SYNTHESIS AND SECRETION OF PLASMA PROTEINS BY EMBRYONIC CHICK HEPATOCYTES
Changing Patterns during the First Three Days of Culture*

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The production of plasma proteins is a major specialized function of the liver, plasma proteins representing roughly one-fifth of the hepatocytes' protein synthetic activity (1, 2). Study of this function not only will add to our understanding of protein biosynthesis in eukaryotic cells, but also will aid in elucidating the process of biochemical differentiation.

In this report the development of a model system for studying the regulation and differentiation of plasma protein synthesis is presented. A model system must be easily set up, reproducible and representative of the in vivo capacity of the hepatocyte to produce plasma proteins, and should allow experiments of several days duration as well as simple and fast measurement of plasma protein synthesis.

Plasma protein synthesis has been investigated in whole animals (1-6), perfused liver (7, 8), liver slices (9), liver cell suspensions (10, 11), and continuous cultures of liver (12) and hepatoma cells (13). For our purpose, whole animal studies are too ambiguous and the perfused liver system is too restricting in the number of conditions that can be varied within any one experiment. Work with whole animals, perfused livers, or liver slices poses difficulties in standardization. Suspensions of liver cells share with perfused livers the problem of being too short-lived. Preferring not to work with the relatively dedifferentiated continuous cell lines, we have concentrated on primary culture of chick embryo liver cells which had been developed in this laboratory and which maintain differentiated functions for several days (14-16).

Quantitation of output in studies of protein synthesis most commonly involves the use of radioactive isotopes and rather elaborate immunological procedures for identification. In contrast, our system eliminates the need for routine radioactive labeling by using a simple immunoassay to determine the synthesis of plasma proteins. The system combines the culture of chick embryo liver cells with Laurell's rocket immunoelectrophoresis technique (17) for identification and quantitation of the plasma proteins secreted into the medium. By an adaptation of this immunological procedure (18, 19), proteins can be assayed in small volumes of medium; sequential sampling and assay establish the kinetics of protein synthesis.

To permit meaningful conclusions to be drawn concerning protein synthesis, a number

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of properties had to be established: (a) the type and number of cells and their viability in
culture; (b) identification of the cultured cells that are responsible for plasma protein
synthesis; (c) stability of the secreted proteins; (d) determination as to whether secretion
reflects a release of stored proteins, or whether synthesis and secretion are continuous;
(e) the overall time required for intracellular transport of plasma proteins synthesized in
culture.

In this paper these points are addressed, and we further demonstrate that the
rate of accumulation of a plasma protein in the medium is equal to the rate of
its synthesis. We show that chick embryo liver cells synthesize proteins at in
vivo rates for at least 72 h in culture and that the proportion of plasma protein
synthesis to total hepatic protein synthesis is comparable to that observed in
vivo. Synthesis of a number of plasma proteins is compared, and it is shown
that decreasing synthesis with time in culture is specific to albumin alone. The
advantages of this system in approaching several problems of biological interest
have been discussed in an earlier report (18).

Materials and Methods

Culture Media and Reagents. The standard medium is modified Ham F-12 (16) containing
10% heat-inactivated (56°C for 30 min) and Millipore-filtered (Millipore Corp., Bedford, Mass.)
fetal bovine serum (Grand Island Biological Co., Grand Island, N. Y.) and mycostatin (E. R.
Squibb & Sons, Princeton, N. J.), 25 U/ml. To improve fibrinogen detection, 15 µg heparin
sodium/ml (Connaught Laboratories, Toronto, Canada) was added to the standard medium where
so indicated. Serum-free medium is modified Ham F-12 supplemented with insulin (crystalline,
bovine pancreas, Sigma Chemical Co., St. Louis, Mo., 24.3 IU/mg) at a concentration of 0.2 µg/
ml.

Acetoxycycloheximide was obtained from the Cancer Chemotherapy National Service Center,
National Cancer Institute, Bethesda, Md. Actinomycin D was obtained from Merck Chemical
Div., Merck & Co., Inc., Rahway, N. J.

L-[4,5-3H]leucine, sp act 6 Ci/mmole, was obtained from Schwarz/Mann Div., Becton, Dickin-
son & Co., Orangeburg, N. Y. A 3H-labeled L-amino acid mixture, as well as Omnifluor and
Protosol, were from New England Nuclear, Boston, Mass.

Primary Culture of Liver Parenchymal Cells. The culturing procedure described previously
(14) was used with minor modifications. Briefly, perfused livers dissected from 16-day-old chick
embryos were trimmed of connective tissue and disaggregated by gentle mechanical and
enzymatic treatment (18). 5 ml of concentrated cell suspension, derived from three livers and
containing single cells and aggregates of 2–10 cells, was routinely diluted 20-fold in medium
before inoculation of 2, 5, or 12 ml, respectively, into 35-, 55-, or 80-mm diameter Falcon Petri
dishes (Falcon Plastics, Div. BioQuest Oxnard, Calif.). In this way, approximately the same ratio
of surface area to volume of inoculum was attained, resulting in similar cell densities after
plating. Less than an hour elapsed between excision of liver and initial incubation of the cells in
a 5% CO2-air atmosphere at 38°C. All viable cells attached within 2 h to the plastic surface of the
dish. Culture medium with cell debris, nonviable cells, and erythrocytes could be aspirated and
replaced with fresh medium as early as 2 h after plating. The ratio of adhering cells to supporting
medium was usually in the range of 1,000,000/ml. Variations made in this general procedure are
noted in legends to the figures.

Antisera. Polyspecific antisera to adult and 17-day embryonic chicken serum, as well as
monospecific antisera to chicken albumin and fibrinogen, were produced in rabbits according to
standard methods (20). The plasma proteins designated "C" and "M" were followed with a
trispecific antiserum whose preparation, characterization, and usage is described elsewhere.1 The

1 The functions of "C" and "M" have not been identified; for simplicity, C and M are used in
this paper.

2 G. Grieninger and J. Pindyck. The application of a modification of rocket immunoelectropho-
resis to the study of plasma protein synthesis in cultured hepatocytes. Manuscript in preparation.
Plasma Protein Synthesis in Cultured Hepatocytes

Antiserum contains anti-albumin, anti-C, and anti-M antibodies. Other antisera used in this study were prepared against various chicken antigens and were kindly provided by the following colleagues: antiplasminogen, Dr. Allan R. Goldberg (The Rockefeller University); anti-12-day embryonic plasma proteins, Dr. Edwin M. Weller (University of Alabama); antilipovitellin, Dr. Robert F. Goldberger (National Institutes of Health); antialbumin, Mr. George Kuzmycz (The Rockefeller University); anti-alpha-macroglobulin, Dr. Deane F. Mosher (University of Wisconsin); and the antiserum against the apoproteins of very low density lipoprotein and against apo-A, the major apoprotein of high density lipoprotein, Dr. David J. Kudzma (University of Miami).

Anti-total chicken plasma proteins, hereafter referred to as anti-TCPP, is a mixture of antisera against various chicken antigens: anti-adult chicken serum, anti-17-day embryonic chicken serum, antifibrinogen, antialbumin, antilipovitellin, anti-12-day embryonic plasma proteins, and the antiserum against the apoproteins of very low density lipoprotein. Anti-mouse albumin was kindly provided by Dr. Hans P. Bernhard (Biozentrum, University of Basel).

The titer and specificity of antisera used were determined by electroimmunoassay and crossed immunoelectrophoresis. No cross-reactions were observed between antisera to chicken plasma proteins and proteins present in fetal bovine serum.

Electroimmunoassay and Crossed Immunoelectrophoresis. The electroimmunoassay (rocket immunoelectrophoresis) of secreted plasma proteins was performed as described previously; a more detailed treatment of the application of the method will be published elsewhere. In the assay procedure, 3 μl of culture medium, containing secreted proteins, was used without preliminary concentration.

Crosed immunoelectrophoresis was performed as described on concentrated samples of medium in which monolayers had been maintained, except that the immunoplates were stained with Coomassie Blue for better photographic contrast.

Determination of Intracellular Plasma Protein Levels by Electroimmunoassay. The monolayers of cells were washed with Earle's balanced salt solution minus magnesium and calcium and detached by adding Na2EDTA (0.02%) in the same solution. A cell pellet was obtained by centrifugation at 180 g for 5 min. Disruption of the cells was accomplished by first freezing then homogenizing the thawed sample at 4°C in 1 ml of sodium barbital buffer (44 mM, pH 8.6) followed by addition of Triton X-100 to a final concentration of 0.5%. Samples were withdrawn for cellular protein determination. After cell debris was removed by centrifugation, the concentrations of cytoplasmic plasma proteins were determined on 3 μl of supernate by electroimmunoassay. The detergent did not disturb the immunoassay or the determination of cellular protein. As an alternate to this procedure, the cells could be disrupted by sonication for 5 min and were found to yield identical intracellular plasma protein levels.

Radioactive labeling of hepatic proteins. In several experiments, short labeling periods were desired, and labeling was terminated by washing the cells four times with Earle's balanced salt solution and then adding fresh, label-free culture medium. Samples were withdrawn at the times indicated and mixed with an equal volume of 16% (wt/vol) trichloroacetic acid and the resulting precipitates were filtered on Whatman GW/C glass fiber filters. The dried precipitates were then washed with 8% trichloroacetic acid containing an excess of the unlabeled amino acid(s). To the dried precipitates a toluene-based scintillation fluid was added containing 0.4% (vol/vol) Omnifluor, 9% (vol/vol) Protosol, and 0.3% (vol/vol) ammonium hydroxide; the samples were counted in a liquid scintillation counter.

In long-term labeling experiments, cells were exposed to a mixture of 3H-labeled amino acids for a period of 48 h. The culture medium was centrifuged to remove any debris and anti-TCPP serum was used to precipitate the radioactively labeled plasma proteins without addition of carrier protein. Preliminary studies had shown that 0.2 ml of the antisera mixture quantitatively precipitated 20 μg of chicken plasma proteins from a final volume of 0.4 ml. Hence, 0.2 ml of anti-TCPP was routinely incubated with 0.2 ml of culture medium for 0.5 h at 25°C and overnight at 4°C. Labeled albumin in the medium was specifically immunoprecipitated in an analogous fashion with anti-chicken serum albumin. Immunoprecipitates were filtered in the cold on two overlaid

3 D. J. Kudzma, J. B. Swaney, and E. N. Ellis. Apolipoproteins of the chicken: effect of estrogen administration. Manuscript submitted for publication.

4 Abbreviations used in this paper: TCPP, total chicken plasma proteins.
Whatman GF/C glass fiber filters and washed thoroughly with cold phosphate-buffered saline containing an excess of the unlabeled amino acid(s) used during labeling. It was found that two filters were required to quantitatively retain the immunoprecipitate. The filters were then dried and counted as described above.

The measurement of label incorporated into cellular protein was accomplished by scraping the cells from the dishes with the aid of a rubber policeman into 8% (wt/vol) trichloroacetic acid and processing the precipitates as described above.

Estimation of Protein, DNA, and Cell Number. Cells were washed and detached from the culture dish with EDTA as described above. The cell suspension was centrifuged, and the cell pellet was resuspended in a small volume of the EDTA solution. Portions of this cell suspension were employed in determination of protein by the method of Lowry et al. (24), and DNA by the method of Burton (25). The cell number was determined in a hemocytometer.

Determinations of cellular protein, DNA content, the incorporation of radiolabeled precursors, and the amount of plasma proteins detected with immunochemical techniques were made on at least three culture dishes.

Results

Hepatocyte Culture. Cells, prepared and inoculated as described in Materials and Methods, formed a monolayer (14) at a density, determined in several experiments, between 2 and \(3 \times 10^5\) cells per cm\(^2\). The cell count was performed on a suspension of cells removed from the dish and was confirmed by counting nuclei in photographs of monolayers. Floating and loosely adhering cells are considered nonviable because they fail to produce detectable plasma proteins when removed and reinoculated 2 h after the original inoculation. Based on our observation that the protein content of \(3 \times 10^6\) cells amounts to approximately 1 mg, we estimate that 30% of the hepatocytes in the intact liver are recovered in culture as viable, attached liver cells.

At the high cell densities used, mitotic figures were scarcely ever seen. Cell count, cellular DNA, and cell protein were found to be constant for at least 72 h in culture demonstrating that the cells do not divide and do not increase in mass. These findings are consistent with studies that showed that mitotic activity decreases in the intact liver both with the age of the chick embryo and with higher cell densities (26). We suggest that in these cultures the embryonic hepatocytes are arrested in the G1 phase of the cell cycle as are mature hepatocytes in vivo (27). Support for this claim is derived from a calculation of the amount of DNA per nucleus based on determinations of cellular DNA and the number of cells. When corrected for binucleated cells (26), a value of 2.5 pg of DNA per nucleus was obtained which agrees with the diploid amount reported for hepatic cells of the domestic fowl (28).

The presence of nonparenchymal cells, particularly fibroblasts, in culture was negligible at the high cell densities used, a phenomenon also observed by others (29, 30). With immunofluorescent staining, it could be shown that uniformly all hepatocytes contained the various plasma proteins studied so far.\(^5\) Fibroblasts were not found to contain plasma proteins with this method. Furthermore, in medium from cultures of fibroblasts on days 1, 2, and 3, no secreted plasma proteins could be detected by electroimmunoassay, even when the medium was concentrated 10-fold. This shows that any fibroblasts contaminating the hepatocyte culture do not contribute to the output of plasma proteins.

\(^5\) R. G. Kalb and G. Grieninger. Immunofluorescent localization of plasma proteins in cultured embryonic chick hepatocytes. Manuscript in preparation.
Stability of the Secreted Plasma Proteins in the Culture Medium. Chick embryo liver cells secrete plasma proteins which accumulate in the culture medium (18). When medium, in which cells had been maintained for 24 h and which contains the secreted plasma proteins, was incubated for another 24 h under the usual culture conditions but now in the absence of cells, no decrease was observed by electroimmunoassay in the amount of three major plasma proteins: albumin and the two plasma proteins designated C and M (18; see Fig. 1 for electrophoretic mobilities). No decreased radioactivity in total plasma proteins (labeled in culture as described in Table Ia) was found by quantitative precipitation with the antibody mixture, anti-TCPP, after a similar 24-h incubation of medium in the absence of cells. When protein synthesis was inhibited by acetoxycycloheximide (31), a potent derivative of cycloheximide, and the cells and medium were left to incubate at 38°C, the levels of albumin, C (Fig. 2), M, and fibrinogen (data not shown) remained unchanged for 20 h. Furthermore, by maintaining the hepatocytes in chemically defined, serum-free medium, it could be demonstrated that the cells do not secrete detectable levels of active proteases (A. R. Goldberg and G. Grieninger, unpublished observations.). These experiments strongly suggest that the cells do not break down the secreted plasma proteins to any significant extent under the culture conditions described. Because the plasma proteins are stable after they have been secreted, the rate of accumulation of a plasma protein in the medium represents its rate of secretion by the cells.

Secreted Proteins Represent Plasma Proteins. The nature of proteins secreted by the embryonic chick liver cells in culture was studied in samples of culture medium collected after a 24-h exposure of the cells to a [3H]amino acid mixture. A measure of secreted proteins was obtained by trichloroacetic acid precipitation of aliquots of the medium. In other aliquots of the same medium, radioactivity in plasma proteins was assessed by precipitation with the antisera mixture anti-TCPP which in preliminary experiments recognized virtually all (adult and embryonic) antigens present in chicken plasma obtained from various developmental stages. The amount of acid-precipitable radioactivity in the medium was found to be identical with the amount immunoprecipitable with anti-TCPP. In other aliquots of the medium, the absence in the immunoprecipitate of proteins other than chicken plasma proteins was demonstrated by precipitating mouse albumin with anti-mouse albumin with the reagents present at concentrations corresponding to those of the reagents in the anti-TCPP precipitation. From the complete correspondence found between acid-precipitated and immunoprecipitated material, it can be concluded that the hepatocytes in culture secrete plasma proteins almost exclusively. Furthermore, it is possible in this cell culture system to interpret trichloroacetic acid-insoluble radioactivity in the medium as representing labeled plasma proteins secreted by the cells.

Spectrum of Secreted Plasma Proteins. Two-dimensional or crossed immunoelectropherograms revealed that a wide spectrum of plasma proteins accumulated in the medium during the first, second, and third days of culture (Fig. 1). The size and intensity of the individual immunoprecipitates ("rockets") indicate the amount of specific protein present. We have shown that the intensity of the immunoprecipitate reflects the amount of antigen by using
FIG. 1. Crossed immunoelectrophoresis of plasma proteins secreted on
days 1, 2, and 3 of culture. Cells were plated in standard medium in 80-
mM diameter dishes. The medium was changed 2.5 h later and every 24
h thereafter. At 2.5, 26.5, and 50.5 h, selected sets of dishes were
transferred to serum-free medium and incubated for an additional 24 h.
The medium was then collected, centrifuged at 500 g for 10 min, and
concentrated 85-fold. Concentrated medium (5 μl), containing plasma
proteins corresponding to the levels secreted by 4.2 × 10⁶ cells in 24 h,
was applied in the sample well visible in the lower left corner of each
panel. Electrophoresis in the first dimension was performed from left to
right and in the second dimension from bottom to top. The antibody-
containing gel contained 10% (vol/vol) anti-adult chicken serum, i.e. 14
μl/cm². The following plasma proteins have been identified immunolog-
ically: peak 2 = plasma protein C, peak 4 = albumin, peak 6 = plasma
protein M, peaks 13 and 14 = lipoproteins, peak 15 = alpha-macroglob-
ulin, peak 19 = transferrin. (a) Secretion between 2.5 and 26.5 h: day 1.
(b) Secretion between 26.5 and 50.5 h: day 2. (c) Secretion between 50.5
and 74.5 h: day 3.
TABLE I
Synthesis of Plasma Proteins during 3 Days of Culture

| Radioactivity in plasma proteins determined by | Labeling period | Day 1 | Day 2 | Day 3 |
|-----------------------------------------------|-----------------|-------|-------|-------|
| (a) Immunoprecipitation                        | 24 h            | 100   | 88    | 79    |
| (b) Acid precipitation                         | 5 min           | 100   | 88    | 98    |

Cells were plated and maintained in 2 ml of medium in 35-mm diameter dishes. Total plasma protein synthesis was determined by two independent methods. Results obtained for days 2 and 3 are expressed relative to the values obtained for day 1. (a) Long-term labeling. At 4 h postinoculation, and every 24 h thereafter, the medium was replaced with fresh medium containing 15 μg heparin/ml. Different sets of dishes were exposed to a 3H-amino acid mixture added to the medium at 2.5 μCi/ml at 4, 28, and 52 h (day 1, day 2, and day 3, respectively). After 24 h of incubation, total plasma protein in the culture medium—amounting to approximately 15 μg—was immunoprecipitated with anti-TCPP as described in Materials and Methods. The value obtained for the first day was 3,450 cpm/0.2 ml of medium. (b) Short-term pulse. 1 h before the addition of label, the cells were washed with medium and then placed in 1.0 ml of medium. At 24, 48, and 72 h of culture (day 1, day 2, and day 3, respectively), different sets of dishes were pulsed for 5 min with 15 μCi of [3H]leucine/ml. After the labeling period, the cells were washed and reincubated in 1.0 ml of label-free medium for 80 min, whereupon trichloroacetic acid-precipitable radioactivity was determined in the entire volume of culture medium. The value obtained for the 1st day was 3,756 cpm/ml of medium.

radioactively labeled plasma proteins and comparing electropherograms with their respective autoradiograms (18). The antiserum used in Fig. 1 was prepared against adult chicken serum and was chosen for its balanced titers of antibodies against various plasma proteins, thereby permitting good discrimination between individual peaks. When compared to anti-TCPP, the antiserum was found to precipitate >80% of the plasma proteins secreted by the cells in culture. The anti-adult chicken serum does not recognize several secreted plasma proteins, namely, fibrinogen, plasminogen, a fetal protein (FP-1) and a phosphoprotein.

In the central panel (b) of Fig. 1, at least 20 different plasma protein peaks can be distinguished and are numbered consecutively from right to left. To match the corresponding peaks in panels a and c, the respective medium samples were directly compared by tandem crossed immunoelectrophoresis (32) with the medium used in b. With this technique, immunologically related peaks derived from two antigen samples fuse to form double peaks, thus permitting direct comparison of different crossed immunoelectrophoresis patterns. With the use of specific antisera, certain peaks have been identified: peak 2 = plasma protein C, peak 4 = albumin, peak 6 = plasma protein M, peaks 13 and 14 = lipoproteins, peak 15 = alpha-macroglobulin. Peak 19 was identified as transferrin by tandem-crossed immunoelectrophoresis with conalbumin, a protein that has a similar polypeptide constitution and is immunologically indistinguishable from transferrin (33).

The proteins secreted by the cultured hepatocytes compare well with plasma proteins present in embryonic blood. By tandem-crossed immunoelectrophoresis it could be demonstrated that most of the proteins present in plasma derived from 15- to 19-day-old chick embryos were secreted in culture with the notable
exception of immunoglobulins, ovalbumin, and another fetal protein, FP-2 (data not shown).

With crossed immunoelectrophoresis, we were able to observe dramatic changes in the spectrum of secreted plasma proteins, the most prominent occurring between the 1st and 2nd days. In Fig 1a, the height and intensity of peak 4 indicates that albumin was the major protein secreted early in culture. Later in culture, plasma protein M and the lipoproteins (peak 6 and peaks 13 and 14, respectively, Fig. 1c) predominated. Fig. 1 also shows that the albumin secreted in fresh medium during day 3 was only a trace of that secreted on day 1. This decrease in albumin secretion was paralleled by an increase in the amount of other plasma proteins secreted.

Electroimmunoassay with monospecific or oligospecific antisera provided a simpler and more precise means for quantification of particular plasma proteins. This is illustrated with assays to determine albumin and plasma protein C, which were performed on unconcentrated culture medium samples taken as described in the legend to Fig. 1. The amount of albumin secreted on days 1, 2, and 3 was found to be 10.5, 1.8, and 0.3 μg/4.2 × 10^6 cells. The same population of cells produced plasma protein C levels of 9.5, 22, and 40 U on these respective days. 10 U correspond to approximately 1 μg of protein.

**De Novo Synthesis of Secreted Plasma Proteins in Culture.** Secreted plasma proteins became radioactivity labeled when chick embryo liver cells were exposed to radiolabeled amino acids in culture, indicating *de novo* synthesis.

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**Fig. 2.** Inhibition of secretion of the plasma proteins albumin and C by acetoxycycloheximide (ACX). Cells, equivalent to 1.8 mg cellular protein, were kept in 55-mm diameter dishes. The secretion of albumin and C was determined by electroimmunoassay and is expressed as micrograms of albumin or units of C in the culture medium per culture dish. 10 U is equivalent to about 1 μg of protein. The experiments in a and b were performed on dishes established in the same culture. (a) Time-course of albumin secretion. Zero time represents the time fresh medium was added 16 h after plating. Acetoxycycloheximide is added 4.5 h later (arrow) to a concentration of 0.15 μg/ml. The *inset* enlarges both scales to show the time period immediately after the addition of acetoxycycloheximide. (b) Time-course of C secretion. Zero time represents the time fresh medium was added 38.5 h after plating. Acetoxycycloheximide is added 6.5 h later (arrow) to a concentration of 0.15 μg/ml.
The contribution of de novo synthesis to the amount of a plasma protein secreted in culture was studied under conditions in which protein synthesis was inhibited. Acetoxycycloheximide was added to the medium at a concentration of 0.15 μg/ml, a concentration that inhibited the incorporation of labeled amino acids into total hepatocellular proteins by about 95%. As seen in Fig. 2, the accumulation of albumin and plasma protein C was interrupted by the addition of acetoxycycloheximide. These results suggest that de novo protein synthesis is required to maintain secretion.

To learn more about the relationship of intracellular proteins to their secreted counterparts, we compared the amounts of plasma proteins released in the medium with the levels maintained intracellularly (Fig. 3). It can be seen that changes in the secretion rate of each protein were reflected in changes in its cellular level: the intracellular amount of albumin decreased with the decline in its rate of secretion (Fig. 3a), whereas the intracellular amount of C increased with the rise in its rate of secretion (Fig. 3b). The initial substantial intracellular C level is not understood. Inasmuch as the actual amounts of both albumin and C inside the cells were small relative to the quantities exported (<5% of the daily secretion), one can conclude that the secreted proteins measured in the culture medium represent de novo synthesis rather than a release from storage pools developed before culture. These findings strongly suggest that the intracellular levels of plasma proteins reflect a steady state between synthesis and secretion.

Stimulation of the rate of secretion of a plasma protein by some given factor(s) might be expected to be accompanied by elevated cellular levels of that protein (i.e. establishment of a new steady-state level). This was explored by evaluating the effect of actinomycin D (Fig. 3a), a drug found to stimulate albumin secretion (34). As shown, the stimulation of the rate of albumin secretion is, indeed, reflected in a simultaneous increase of the intracellular albumin level.

An independent estimate of the level of albumin in the cells can be obtained from experiments described above. Immediately after the addition of acetoxycycloheximide, the cells continue to secrete albumin for 1 h before accumulation ceases — 3.7 μg/dish (see inset, Fig. 2a), an amount equivalent to 2 μg/mg cell protein. Peters and Peters (2), working with the liver of the intact rat, similarly found that in the absence of protein synthesis, secretion of previously synthesized albumin continues. We assume that the albumin (2 μg/mg cell protein) that continues to be secreted by the embryonic chick liver cells in the presence of acetoxycycloheximide is the steady-state albumin present intracellularly at the time the inhibitor was added (20.5 h). This is confirmed by direct measurement of cellular albumin in the experiment described in Fig. 3a, i.e. after 20 h of culture the intracellular albumin amounts to approximately 2 μg/mg cellular protein.

The failure to observe a parallel gradual cessation of accumulation in the case of plasma protein C can be attributed to this circumstance: the intracellular level of C at 46 h (2 U/mg cell protein, Fig. 3b) corresponds to <4 U/dish, a value too low to be resolved in Fig. 2b. Hence C secretion appears to stop abruptly upon the addition of acetoxycycloheximide.

Intracellular Transit Time. The rapidity of secretion after synthesis is
Fig. 3. Intracellular and secreted levels of albumin and plasma protein C. The cells were prepared as described in Materials and Methods except that the concentrated cell suspension was washed with medium by centrifuging at 180 g for 5 min before further dilution. The cells were maintained in 80-mm diameter dishes and culture medium was changed at 4, 11, and 23 h. Cellular protein was determined at 11, 23, and 47 h and was found to remain constant at 4.2 mg per dish. Albumin and plasma protein C levels in the cells as well as in the medium were determined by electroimmunoassay and are expressed per milligram of cellular protein. Zero time values were determined on a sample of the cell inoculum. The data shown in a and b for control cells (no drug) were obtained from the same dishes. (a) Albumin, in micrograms per milligram of cell protein, intracellularly (upper panel) and secreted into the medium (lower panel; cumulative levels). At 11 h, actinomycin D (AD) was added to some dishes to a final concentration of 0.25 μg/ml (arrow). The actinomycin D was replenished in these dishes at the next medium change which occurred 23 h after plating. Addition of the drug resulted in a reduction of cell protein from 4.2 mg to 3.8 and 3.4 mg per dish at 23 and 47 h, respectively. ●–●, Control cultures; ○--○, cells treated with actinomycin D. (b) Plasma protein C, in units per milligram cell protein, intracellular (upper panel) and secreted into the medium (lower panel; cumulative levels). 10 U is equivalent to about 1 μg of protein.

limited by the time required for the transport of the proteins along the secretory pathway from their assembly at the polysomes to their release from the cell. The intracellular transit time of the total population of plasma proteins in cultured chick hepatocytes was explored in the experiment described in Fig. 4. The cells were labeled with [3H]leucine for 70 min, a time found in preliminary experiments to be sufficient for establishing a steady state of radiolabel in intracellular plasma proteins. The cells were then washed and incubated again in unlabeled medium. At frequent time intervals, the trichloroacetic acid-precipitable radioactivity accumulating in the medium was determined. In the figure, it can be seen that within 60 min the accumulation of acid-precipitable
counts has ceased. We interpret these data to mean that all labeled plasma proteins have left the cells within 1 h after the termination of the labeling period and that about 80% of the overall plasma proteins have been secreted within 30 min of their synthesis.

The time required for completed transit of albumin can be estimated from an experiment described earlier. From Fig. 3a, it is possible to say that at any given time the albumin present in the cultured hepatocytes constitutes less than the amount secreted per hour. In other words, the intracellular albumin level constitutes <5% of the daily secretion of this protein. From this, one may estimate that albumin molecules, once synthesized, should all be exported within 1 h.

Rate of Total Plasma Protein Synthesis in Culture. Total plasma protein synthesis in culture was quantified in two ways. First, cells were exposed to a mixture of radiolabeled amino acids for 24 h beginning at different times in culture, and chicken plasma proteins in the medium were quantitatively immunoprecipitated (Table Ia). The radioactivity detected in the immunoprecipitate is a measure of the daily synthesis of total plasma proteins. The use of long-term labeling is valid in this case because of the stability of the secreted plasma proteins. A slight decrease in incorporation is observed over 76 h.

Second, cells were labeled with [3H]leucine for only 5 min at different times in culture, washed, and transferred to unlabeled medium. After 80 min, the
trichloroacetic acid-precipitable radioactivity in this medium was determined. Because the half-time of intracellular transit for the total population of plasma proteins was found to be about 10 min (Fig. 4), a labeling period of 5 min was chosen to assure that all labeled plasma proteins were still contained within the cells at the medium change. The subsequent 80-min period was shown to be sufficient for complete secretion of all the plasma proteins labeled during the 5-min pulse (compare with Fig. 4). In Table 1b, it can be seen that acid-precipitable radioactivity, representing labeled plasma proteins, does not change substantially over 3 days. The results of both experiments demonstrate that the nonproliferating chick hepatocytes produce overall plasma protein at a nearly constant rate for 3 days in culture.

Relationship of Plasma Protein Synthesis to Total Hepatic Protein Synthesis. An estimate of the percentage of total hepatic protein synthesis in culture contributed by plasma protein synthesis was obtained in experiments designed as follows. After a 5-min exposure to [3H]leucine (pulse period), the washed cells from one group of dishes were used to determine radiolabel incorporated into cellular protein which served as a measure of total hepatocellular protein synthesis. Fresh label-free medium was added to the washed cells from a second group of dishes that were then incubated for 80 min to permit secretion of all proteins that had become labeled during the pulse. At the end of this time, the medium was assayed for trichloroacetic acid-insoluble radioactivity to provide a measure of total plasma protein synthesis. In several independent trials, plasma proteins represented about 20% of the hepatocellular proteins synthesized during the pulse. This relationship was maintained over a 3-day culture period.

During the 1st culture day, 90 ~g of albumin was synthesized per milligram of cell protein as determined by electroimmunoassay (Fig. 3 and reference 18). By quantitative immunoprecipitation of labeled plasma proteins in the medium with anti-TCPP as well as with antialbumin, it was shown that albumin synthesis comprises almost 50% of plasma protein synthesis during that period (see also Fig. 1a). Hence, total plasma protein synthesis, previously shown to be constant (Table I), is approximately 200 ~g/mg of cell protein daily. Because plasma protein synthesis, in turn, is about 20% of total hepatic protein synthesis, we conclude that the liver cells in culture synthesize protein in the range of 1 mg/day per mg of cellular protein.

Discussion

This paper demonstrates the suitability of embryonic chick liver cells in primary culture as a model system for the study of plasma protein synthesis.

Cells in Culture. Primary cultures of embryonic chick hepatocytes have the advantage of being both rapidly achieved and highly reproducible. The cells generated from only a few embryo livers are sufficient to permit multiple observations and, because of the excellent yield of cultured cells per liver, we believe that the cultures contain a population of hepatocytes that are representative of those in the intact tissue. Optimal plasma protein production by the cells occurs even at the high cell densities chosen for routine cultures. The

* It was found that the secreted amounts of albumin, C and M, are directly proportional to the number of cells (per dish) over a range of cell densities from 0.1 to 3.3 × 10⁶ cells per cm².
homogeneously populated monolayers of nonproliferating hepatocytes remain viable for at least a week. After the 4th day of culture, we observed a general decrease in protein synthesis, probably due to prolonged maintenance of the cells in a confluent monolayer. However, throughout the first 3 days, the amount of plasma proteins synthesized was found to be constant (Table I). Moreover, the amount of plasma proteins secreted in a given experimental period can be related to a fixed cell number and cell mass because the cells are not dividing. These features of the culture permit its straightforward application to study of the rate of plasma protein secretion over prolonged periods of time.

**Secretory Proteins.** Because all the labeled proteins that are secreted into the medium, as measured by trichloroacetic acid precipitation, are precipitable with the antisera mixture anti-TCPP, it can be concluded that the hepatocytes in culture secrete plasma proteins almost exclusively. The secreted proteins studied are stable in the medium. To date, 24 of these plasma proteins have been distinguished and quantified (Fig. 1). Among them are most of the plasma proteins represented in the blood of the chick embryo with the exception of certain proteins, namely, the immunoglobulins, ovalbumin, and one particular fetal protein (FP-2), which are presumably synthesized in the embryo by either the lymphocytes or the yolk sac. In addition, the cultured embryonic liver cells synthesize several adult-type plasma proteins (corresponding to peaks 6, 7, and 20 in Fig. 1) which are absent in the developing embryo (18) and which appear in the blood of the chick only after hatching (G. Grieninger, unpublished observations). Apparently these embryonic hepatocytes differentiate in culture.

**Synthesis and Secretion.** About 80% of the plasma proteins were found to be secreted within 30 min of their synthesis. In other words, the time required for plasma protein movement along the secretory pathway is short, the overall half-time being <10 min (Fig. 4). Our findings are in excellent agreement with studies performed on intact animals (35, 36).

In the cultured chick hepatocytes, cellular levels of plasma proteins are low. The cells secrete all plasma proteins within 1 h of synthesis (Fig. 4), from which it follows that internal steady-state levels are equivalent to <5% of the daily secretion. Intracellular levels determined directly for albumin and C support this conclusion (Fig. 3).

Inasmuch as intracellular levels vary directly with the changing rates of secretion (Fig. 3), it can be concluded that the degree of secretion of a plasma protein is determined by its degree of synthesis. In our routine experiments, therefore, the rate of secretion into the medium, as determined by sequential immunoassay of accumulating plasma protein, gives a valid estimate of the rate of synthesis of that plasma protein.

**Hepatic Protein Synthesis.** The rate of total protein synthesis (including plasma proteins) in embryonic chick liver cells in culture was estimated to be approximately 1 mg/mg cell protein per day. This is in excellent agreement with the values derived for total hepatic protein synthesis in the intact chicken (M. Gruber, personal communication), rat (1), and mouse (37). The daily rate at which the liver cell synthesizes the equivalent of its own protein content seems to represent an upper limit of the rate of protein synthesis in the liver. For
example, injection of a chicken with estradiol causes the liver to produce large quantities of the egg yolk precursor protein, vitellogenin, comprising up to 10% of total protein synthesized (38); but total protein synthesis remains constant (38, 39). These observations suggest that with respect to protein synthesis the liver is working at full capacity and that the rate of protein synthesis is limited by the amount of protein synthetic machinery. This is further supported by the observation that most liver ribosomes are active (37).

In light of the evidence that the liver cells in culture do not grow, all of the protein synthesized must be exported or turned over. In experiments reported here, exported proteins were estimated to be 0.2 mg plasma proteins/mg of cell protein per day as well as 20% of total protein synthesis. It follows that in culture 80% of the cellular protein is replaced per day. Similar replacement rates have been calculated for the intact liver (40, 41) using data obtained from the rat and mouse.

**Albumin synthesis.** Albumin is the major plasma protein synthesized early in culture. During the 1st day, albumin comprises nearly 50% of total plasma protein synthesis, i.e. 10% of hepatic protein synthesis in culture. These values completely confirm in vivo data from other systems: albumin comprised about half of total plasma protein synthesis in the intact rat (1) and 10% of hepatic protein synthesis in the chicken (37).

The decline in albumin secretion in culture was accompanied by falling intracellular levels (Fig. 3). With immunofluorescent staining it could be shown that the albumin level decreases uniformly in all cells. To explain the declining internal levels, protein degradation during intracellular transport, specific for albumin, was considered. However, two lysosomotropic drugs, chloroquine and neutral red, which inhibit intracellular degradation of protein (42), did not stimulate albumin secretion. In addition, no radiolabel in cellular albumin was detectable after a short pulse at 72 h (G. Grieninger, unpublished observations), at a time when albumin secretion has virtually ceased, hence we conclude that the decreasing rate of secretion reflects a true decrease in albumin synthesis.

When viewed against the simultaneous increased synthesis of other plasma proteins, particularly the induction of adult-type plasma proteins, the dwindling rate of albumin synthesis cannot be attributed to a general dedifferentiation of the liver cell's ability to synthesize plasma proteins. Furthermore, it is unlikely that the change observed in the spectrum of plasma proteins synthesized is due to the appearance of new cell populations selected by the culturing process because we have shown that the cells do not proliferate in culture.

The specific decrease of albumin synthesis is apparently introduced by the culturing process and is not related to embryonic development because it is also observed with liver cell cultures obtained from either 10- or 19-day-old chick embryos (G. Grieninger, unpublished observations). This "phenotypic change" (43) should, therefore, be reversible or preventable with appropriate modification of the culture conditions. Recent experiments have confirmed this hypothesis, demonstrating continued albumin synthesis when cell cultures are initiated and maintained in a chemically defined medium in the absence of fetal bovine serum (44). We are currently exploring the nature of this apparent suppression of albumin synthesis.
We observed that for 72 h the rate of total plasma protein synthesis remains constant in culture despite drastic changes occurring in the synthesis of individual plasma proteins during this time. Under the described culture conditions, the enhanced synthesis of several plasma proteins, such as C and M, occurs most strikingly during the first 2 days of culture, just when the most dramatic decrease of albumin synthesis is observed (Fig. 1), suggesting a possible relationship between these events: increased synthesis of other plasma proteins seems to compensate quantitatively for declining albumin synthesis. Based on the evidence presented and indications that each hepatocyte has the capacity for synthesizing the entire spectrum of plasma proteins, we interpret our results in the following way: in culture, the hepatocyte changes its program for the synthesis of specific plasma proteins while continuing the overall production of plasma proteins at full capacity. The molecular basis for this change of program will be addressed in a subsequent paper.

Summary

A simple model system is described for studying synthesis of plasma proteins. The system is based on chick embryo hepatocytes in primary monolayer culture which synthesize a broad spectrum of plasma proteins and secrete them into the culture medium. The secreted proteins are stable and consist almost exclusively of plasma proteins. The cultured cells are nonproliferating hepatic parenchymal cells whose cell mass remains constant in culture. By a modification of Laurell's rocket immunoelectrophoresis, the secreted plasma proteins can be detected in nanogram amounts in 3 μl of unconcentrated culture medium. Kinetics of secretion are obtained by sequential assay of proteins accumulating in the medium.

In this system it is demonstrated that: (a) intracellular plasma protein levels are equivalent to <5% of the daily secretion; (b) synthesis and secretion are continuous; and (c) the overall half-time for plasma protein movement along the secretory pathway is <10 min. From these results, it follows that the rate at which the plasma proteins are secreted gives a valid estimate of their rate of synthesis. This feature of the culture and the sensitivity of the assay allow routine measurements of plasma protein synthesis without disruption of the cells and without the use of radioisotopes. It is shown, furthermore, that the overall rate of plasma protein synthesis in cultured hepatocytes is constant over a 3-day period and is similar to that of the intact liver. 3,000,000 cells, containing 1 mg cell protein, synthesize 0.2 mg of plasma proteins daily, amounting to one-fifth of hepatocellular protein synthesis. Under the conditions used, albumin synthesis steadily decreases with culture time whereas the synthesis of many other plasma proteins increases. The observed phenotypic changes and reorganization of plasma protein synthesis illustrate how the system may be exploited for studying the regulatory processes governing plasma protein synthesis.

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