SYNTHESIS OF ANGIOTENSINOGEN BY ISOLATED RAT LIVER CELLS AND ITS REGULATION IN COMPARISON TO SERUM ALBUMIN*

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SUMMARY: Angiotensinogen (renin substrate) and albumin are synthesized by isolated hepatocytes almost linearly for 5 hr. The incorporation of radioactive leucine into total protein proceeded linearly for 3 hr. Without addition of amino acids to the incubation medium the synthesis of both proteins was still linear but fell off to 40% compared to the synthesis rate obtained by incubation with amino acids in serum concentrations. Higher amino acid concentrations could not further stimulate the synthesis. Addition or withdrawal of tryptophan had no effect on the synthesis rate of both proteins. After 5 hr incubation hydrocortisone had stimulated the incorporation of radioactive leucine into total protein by 13%, the albumin synthesis by 43%, and the angiotensinogen synthesis by 142%.

Despite the important role of the serum glycoprotein angiotensinogen in the regulation of blood pressure in physiological and pathological states (for review see 1) only a few investigations of its net synthesis and regulation are available (2,3). Angiotensinogen is synthesized by the liver and it was assumed that it is produced by the parenchymal cells. By using liver slices (2) or the perfused liver (3) the non-parenchymal cells as the site of its synthesis cannot be excluded. Enzymatically isolated rat liver cells preserve the capacity to synthesize specific cell proteins (4) and to synthesize and secrete serum proteins (5-10). Cell suspensions allow the investigation of several identical samples and are therefore a valuable tool for the study of regulatory factors. In the following report the synthesis and secretion of angiotensinogen by isolated hepatocytes is described.

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In addition some regulatory factors are investigated and compared with those of the albumin synthesis.

MATERIALS AND METHODS

Animals and chemicals: Adult male rats of the strain: Chbb: Thom (SPF) with a body weight of 220-280 g were used. Animals were fed with Altromin standard diet and water ad libitum and were kept at 22°C under automatic lighting. 18 hr prior to sacrifice the diet was withdrawn. Collagenase (grade II purity) was obtained from Worthington Biochem. Corp. (Freehold, New Jersey, USA). All other chemicals were of analytical grade.

Isolation and incubation of hepatocytes: Cells were isolated by the method of Berry and Friend (11) modified as described previously (5,6). $2.39 \pm 0.17 \times 10^6$ cells per ml were incubated in Krebs-Ringer-carbonate-buffer, pH 7.4, at 37°C. The buffer contained 10 µg/ml D(-)-alpha-aminobenzylpenicillin, 20 µg/ml streptomycin and amino acids in concentrations as described (12). Samples of 20 ml were shaken 84 times per minute in a Warburg-apparatus in 200 ml Erlenmeyer flasks in an atmosphere of 95% oxygen and 5% carbon dioxide. Incubation was stopped by rapid cooling and subsequent centrifugation for 2 min at 100 g. The supernatant was centrifuged again for 10 min at 12,000 g.

Measurement of albumin and angiotensinogen:: Albumin was determined by the radial immunodiffusion of Mancini (13). Albumin used for the immunization of rabbits and as standard was purified as described (5). For the albumin determination in the medium, the supernatant was concentrated by precipitation with trichloroacetic acid to a final concentration of 5%, redissolved in one-twentieth of the initial volume and extensively dialyzed against 0.01 M Tris-HCl buffer, pH 7.4. The cells were washed once, treated with sodium desoxycholate (0.8%), homogenized, and also extensively dialyzed after centrifugation.

Angiotensinogen was measured as angiotensin I with specific radioimmunoassays. Angiotensin I was liberated from angiotensinogen by incubation of 0.5 ml of the sample with 2 Goldblatt units of hog renin (Nutritional Biochem. Corp., Cleveland, Ohio, USA) in 30 mM phosphate buffer, pH 6.5, plus 2.2 mM EDTA and 0.1 M diisopropylfluorophosphate for 15 min at 37°C. The enzyme was inactivated by heating and subsequent centrifugation. The same results were obtained with rat renin. However, no angiotensin I could be liberated by incubation without or with human renin. Most of the samples were measured with the commercial radioimmunoassay kit from CEA-Ire-Sorin (Saluggia, Italy). The 5 hr incubation experiments were determined with the Sqibb-kit (Chem. Fabrik von Heyden GmbH, München, Germany). The binding to the antiserum was 50-60% for both kits. After incubation for 20 hr at 4°C lyophylized bovine serum and activated charcoal was added, the samples centrifuged and the radioactivity determined in a gamma-counter (Fa. Picker, Frankfurt, Germany).

Incorporation experiments: Total protein synthesis was estimated by incorporation of 1-^{14}C-leucine (1 µCi/ml; 59 mCi/mmole) (Radiochemical Centre Amersham, United Kingdom). Incubation was stopped by pouring aliquots into non-radioactive leucine to a final concentration of 0.75%. Radioactivity in protein was determined by the method of Mans and Novelli (14) and counting was performed in a Packard scintillation counter model 3380. Protein was determined by biuret (6) with bovine serum albumin as standard. Cell counts were made in a Bürker chamber and the viability of the cells estimated by 0.2% trypan blue staining was 89 ± 3%.

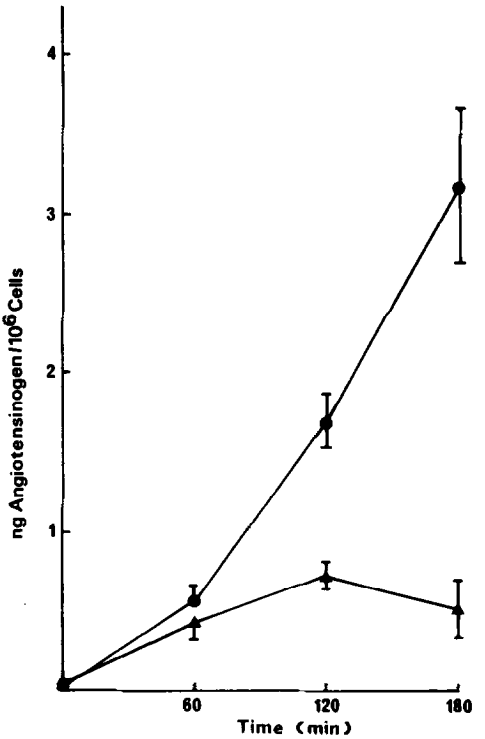

Figure 1. Angiotensinogen measured by radioimmunoassay in the medium of cell suspensions incubated with amino acids in serum concentrations without (●--●) or with (▲--▲) 0.7 mM puromycin. Each point represents the mean ± SE of 6 experiments. Abscissa: Time in min. Ordinate: ng angiotensinogen synthesized per 10^6 cells.

RESULTS

Angiotensinogen in the medium increased from non-measurable amounts at the beginning of the incubation to a concentration of 3.2 ± 1.2 ng per 10^6 cells (n=6) after an incubation period of 3 hr (Fig. 1). From the second to the fifth hr the increase was almost linear (Fig. 3). During the first hr the synthesis rate was somewhat lower. The wide standard deviations are explained in part by the variability of the assay which was found to be 13-19%. The incorporation of radioactive leucine into total protein (Fig. 4) and the net synthesis of albumin (Fig. 3) showed a linear rate for 3 and 5 hr, respectively. Addition

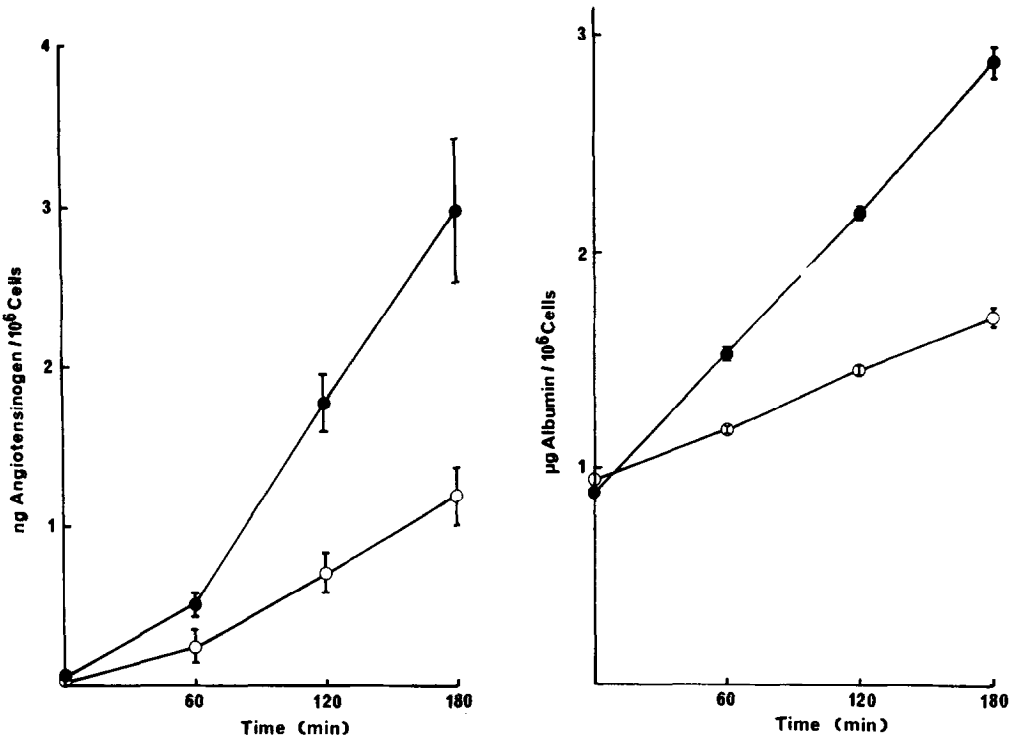

Figure 2. Synthesis of angiotensinogen (left) and albumin (right) by liver cell suspensions with amino acids in serum concentrations (●--●) or without amino acids (O--O). Each point represents the mean ± SD of 5 (angiotensinogen) or 3 (albumin) experiments. Abscissa: Incubation time in min. Ordinate: ng angiotensinogen and µg albumin synthesized per 10^6 cells.

of 0.7 mM puromycin inhibited the increase of angiotensinogen during 3 hr of incubation by 84% (Fig. 1). A similar result was found by addition of 35 mM cycloheximide. These concentrations are rather high and sufficient for complete inhibition of the incorporation of radioactive leucine into protein (15) and of albumin synthesis (6). The measured amount of angiotensinogen which cannot be suppressed by addition of puromycin is most likely due to a non-specific reaction. It cannot be explained by release of angiotensinogen from intracellular storage as when angiotensinogen was determined within the cells and in the medium separately less than 0.1 ng of angiotensinogen per 10^6

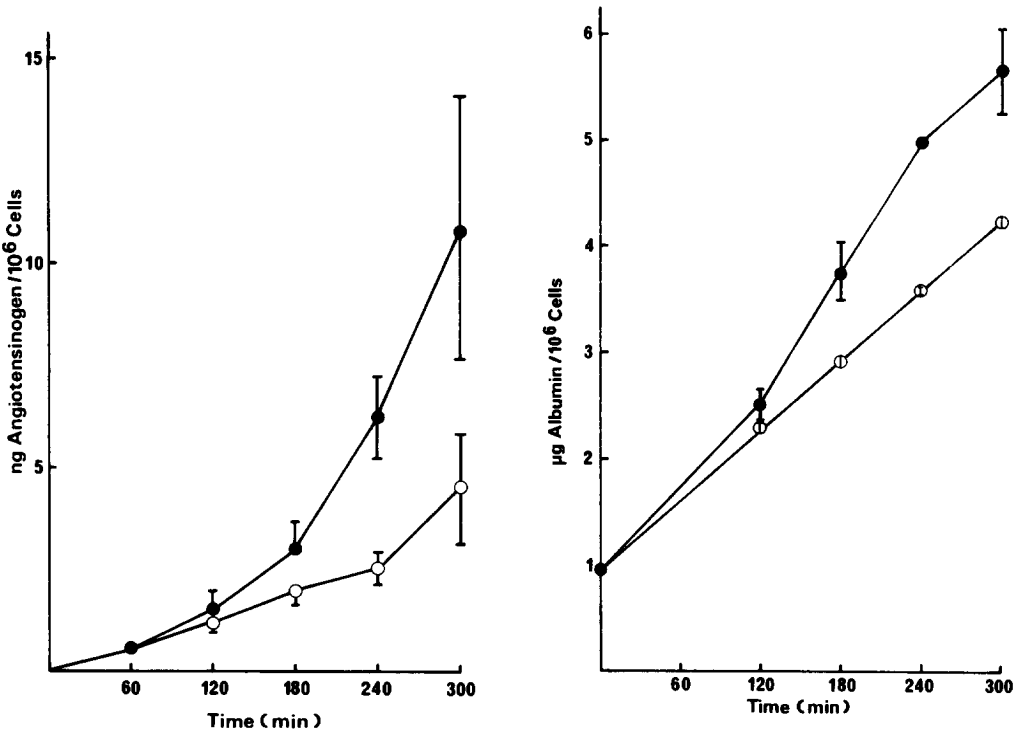

Figure 3. Synthesis of angiotensinogen (left) and albumin (right) with amino acids in serum concentrations and without (0--0) or with 10^{-4} M hydrocortisone (●--●). Each point represents the mean ± SD of 5 (angiotensinogen) or 3 (albumin) experiments.

cells was found during the whole incubation period. By subtracting the non-specific part, a net synthesis of angiotensinogen of 0.9 ng/10^6 cells/hr can be calculated.

Without addition of amino acids to the incubation medium, the angiotensinogen synthesis was considerably lower (Fig. 2a). After 3 hr only 1.2 ± 0.4 ng/10^6 cells had been synthesized compared to 3.0 ± 1.4 ng/10^6 cells for the control. A similar result was found for the albumin synthesis. The albumin content increased from 0.90 ± 0.02 to 2.88 ± 0.06 µg/10^6 cells/3 hr in the cell suspension incubated with amino acids, compared to an increase from 0.92 ± 0.01 to 1.70 ± 0.04 µg/10^6 cells incubated without amino acids (Fig. 2b). For both proteins the differences

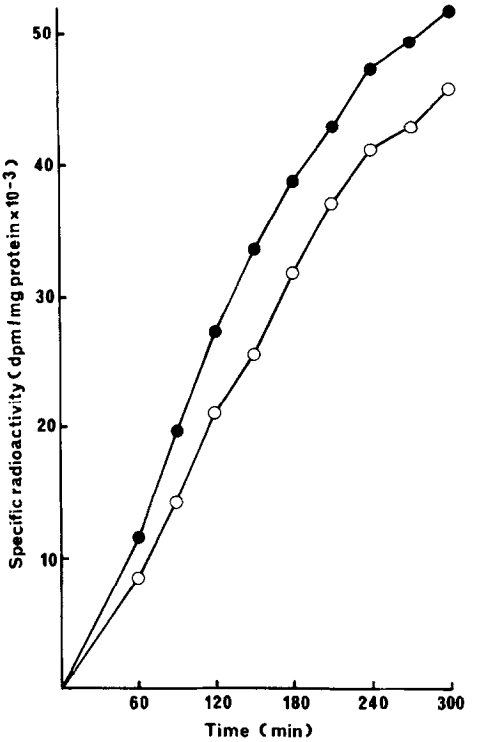

Figure 4. Incorporation of 1-^{14}C-leucine into total protein (1 μCi/ml; 59 mCi/mmole) with amino acids in serum concentrations (except leucine) and without (O--O) or with (●--●) 10^{-4} M hydrocortisone. Abscissa: Incubation time in min. Ordinate: Specific protein radioactivity (dpm/mg protein x 10^{-3}).

were highly significant ($p < 0.01$). By increasing the amino acid concentration in the medium above normal serum concentrations the angiotensinogen and albumin synthesis could not be further stimulated (data not shown). The addition or the withdrawal of tryptophan had no effect on the synthesis rate of albumin and angiotensinogen. Elimination of tryptophan from the amino acid mixture resulted in the same synthesis rate as with the complete amino acid mixture. Addition of tryptophan (2×10^{-4} M) without the other amino acids could not enhance the synthesis rate (data not shown).

Without cortisone the cells synthesized angiotensinogen and albumin for 5 hr almost linearly. Addition of 10^{-4} M hydrocortisone stimulated the synthesis rate of both serum proteins after 2-3 hr. After 5 hr the

cells incubated with hydrocortisone had synthesized 10.8 ± 7.3 ng/10^6 cells compared to 4.6 ± 2.5 ng/10^6 cells for the control, corresponding to an increase of 142% (Fig. 3a). The difference in the synthesis rate was not statistically significant after 3 hr incubation but was after 4 hr ($p < 0.01$). The albumin synthesis was also stimulated by hydrocortisone, but only by 43% ($p < 0.01$) (Fig. 3b). In contrast, the synthesis of total liver protein was not significantly elevated by cortisone. The incorporation of radioactive leucine into total liver protein increased by 13% only (Fig. 4).

DISCUSSION

Angiotensinogen is synthesized by isolated hepatocytes from which non-parenchymal cells have been removed by centrifugation. The final suspensions are contaminated by 4% Kupffer cells only (16), so it is unlikely that such cells could account for the angiotensinogen synthesis. Assuming that only cells excluding vital dye are capable of producing serum proteins, it may be calculated that 10^6 viable cells produced 1.01 ng (0.0178 pmol) angiotensinogen per hr and 0.75 µg (11.5 pmol) albumin per hr. As 1 g liver (wet weight) contains about 1.7 x 10^8 cells (17), 0.172 µg angiotensinogen/g/hr was synthesized compared to 128 µg albumin/g/hr. In fasted rat liver *in vivo* a synthesis rate of 340 µg albumin/g/hr has been reported (18). Assuming that the synthesis of angiotensinogen in isolated liver cells is diminished to the same extent as the synthesis of albumin, a synthesis rate of 0.46 µg angiotensinogen/g/hr for normal rat liver can be calculated. This compares with reported values of 0.12 µg/g/hr for liver slices(2) and 0.012 µg/g/hr for the perfused liver (3).

Removal of amino acids from the incubation medium decreased the synthesis rate of both angiotensinogen and albumin by 60%. The synthesis however, was linear during the experimental period. This indicates that sufficient amino acids are liberated to maintain this synthesis rate,

either by breakdown of damaged cells or by release of amino acids from cell proteins with a rapid turnover. The fact that fasting decreases the albumin synthesis by 53% (19) strongly argues for the latter possibility. It was demonstrated in the perfused liver that addition of tryptophan stimulated albumin synthesis (20), although some other workers could not confirm this (21,22). Tryptophan has also been found to be important for polysome aggregation (23). In these isolated cell suspensions tryptophan had no effect on the synthesis of angiotensinogen and albumin. Further studies must be undertaken to see whether the level of free tryptophan is higher in isolated cells than in the perfused liver.

The simultaneous investigation of various proteins allows distinction between non-specific and specific regulatory mechanisms. Hydrocortisone stimulated the total liver protein synthesis only slightly, whereas the synthesis of angiotensinogen and albumin was stimulated by 142% and 43%. In the perfused rat liver the albumin synthesis was stimulated by addition of cortisone in the same molarity as in this study although not to the same extent as in the isolated cells (24). The rapid increase of angiotensinogen after hydrocortisone may play a role in the elevated blood pressure after stress.

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REFERENCES

1. Oparil, S. and Haber, E. (1974) New Engl. J. Med. 291, 389-401.
2. Freemann, R.H. and Rostorfer, H.H. (1972) Amer. J. Physiol. 223, 364-370.
3. Nasjletti, A. and Masson, G.M.C. (1972) Suppl. II Circulation Res. 30 & 31, II 187-198.
4. Lakshmanan, M.R. Nepokroeff, C.M., Kim, M. and Porter, J.W. (1975) Arch. Biochem. Biophys. 169, 737-745.
5. Weigand, K., Müller, M., Urban, J. and Schreiber, G. (1971) Exptl. Cell Res. 67, 27-32.
6. Weigand, K. and Otto, I. (1974) FEBS Letters 46, 127-129.
7. Crane, L.J. and Miller, D.L. (1974) Biochem. Biophys. Res. Commun. 60, 1269-1277.
8. Jeejeebhoy, K.N., Ho, J., Breckenridge, C., Bruce-Robertson, A., Steiner, G and Jeejeebhoy, J. (1975) Biochem. Biophys. Res. Commun. 66, 1147-1153.

9. Jeejeebhoy, K.N., Ho, J., Gordon, R., Greenberg, G.R., Phillips, M.J., Bruce-Robertson, A. and Sodtke, U. (1975) Biochem. J. 146, 141-155.
10. Van Bezooijen, C.F.A., Grell, T. and Knook, D.L. (1976) Biochem. Biophys. Res. Commun. 71, 513-519.
11. Berry, M.N. and Friend, D.S. (1969) J. Cell Biol. 43, 506-520.
12. Scharff, R. and Wool, I.G. (1964) Nature 202, 603-604.
13. Mancini, G., Carbonara, A.O. and Heremans, J.F. (1965) Immunochemistry 2, 235-254.
14. Mans, R.J. and Novelli, G.D. (1960) Biochem. Biophys. Res. Commun. 3, 540-543.
15. Schreiber, G. and Schreiber, M. (1972) J. Biol. Chem. 247, 6340-6346.
16. Katz, N. and Jungermann, K. (1976) Hoppe-Seyler's Z. Physiol. Chem. 357, 359-375.
17. Weibel, E.R., Stäubli, W., Gnägi, H.R. and Hess, F.A. (1969) J. Cell Biol. 42, 68-91.
18. Peters, Th. Jr. and Peters, J.C. (1972) J. Biol. Chem. 247, 3858-3863.
19. Rothschild, M.A., Oratz, M., Mongelli, J. and Schreiber, S.S. (1968) J. Clin. Invest. 47, 2591-2599.
20. Rothschild, M.A., Oratz, M., Mongelli, J., Fishman, L. and Schreiber, S.S. (1969) J. Nutr. 98, 395-403.
21. Miller, L.L. and Griffin, E.E. (1971) Amer. J. Clin. Nutr. 24, 718-729.
22. Kelman, L., Saunders, S.J., Wicht, S., Frith, L., Corrigall, A., Kirsch, R.E., and Terblanche, J. (1972). Biochem. J. 129, 805-809.
23. Munro, H.N. (1970) Mammalian Protein Metabolism, Vol. IV, pp. 299-386, Academic Press, New York.
24. John, D.W. and Miller, L.L. (1969) J. Biol. Chem. 244, 6134-6142.