (Northemann et al., 1980) and 30 °C in the case of the reticulocyte lysate. Protein synthesis was terminated by addition of sodium dodecyl

[1] Abbreviations: HPLC, high-pressure liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; BSA, bovine serum albumin; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(4-methyl-5-phenyloxazol-2-yl)benzene.

sulfate to a final concentration of 4% and subjected to a heat treatment at 95 °C for 2 min.

Measurement of Total Protein and Albumin Synthesis in Hepatocyte Suspensions and in the Cell-Free System. Total protein synthesis was measured as hot trichloroacetic acid insoluble radioactivity (Mans & Novelli, 1961) on filter paper disks and counted in toluene plus 0.4% PPO and 0.005% POPOP. The counting efficiency on disks was 70%, and the absolute counting efficiency was estimated with partially purified, calibrated ^{14}C-labeled rat serum albumin (Schreiber et al., 1969). In the incubation medium of the cells rat serum albumin was determined with the rocket electrophoresis technique (Laurell, 1966) using monospecific antiserum. Albumin used as a standard and for immunization was isolated from rat serum and purified as described previously (Schreiber et al., 1969; Weigand et al., 1971). The purification procedure included ammonium sulfate fractionation, ethanol extraction, Sephadex–cellulose and DEAE-cellulose chromatography, and preparative polyacrylamide gel electrophoresis. Purity was confirmed by analytical polyacrylamide gel (Ornstein, 1964; Davis, 1964) and sodium dodecyl sulfate electrophoresis (King & Laemmli, 1971). For calibration the specific extinction was determined and found to be 0.61 for 1 mg/mL in aqueous solution at 280 nm. The albumin concentration within the cells was measured by an enzyme-linked immunosorbent assay (Elisa). The Elisa was performed with alkaline phosphatase coupled to purified rat serum albumin and 4-nitrophenyl phosphate as enzyme substrate. Alkaline phosphatase was coupled to albumin by *N*-succinimidyl 3-(2-pyridyldithio)-propionate as described by Carlsson et al. (1978). Polystyrene tubes were coated with 1:500 diluted rabbit anti-rat serum albumin immunoglobulin G, purified by affinity chromatography, as described previously (Weigand et al., 1981). Subsequently the tubes were incubated overnight with the samples or standard dilutions and the conjugated RSA simultaneously. The inhibition curve was almost linear from 30 to 120 ng of albumin/mL.

Albumin synthesis in the cell-free system was determined by the following procedure: After 40-fold dilution of the translation mixture with 20 mM Tris-HCl buffer, pH 7.6, containing 140 mM NaCl, 5 mM EDTA, and 1% Triton X-100 and addition of proteinase inhibitors (0.1 mg/mL kallikrein trypsin inhibitor and 0.6 mM phenylmethanesulfonyl fluoride), 8 μg of anti-rat serum albumin immunoglobulin G, purified by affinity chromatography, was added. The preproalbumin IgG complex was bound to 10 mg (dry weight) of protein A–Sepharose CL-4B, washed, and subsequently eluted from protein A–Sepharose with 0.1 M Tris-HCl, pH 8.0, containing 2.5 % sodium dodecyl sulfate (Maccecchini et al., 1979). The supernatant was subjected to NaDodSO$_4$–polyacrylamide slab gel electrophoresis, according to King & Laemmli (1971). After electrophoresis, the gels were prepared for fluorography according to Bonner & Laskey (1974). The preproalbumin band was localized by fluorography and cut out, and the radioactivity in this band was determined.

Purification of α_1-Antitrypsin from Rat Serum and Antiserum Preparation. Rat serum was subjected to a 50% and a subsequent 80% ammonium sulfate precipitation, followed by affinity chromatography first on activated thiol–Sepharose 4B as described by Laurell et al. (1975), second by affinity chromatography on concanavalin A–Sepharose according to Saklatvala et al. (1976), and third by preparative NaDodSO$_4$–polyacrylamide slab gel electrophoresis (King & Laemmli, 1971).

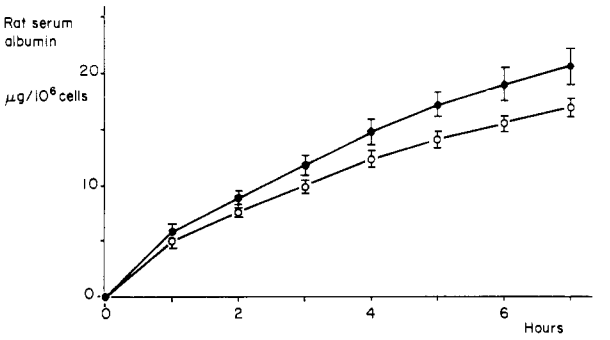

FIGURE 1: Net synthesis of rat serum albumin in hepatocyte suspensions. The hepatocytes were incubated in buffer containing amino acids, either with 1% BSA (●) or without BSA (○). Each point represents the mean ± SEM of seven and ten experiments for the upper and the lower curve, respectively. After 5 h of incubation the difference in the synthesis rate became significant ($p < 0.05$).

The polyacrylamide band containing the α_1-antitrypsin was cut from the gel, homogenized, mixed with Freund's complete adjuvant, and used for the immunization of rabbits.

Results

The freshly isolated hepatocytes had a viability of 85 ± 2% (SD) as estimated by Trypan blue staining. After 4 h of incubation, still 69 ± 2% (SD) of the cells remained unstained. Thereafter, counting became impossible because of an increasing tendency of the intact cells to form aggregates. Although the long-term survival of the cells was good, the 16% decrease in viability during the incubation period may be responsible for the nonlinear increase in the albumin concentration of the medium (Figure 1). However, despite the lack of linearity, the synthesis rate of albumin was high. In the protein-free medium the hepatocytes synthesized 17 ± 0.8 (SEM) μg of albumin/10^6 cells, within 7 h. Addition of 1% bovine serum albumin stimulated rat albumin synthesis to 21 ± 1.6 (SEM) μg/10^6 cells. Since 1 g of liver wet weight contains 1.7×10^8 hepatocytes (Weibel et al., 1964), the cell suspensions synthesized and secreted albumin at a rate of 510 μg g^{-1} h^{-1}. In comparison, 470 μg was reported for fasted rat liver in vivo (Peters & Peters, 1972). The stimulation of albumin synthesis in the presence of BSA in the medium has not been described yet and may be due either to a protective effect on the cell membrane of the isolated hepatocytes, to a better substrate delivery to the cells, or to binding of potentially toxic metabolites produced by the cells.

Liver cell suspensions allow the simultaneous incubation of several identical samples. Therefore, even small regulatory effects can be measured reliably. For elimination of interindividual variations in the synthesis rate of albumin, each measured albumin concentration is given in percent of the corresponding control. Addition of 3.4×10^{-5} mol/L proalbumin hexapeptide every 30 min to cell suspensions, incubated in bovine albumin free medium, did not influence the rate of rat albumin synthesis (Figure 2a). In contrast, addition of the hexapeptide in the same concentration and the same time intervals to cell suspensions, incubated in the presence of 1% bovine serum albumin, inhibited the net increase of rat albumin in the medium (Figure 2b). After 4 h the inhibition was 10.0 ± 1.6% (SEM) compared to the control suspensions, incubated without the hexapeptide. This difference was highly significant ($p < 0.002$) and continued to be for the remaining incubation period of 3 h. Since the metabolism of the free proalbumin hexapeptide has not been determined so far, the hexapeptide was added to the medium every 30 min in order to compensate for a possible rapid breakdown. But, when the hexapeptide

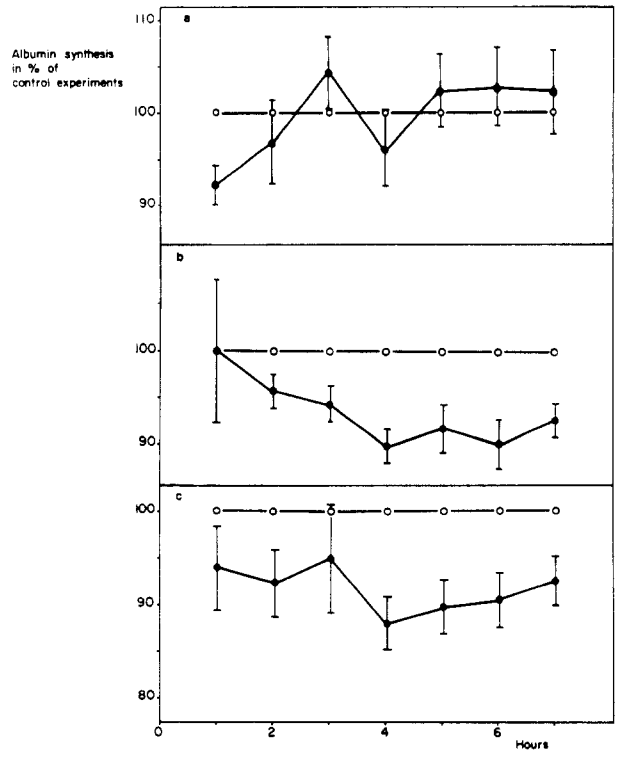

FIGURE 2: Effect of the hexapeptide on net albumin synthesis in hepatocyte suspensions. The hepatocytes were incubated in buffer containing amino acids without (a) or with (b and c) BSA. The hexapeptide was added either every 30 min in a concentration of 3.4×10^{-5} mol/L (a and b) or only at the beginning in a concentration of 1.1×10^{-4} mol/L (c). The albumin concentrations in the medium of the suspensions to which the hexapeptide was added (●) are plotted in percent of the corresponding controls (○) (for details see Materials and Methods). Each point represents the mean ± SEM of the results obtained from four (b and c) or six (a) separately isolated cell suspensions. By t test analysis, the difference to the control became significant after 4 h [$p < 0.002$ and $p < 0.01$ for (b) and (c), respectively].

was added as a single dose of 1.1×10^{-4} mol/L at the beginning of the incubation, a very similar inhibition curve of albumin secretion was found (Figure 2c). After 4 h the inhibition was 11.8 ± 2.8% (SEM) and significant ($p < 0.01$). Thereafter, the secretion rate of albumin showed a slight increase by time. The same inhibition by a single dose may indicate that the half-life time of the hexapeptide or an active metabolite is rather long, at least several hours.

Albumin is continuously synthesized and secreted by the liver, and normally the albumin concentration within the hepatocyte is very low (Weigand & Otto, 1974). The diminished increase of the albumin concentration in the medium, after addition of the hexapeptide, can be due to an inhibition of either albumin synthesis, its secretion, or both. The albumin content of the cells was measured to clarify the underlying mechanism. The cell pellets obtained from the aliquots of each experiment were washed once with buffer, suspended in phosphate-buffered saline, pH 6.8, containing 0.05% Tween and 0.02% sodium azide, and treated for 1 min with ultrasonic. The suspension was centrifuged for 2 min at 12000 rpm, and subsequently albumin was measured in the supernatant by Elisa. After 7 h the albumin concentration in the medium of cell suspensions, incubated in the presence of the hexapeptide, was 2 $\mu g/10^6$ cells less than in the medium of the controls. If the impaired albumin release had been due to an inhibition of secretion only, the albumin content within the cells should have been increased by this amount. However, during the whole incubation period, the intracellular albumin concentration remained constant at a level of about 1 $\mu g/10^6$ cells (Figure 3), which is in accordance with previously published data (Weigand & Otto, 1974). No influence of the hexapeptide could be observed. Since the microsomal albumin content within the liver is directly related to the rate of albumin synthesis (Peters & Peters, 1972), a decreased albumin synthesis should also result in a lower albumin content within the cells. From the slope of the published correlation curve it can be calculated that a 12% decrease in the rate of albumin synthesis should lead to a 8% fall in the intracellular albumin concentration, corresponding to 0.08 $\mu g/10^6$ cells within 7 h. This small amount is within the variation of the Elisa technique. The constant albumin concentration within the cells therefore strongly suggests that the hexapeptide primarily influenced albumin synthesis.

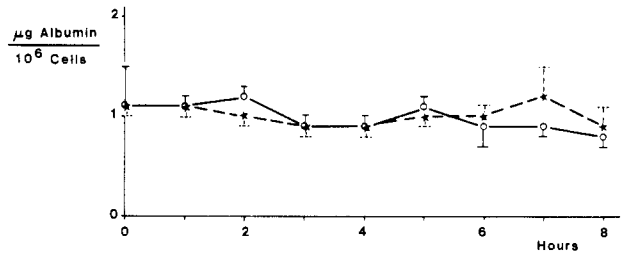

FIGURE 3: Intracellular albumin concentration of rat hepatocytes incubated in Krebs–Ringer buffer for 8 h. The buffer of the control hepatocytes (○) and the hepatocytes incubated with 110 μM peptide (★) contained 1% BSA (mean ± SEM, $n = 4$).

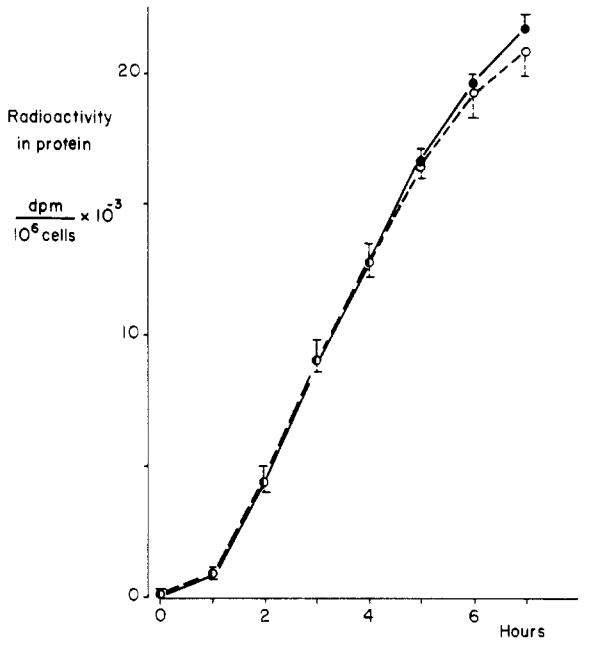

FIGURE 4: Effect of the hexapeptide on the incorporation of [^{14}C]-leucine into secretory proteins of hepatocyte suspensions. The hepatocytes were incubated in buffer containing amino acids and 1% BSA. The hexapeptide was added as a single dose of 1.1×10^{-4} mol/L at time zero (●). Controls were incubated without the hexapeptide (○). In hourly intervals the protein radioactivity in the medium was measured. Each point represents the mean ± SEM of the results of five separately isolated cell suspensions.

For investigation of whether the inhibition by the hexapeptide is specific for albumin or affects other secretory proteins also, the incorporation of [^{14}C]leucine into secretory proteins in the medium of suspensions was studied. No effect of the hexapeptide on the incorporation of [^{14}C]leucine into total secretory proteins was found (Figure 4), indicating that the inhibition was specific with respect to serum proteins.

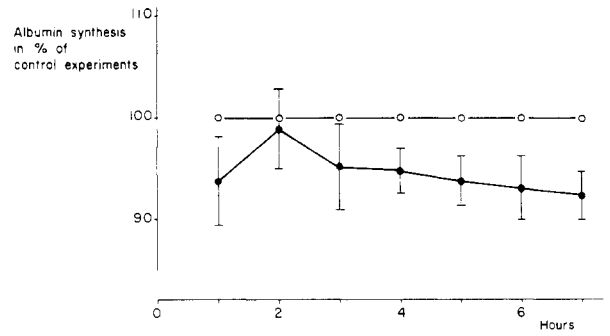

FIGURE 5: Inhibition of net albumin synthesis by the tetrapeptide in hepatocyte suspensions. The hepatocytes were incubated in buffer containing amino acids and 1% BSA. The albumin concentrations in the medium of the suspensions to which the tetrapeptide was added every 30 min in a concentration of 3.4×10^{-5} mol/L (●) are plotted in percent of the corresponding controls (○). Each point represents the mean ± SEM of the results obtained from four separately isolated cell suspensions. The difference in the albumin concentration became significant after 5 h ($p < 0.05$).

Since in vivo albumin accounts for about half of the secretory proteins produced by the liver, a 5% lower incorporation of radioactive leucine into total secretory proteins should be expected. The lack of inhibition can be explained in two ways. In isolated hepatocytes the percentage of albumin on total secretory protein synthesis might be considerably lower than that in vivo, and therefore the difference in incorporation might be too small to be measurable. A stimulation of the synthesis of some other secretory proteins by the hexapeptide, in contrast to albumin, could be another explanation.

The tetrapeptide Arg-Gly-Val-Phe is a possible degradation product of the proalbumin hexapeptide. Addition of the tetrapeptide also inhibited albumin synthesis, but only half as effective as the hexapeptide. After 4 h of incubation the albumin synthesis was inhibited by 5.2 ± 2.2% ($n = 6$; $p < 0.1$). After 5 h the inhibition was 6.2 ± 2.4 and statistically significant ($p < 0.05$) (Figure 5). The tetrapeptide showed the same inhibitory effect on albumin synthesis whether it was given as a single bolus or sequentially every 30 min, in the presence of 1% BSA. In contrast, no inhibition could be observed when BSA was omitted from the incubation medium.

The hexapeptide is released from proalbumin within the Golgi apparatus. It is not known whether it remains within the cell or is secreted together with albumin. A limited uptake of the hexapeptide from the incubation medium may explain the relatively weak effect on albumin synthesis in the isolated hepatocytes. Therefore, we studied its influence on albumin synthesis in cell-free protein synthesizing systems. Addition of the hexapeptide to the cell-free system, in a concentration up to 10^{-6} mol/L, had no influence on either total protein or albumin synthesis. However, higher hexapeptide concentrations resulted in a strong inhibition of albumin synthesis, whereas total protein synthesis was less affected (Figure 6). A hexapeptide concentration of 10^{-4} mol/L inhibited total protein synthesis only by 9.7 ± 2.6% (SEM); albumin synthesis, however, was inhibited by 37.8 ± 5.8% ($n = 5$; $p < 0.01$). At a concentration of 10^{-3} mol/L, total protein synthesis was inhibited by 31.4 ± 7.5% and albumin synthesis by 59.3 ± 10.0% ($n = 5$; $p < 0.05$). Thus, in lower concentrations the inhibition by the hexapeptide showed specificity for albumin. On fluorography only small amounts of incomplete peptide products were seen (Figure 7), and no difference was found between the wheat germ system and the reticulocyte system. The specificity of the hexapeptide inhibition for albumin was further confirmed by the lack of effect of the hexapeptide on the synthesis of α_1-antitrypsin (Figure 8). Furthermore, we

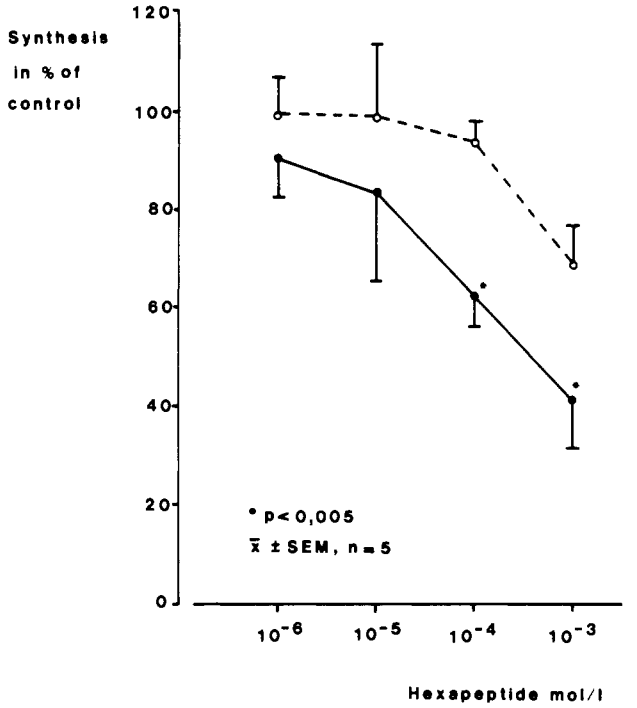

FIGURE 6: Inhibition of albumin and total protein synthesis by the albumin propeptide in cell-free albumin synthesizing systems. Rat liver mRNA was translated with the wheat germ system in the presence of 0.2 μCi/μL [^{3}H]leucine (three experiments) or with the rabbit reticulocyte system in the presence of 0.9 μCi/μL (0.44 μCi/μL) [^{35}S]methionine (two experiments) in the absence or presence of the propeptide (10^{-6}–10^{-3} mol/L). The mean [^{3}H]leucine incorporation into total acid-precipitable protein (○) of the control (without the hexapeptide) was 247 000 (95 000–381 000) cpm with and 10 400 (7200–14 300) cpm without mRNA. The control of the reticulocyte system incorporated 6.7×10^5 (11×10^6) cpm with and 3.5×10^5 (2×10^6) cpm without mRNA, for the first (the second) experiment. After immunoprecipitation, binding to and elution from protein A–Sepharose as described under Materials and Methods, the samples were subjected to NaDodSO$_4$–polyacrylamide gel electrophoresis and fluorography (Figure 7). The albumin bands on the gel, localized on the fluorogram, were cut out, and their radioactivity was determined to be 1.3×10^3–1.4×10^5 cpm in the absence of the peptide (●). The data are expressed in percent of the corresponding controls. Each point represents the mean ± SEM of all five experiments. The upper curve (○) gives the radioactivity in total protein and the lower curve (●) the radioactivity in albumin. At hexapeptide concentrations of 10^{-4} and 10^{-3} mol/L, the difference between total protein and albumin radioactivity is statistically significant ($p < 0.005$).

have shown that the presence of hexapeptide in its highest concentration used did not influence the antibody–antigen interaction. Addition of either arginine or bradykinin stimulated total protein synthesis slightly but had no effect on albumin synthesis.

Discussion

A common feature of most of the secretory proteins is the synthesis via early precursor molecules. These so-called preproteins differ from their mature forms by a peptide extension at the N terminus, which is required for the initiation of the secretory process (Blobel et al., 1979). In contrast, direct intracellular precursors, called proproteins, have been demonstrated for a few secretory proteins only. Some of them are characterized by an additional peptide extension at the N terminus [for review, see Dean & Judah (1980)]. Among 16 plasma proteins investigated, albumin was found to be the only secretory protein produced by the liver with a pro form characterized by a peptide extension (Schreiber et al., 1981). Since only a few proteins are produced via pro forms, it is

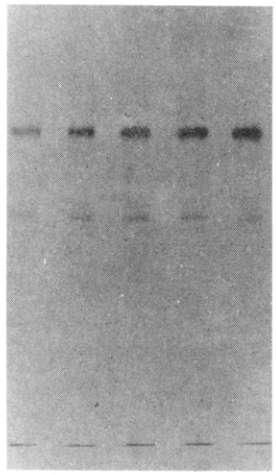

FIGURE 7: Effect of increasing hexapeptide concentrations on the in vitro translation of albumin. The preparation of mRNA, the translation with the reticulocyte system, the conditions of immunoprecipitation with anti-albumin immunoglobulins, the $NaDodSO_4$ gel electrophoretic separation, and the fluorography are described under Materials and Methods. Control translation in the absence of the hexapeptide (lane a) and translations in the presence of the hexapeptide in concentrations of 10^{-6} (b), 10^{-5} (c), 10^{14} (d), and 10^{-3} (e) mol/L.

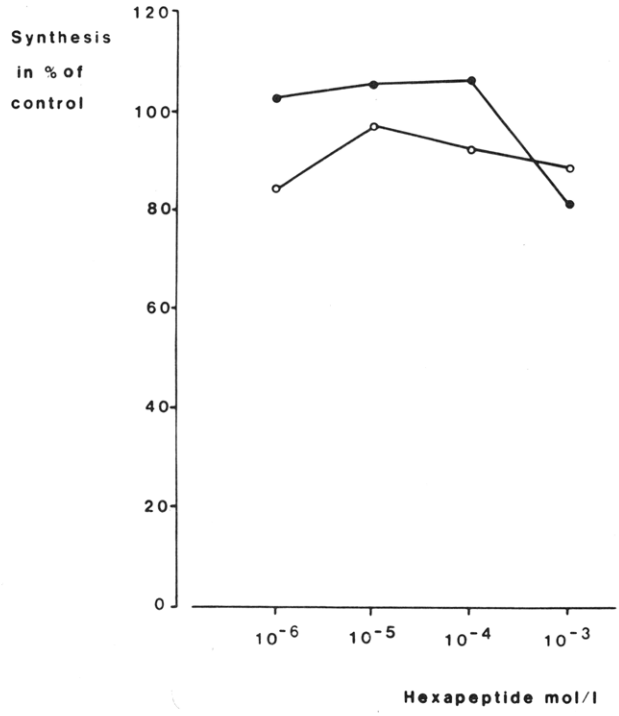

FIGURE 8: Effect of the albumin propeptide on α_1-antitrypsin and total protein synthesis in the rabbit reticulocyte system. Rat liver mRNA was translated in the presence of [^{35}S]methionine with or without the propeptide (10^{-6}–10^{-3} mol/L). The data of the incorporation into total acid-precipitable protein are given in the legend to Figure 6 (second experiment with the reticulocyte system). After immunoprecipitation, binding to and elution from protein A–Sepharose as described under Materials and Methods, the samples were subjected to $NaDodSO_4$–polyacrylamide gel electrophoresis and fluorography. The α_1-antitrypsin bands on the gel, localized on the fluorogram, were cut out, and their radioactivity was determined to be 1.2×10^5 in the absence of the hexapeptide. The radioactivity of total protein (○) and of α_1-antitrypsin (●) is expressed in percent of the corresponding controls. No difference between total protein and α_1-antitrypsin radioactivity was found.

unlikely that the propeptides are also involved in the secretory mechanism. Also no other functions of the proproteins have been found so far.

The N-terminal propeptide of collagen type III, which is cleaved from the collagen molecule extracellularly and a pentapeptide derived from this propeptide (Hoerlein et al., 1981), inhibits collagen synthesis in cell cultures (Wiestner et al., 1979) and in the cell-free system (Paglia et al., 1979). A peptide, released from a protein intracellularly, might have even a more efficient regulatory function. And indeed, the inhibition of albumin synthesis by the hexapeptide, both in the cell-free system and in isolated hepatocytes, strongly suggests that the synthesis of preproalbumin is regulated at the translational step by a feedback mechanism with its own propeptide as inhibitor. The relatively weak inhibition of 10% only on the synthesis of albumin in the hepatocyte suspensions, compared to a 40% inhibition in the cell-free system, may indicate that the uptake of the highly charged hexapeptide into the cells is limited. The lack of any effect in the absence of BSA, which seems to be necessary for the binding or the uptake of the hexapeptide by the cell, and the lag period of 3 h support this assumption. The results are compatible with the hypothesis that the released hexapeptide inhibits the albumin synthesis of its own cell. It is less likely that the hexapeptide produced by one cell inhibits albumin synthesis of another hepatocyte. The hexapeptide does not inhibit unspecifically the synthesis of all secretory proteins, as is shown by the incorporation of radioactive leucine into total secretory proteins and by the lack of inhibition of α_1-antitrypsin synthesis in the cell-free system. In contrary, the synthesis of some secretory proteins may even be stimulated. In the cell-free system total protein synthesis is also inhibited by the hexapeptide, but only about half as effective as albumin, indicating that, at lower hexapeptide concentrations, only the synthesis of albumin is inhibited. at higher concentrations, however, the synthesis of some cellular proteins is also inhibited. From the experiments with the tetrapeptide it may be concluded that the structure required for inhibition includes a basic constituent in conjunction to the lipophilic tripeptide counterpart. Additional experiments with peptides of similar structure have to be done to define precisely the active site. The basic peptide bradykinin, or arginine, alone had no inhibitory effect, either on total protein or on albumin synthesis in the cell-free system, excluding a general effect of basic amino acids or peptides on protein synthesis.

The plasma level of albumin is kept fairly constant, but the underlying regulatory mechanism of synthesis has not been determined as yet. The small molecular weight hexapeptide may be responsible for the transfer of information from the albumin in plasma to its intracellular site of synthesis. This offers a new concept for the regulation of the synthesis of albumin, and perhaps for some other plasma proteins also. The plasma concentration of albumin is approximately 5×10^{-4} mol/L. Since the hexapeptide is synthesized in equimolar amounts as albumin, the relatively high hexapeptide concentrations required for inhibition (10^{-5}–10^{-3} mol/L) seem to be physiologically meaningful. Moreover, by high-pressure liquid chromatography we found in a liver homogenate a peak that is identical with the peak of the synthesized hexapeptide (Figure 9). This finding strongly suggests that the hexapeptide is present in liver. Although the estimated concentration of the hexapeptide found in liver is lower than the concentration necessary for inhibition, which can be due to a pool effect or to degradation during preparation, it may be suggested that the hexapeptide plays a role in the regulation of albumin synthesis.

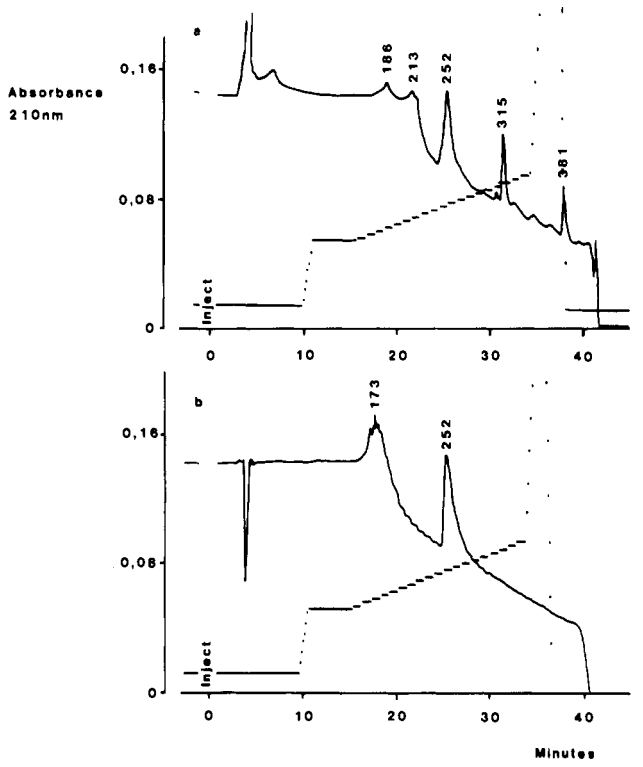

FIGURE 9: HPLC analysis of rat liver homogenate (a) and the chemically synthesized hexapeptide (b). The absorption at 210 nm is plotted against time. The dotted lines indicate the gradient. For details see Materials and Methods. The hexapeptide showed a single peak at 25.2 min. In liver homogenate a peak with the same retention time was detected. At the absorbance of 260 nm a smaller peak appeared at the same time, indicating that the peptide contained phenylalanine. When the height of the peaks are compared, a hexapeptide concentration of 20 nmol/g liver wet weight can be estimated.

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