

PLASMA PROTEIN SYNTHESIS BY ISOLATED RAT HEPATOCYTES

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ABSTRACT

A system of preparation of rat hepatocytes with extended viability has been developed to study the role of hormones and other plasma components upon secretory protein synthesis. Hepatocytes maintained in minimal essential medium reduce the levels of all amino acids in the medium except the slowly catabolized amino acids leucine, isoleucine, and valine, which steadily increase as the result of catabolism of liver protein. Although the liver cells catabolize 10–15% of their own protein during a 20-h incubation, the cells continue to secrete protein in a linear fashion throughout the period. The effects of insulin, cortisol, and epinephrine on general protein synthesis, and specifically on fibrinogen and albumin synthesis, have been tested on cells from both normal rats and adrenalectomized rats. Cells from normal animals show preinduction of tyrosine amino transferase (TAT), having at the time of isolation a high level of enzyme which shows only an increase of approximately 60% upon incubation with cortisol. In contrast, cells from adrenalectomized animals initially have a low level of enzyme which increases fourfold over a period of 9 h. The effects of both epinephrine and cortisol on protein synthesis are also much larger in cells from adrenalectomized animals. After a delay of several hours, cortisol increases fibrinogen synthesis sharply, so that at the end of the 20-h incubation, cells treated with hormone have secreted nearly 2.5 times as much fibrinogen as control cells. The effect is specific; cortisol stimulates neither albumin secretion nor intracellular protein synthesis. The combination of cortisol and epinephrine strongly depresses albumin synthesis in both types of cells. Insulin enhances albumin and general protein synthesis but has little effect on fibrinogen synthesis.

One of the specialized functions of the liver is the synthesis and secretion of a large number of plasma proteins, including some of those involved in the coagulation process (37). The plasma levels of several of these proteins seem to be tightly regulated (3, 5). Control of synthesis of the proteins involved in coagulation offers a unique problem to the organism; that is, the location and activity of these proteins are entirely remote from their site of synthesis and from at least one of their sites of degradation (13, 43).

Fibrinogen, a relatively stable protein, is one of

the best characterized proteins in the coagulation system (6, 14, 31, and references therein). It is synthesized at a sufficient rate to be readily detectable (25), and its synthesis is controlled in an inducible fashion (43, 44). Albumin synthesis by the liver has been studied extensively and is reasonably well characterized (40, 42, and references therein). Although the synthesis of albumin is less sensitive to many specific physiological mediators than is fibrinogen synthesis, the synthesis of albumin is affected to a greater extent by general changes in the cellular environment (25, 41, 50).

We have previously reported the synthesis and secretion of fibrinogen and albumin (10), and also of transferrin (9), by isolated rat hepatocytes maintained in suspension for longer than 20 h. We now report a more thorough investigation of the cell preparation throughout the entire incubation, including the effects of several hormones on the synthesis of fibrinogen and albumin as well as on general protein synthesis. Two of these hormones, epinephrine and cortisol, are of particular interest because of the implication that these hormones play a role in the hyperfibrinogenemic response that occurs in some physiological stress conditions (21, 34, 35). Abolition of this response has been reported in adrenalectomized rats after surgical trauma (21) or the injection of Celite (34). Direct injection of either epinephrine or cortisol elicits hyperfibrinogenemia (2, 24, 35). Even the mild stress accompanying saline injection has been reported to be sufficient to trigger the response (39). These responses appear also in perfused livers, either in livers from normal animals stimulated by talc injection (17) or in livers perfused by hormone mixtures containing cortisol (20).

Because of the complexities involved in analyzing such hormonal responses *in vivo*, we have undertaken to define a system of isolated cells in suspension that reproducibly respond to hormones in the regulation of plasma protein synthesis in a manner resembling that seen in the intact animals. It is the purpose of this paper to describe the preparation and general characteristics of the system of cells we have developed as well as to present the response patterns of these cells to the hormones insulin, epinephrine, and cortisol.

MATERIALS AND METHODS

Animals

Male rats (200–300 g) of the Long-Evans and Sprague-Dawley strains fed *ad lib.* on Purina Rat Chow (Ralston Purina Co., St. Louis, Mo.) have been used as liver donors. Adrenalectomized Sprague-Dawley rats were maintained at 28°C on normal saline drinking water containing tetracycline.

Liver Cell Preparation

Our technique for preparing liver cells, adapted from the collagenase perfusion method of Berry and Friend (4), consists of cannulating and exsanguinating the liver *in vivo* and perfusing the liver with collagenase in a recirculating system. The rat is anesthetized by inhalation of Penthrane (Abbott Laboratories, N. Chicago, Ill.). The abdominal cavity is opened, the inferior vena cava ligated, and the superior vena cava cannulated via the

atrium. The portal vein is severed, and perfusion is begun (in the direction counter to blood flow) with a modified, calcium-free Krebs-Ringer bicarbonate buffer (12) containing 3 mg/100 ml heparin, 7 mg/100 ml streptomycin sulfate, 10,000 U/100 ml penicillin G, and 2% dialyzed bovine serum albumin. The buffer is oxygenated and its pH controlled at 7.4 by passage through a perfusion lung in an O₂-CO₂ atmosphere.

During the one-way perfusion maintained at 30 ml/min, the liver is excised and transferred to a dish thermostated at 37°C. 20 mg Boehringer collagenase grade II (C. F. Boehringer and Sons, Mannheim, Germany) or 50 mg Worthington collagenase type IV (Worthington Biochemical Corp., Freehold, N. J.) and 5 mg soy trypsin inhibitor (previously dialyzed for several hours vs. 0.15 M NaCl) is added to the perfusion medium (total volume, 100 ml) now being recirculated, and perfusion is continued for 30–45 min or until the liver begins to visibly disintegrate. The liver is then gently minced in 100 ml cold Joklik-modified minimal essential medium (Gibco cat. no. F-13; Grand Island Biological Co., Grand Island, N.Y.) containing 10% fetal calf serum, and passed through a 100-mesh silk screen supported in a powder funnel.

The cell suspension is centrifuged at approximately 50 g for 3 min at 4°C, the cells are suspended in fresh medium, and the washing process is repeated twice. The cells are then suspended for incubation at a density of 2–3 × 10⁶ cells/ml. The liver of a 300-g rat yields about 10⁸ cells, 80–95% of which exclude 0.5% trypan blue at all stages of the procedure.

To minimize microbial contamination, the equipment, perfusion apparatus, and salt solutions are sterilized by heat or UV irradiation; exposure of the liver to the atmosphere is limited; and the media and collagenase solutions are Millipore filtered (Millipore Corp., Bedford, Mass.).

Incubation Conditions

6–14 × 10⁶ cells in 4 ml of normal medium (0.1 mg/ml heparin, 0.1 mg/ml streptomycin, and 0.05 mg/ml penicillin G in F-13 medium containing 10% fetal calf serum) were incubated at 37°C in 25-ml polypropylene Erlenmeyer flasks while kept in suspension by gentle gyratory shaking in a 95% O₂-5% CO₂ atmosphere sufficient to maintain the pH of the medium at 7.3–7.4. At each assay time, a flask was withdrawn, the cell viability determined by trypan blue exclusion; the intact cells were pelleted at low speed and reserved for assay.

Methods of Assay

The assay for tyrosine amino transferase (TAT) activity was performed by the method of Granner and Tomkins (19) on cells sonicated or treated with 0.5% deoxycholate (DOC) and 0.5% Triton-X-100 (TX-100) in 0.1 M potassium phosphate buffer, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol (DTT), and 5 mg/ml bovine albumin. Both methods of solubilization gave comparable enzyme

activity measurements. Total cellular protein was determined by the method of Lowry et al. (33) on TCA-precipitated cellular protein.

Total protein synthesis was measured by the incorporation of [^3H]leucine or [^3H]valine into material precipitable by hot TCA. The labeled amino acid was added to a concentration of 1 $\mu\text{Ci/ml}$, giving an initial specific activity of about 1 cpm/pmol. The change in the specific activity of [^3H]leucine in the medium during the incubation was monitored by amino acid analysis on aliquots of medium deproteinized with 10% TCA. [^{14}C]Leucine was added to this solution as an internal standard, and the $^3\text{H}/^{14}\text{C}$ ratio measured. The major tritium-containing metabolites of leucine are volatile in weak acid (D. L. Miller, unpublished observations); the extent of metabolism of leucine was, therefore, more conveniently determined by measuring the $^3\text{H}/^{14}\text{C}$ ratio before and after lyophilization of the TCA-treated supernate.

To measure amino acid incorporation into secreted protein, 50 μl of the supernatant medium was pipetted onto a TCA-soaked filter that was then dried and washed in boiling 5% TCA. Incorporation into intracellular protein was determined in the same manner after disrupting the cells with 0.5% sodium DOC and 0.5% TX-100. Before measuring their radioactivity, the precipitates were solubilized in 1 ml of 0.15 N NaOH containing 10% TX-100 and 2% sodium dodecyl sulfate, and then 10 ml of Aquasol (New England Nuclear, Boston, Mass.) was added.

The radioimmunoassay for fibrinogen using Sepharose-bound antibody and [^{14}C]methylated standard fibrinogen has been described elsewhere (11). The assay for albumin was identical to this. The antibody raised to rat albumin does not cross-react with bovine albumin.

Preparation of Purified Fibrinogen and Albumin

Standard rat fibrinogen was prepared from rat plasma by the method of Straughn and Wagner (49). Electrophoretically pure albumin was prepared from rat plasma from which fibrinogen had been removed by β -alanine precipitation. Albumin was precipitated from the subsequently dialyzed solution by 40–50% ethanol as described by Cohn et al. (8). The precipitate was dialyzed against distilled water, precipitated again in 50–70% saturated ammonium sulfate, then extracted with 60% saturated ammonium sulfate. The soluble fraction, after dialysis, was extracted with 2 g of charcoal at pH 3. The remaining preparation was homogeneous by disc gel electrophoresis.

RESULTS

Effects of Variables in the Cell

Preparation Procedure upon Cell Viability and Plasma Protein Synthesis

In this study, the cells have been assessed primarily for their capacity to synthesize and secrete

albumin and fibrinogen and to respond to hormones in a manner resembling the intact liver. They have also been examined for induction of TAT in response to cortisol, for exclusion of trypan blue, and for retention of cytoplasmic enzymes.

SELECTION OF THE PERFUSION MEDIUM: The early cell preparation methods used calcium-free Hanks' solution (4, 23), while a more recent report has favored a supplemented Krebs-Ringer solution to better preserve the metabolic functions of the cells (7). We have not detected any significant differences between the cells prepared in these two media. 2% albumin is included in the perfusion medium to supply osmotic support and to limit the destruction of the cell membranes by proteases. Considerable cell destruction has occurred with some preparations of defatted albumin, and we now use normal fraction V albumin dialyzed for 2 days vs. 0.15 M NaCl. Although fetal calf serum would seem to be a desirable osmotic support because of its beneficial effects on cell viability, we find that digestion of the liver does not occur when 10% fetal calf serum is added to the perfusion medium, presumably because of the presence of proteolytic inhibitors.

Although the perfusion medium is calcium free, a low level of this metal ion is apparently required for digestion to occur. If EGTA (0.1 mM) is included in the perfusion medium, we find that the liver remains intact.

ATTEMPTS TO ANALYZE AND IMPROVE THE DIGESTIVE ENZYME MIXTURE USED FOR DISRUPTION OF THE INTRACELLULAR MATRIX: The efficacy of the collagenase preparation in releasing single cells from the liver is apparently dependent not only upon the collagenase activity but also upon other enzyme activities. We find that collagenase purified by chromatography on Sephadex G-100 and DEAE-cellulose is ineffective in releasing liver cells. No phospholipase activity was detected in the Worthington type IV collagenase preparation analyzed (H. Weissbach, personal communication), but there are several proteases of varying substrate specificities. Although some protease activity is probably necessary to release the cells, we have been concerned that the proteases would damage cell membranes and destroy hormone receptors (27). In an attempt to eliminate the trypsin-like activity found in crude collagenase, we included soybean trypsin inhibitor in the perfusion medium. In the single test of its effectiveness, we found a substantial improvement in long-term viability.

The best procedure may be to use purified collagenase plus a suitable purified protease, as Amsterdam and Jamieson (1) have demonstrated successfully in the isolation of pancreatic exocrine cells. In anticipation of this possibility, we have incubated low concentrations of chymotrypsin (0.1 mg/ml) and elastase (10 μ g/ml) in suspensions of liver cells. Although chymotrypsin slowly rendered the cells permeable to trypan blue, elastase had no apparent effect on viability by that criterion. However, when we combined in a perfusion experiment this level with the purified collagenase described above, considerable damage to the cells resulted.

Pending development of a better digestive mixture, we have continued to screen commercially available preparations by incubating small amounts with cells to find those that permit long-term viability, hormonal responsiveness, and minimal agglutination.

RELEASING THE CELLS AFTER PERFUSION: Two methods are commonly used to separate cells after mincing the liver. The minced liver may be shaken in a collagenase solution until a sufficient number of cells have been released. Alternatively, the minced liver is gently stirred in cold medium and filtered through a silk screen without the secondary digestion. Although the additional digestion with shaking gives a higher initial percentage of cells impermeable to trypan blue (98–99%) in our hands, the total yield has been lower, and the cells synthesized protein at lower rates.¹

MAINTENANCE OF THE CELLS: The cells are maintained in suspension by gyratory agitation (80 rpm) to insure an adequate oxygen and nutrient supply. We have found that reciprocal agitation tends to break up the cells. In addition to the usual supply of nutrients found in the Gibco F-13 culture medium, we have tried several combinations of additives such as vitamins, other minerals, and additional amino acids. As is shown in Fig. 1, the only change substantially affecting the viability of the cells measured by dye exclusion was the omission of fetal calf serum from the incubation medium. We, therefore, tested sera from several

¹ The apparently higher initial viability of the cells prepared by the shaking procedure may be misleading. Cells damaged during perfusion and mincing are preferentially destroyed during the shaking procedure, leaving no indication of how much liver cell damage occurred during perfusion.

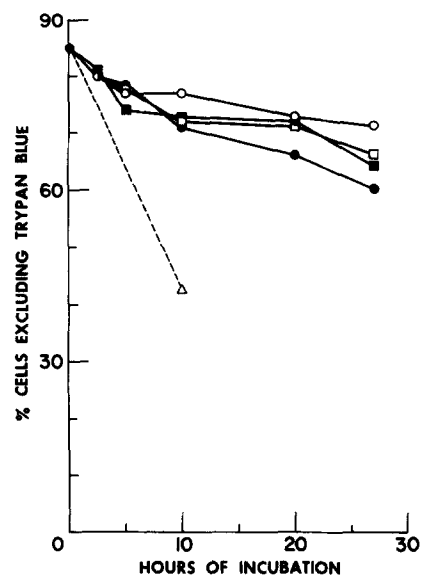


FIGURE 1 Effect of supplementation of the culture medium upon the viability of hepatocytes in suspension. Cells were prepared as described above, then suspended at a density of 2×10^6 cells/ml in Gibco F-13 culture medium supplemented, unless stated otherwise, with fetal calf serum to a final concentration of 10%. Additional supplementation was as follows: (■—■) none; (●—●) supplementary medium A (2 mM sodium pyruvate, 1.5 mM arginine HCl, 1.0 mM glutamine, 10^{-7} M CuSO_4 , 3×10^{-5} M ZnCl_2 , 3×10^{-5} M FeSO_4 , vitamins); (○) supplementary media A, + B (0.4 mM histidine, 0.4 mM tyrosine, 0.4 mM phenylalanine, 0.2 mM tryptophan); (□) supplementary media A + B, + C (0.4 mM serine, 0.4 mM asparagine, 0.4 mM proline, 0.4 mM alanine, 0.4 mM aspartic acid, 0.4 mM glycine); (△) fetal calf serum deleted from medium. Viability was measured as percent cells excluding 0.5% trypan blue as determined by light microscope examination.

different species to find the best source in terms of maintaining viability and supporting protein synthesis. At the level of 10% supplementation, fetal calf, chicken, rabbit, and horse serum (all Gibco) were indistinguishable in maintaining (a) the viability of the cells, (b) their levels of general protein synthesis, and (c) fibrinogen synthesis. Albumin synthesis was not measured in these experiments. We did not find that any of the several lots of fetal calf serum were toxic to the cells, contrary to the experience of Jeejeebhoy et al. (26).

Induction of TAT

Originally, our intent was to use this determination as yet another indicator of cellular viability

and functionality, having observed with several preparations that the ability of cells to secrete protein and the ability to respond to hormones was not necessarily retained or lost simultaneously. We, therefore, intended to monitor the induction of TAT by cortisol as a measure of hormonal responsiveness not dependent upon retention of an intact protein secretory apparatus. Fig. 2 shows the profile of TAT activity found in cells from normal animals. The apparent lack of inducibility and the high initial level of the enzyme in cells from normal rats led us to the tentative conclusion that our cells were responding to previous exposure to hormone before preparation of the suspension. Although there is an increase in enzyme activity in the cells as a function of time in response to the addition of cortisol succinate to the cell suspension, the initial specific activity ($0.046 \text{ U/mg protein}$)² is much higher than would be expected for uninduced cells. (Lin and Knox [32] have reported a specific activity of $0.016 \text{ U/mg protein}$ in livers from normal rats). Cells prepared from animals adrenalectomized 14 days before the cell preparation show an induction profile (Fig. 2) more like that seen in intact animals; the initial level is very low, and the stimulation is approximately fourfold over a 9-h period (18, 28). If the cortisol is added to the cell suspension after 10 h of incubation, the enzyme is once again induced and increases in a similar fashion, although to a lesser extent than if induced initially.

Metabolism of Amino Acids

The liver cells deplete the medium of a large fraction of the amino acids initially available during a 24-h incubation (Fig. 3). Isoleucine, leucine, and valine are exceptions to this general pattern, and their concentrations increase throughout the incubation (Fig. 4). This behavior indicates that liver protein is being degraded faster than it is being synthesized and that the slowly catabolized amino acids accumulate. Similar behavior has been observed in the perfused liver. When plasma levels of amino acids are included in the perfusate, the aliphatic amino acids are released by the liver at rates similar to those observed in the present study (53). Degradation seems to be independent of protein synthesis, for the increase in aliphatic amino acids occurs also in the presence of suffi-

² This value was obtained by dividing the value shown in Fig. 2 by the cellular protein content ($2.2 \text{ mg}/10^6 \text{ cells}$).

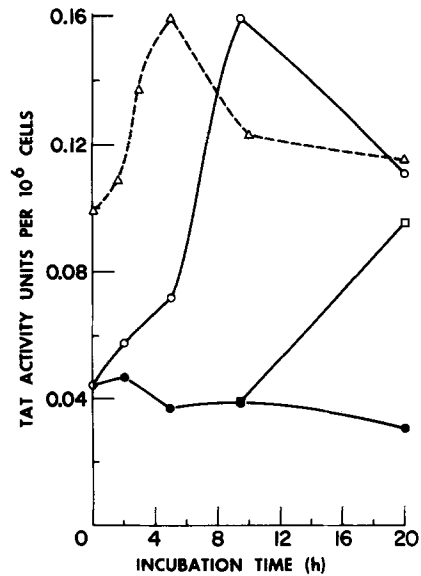


FIGURE 2 Induction of TAT synthesis by hydrocortisone-21-sodium succinate. Hepatocytes prepared as described from either normal or adrenalectomized rats were incubated at a density of $2 \times 10^6 \text{ cells/ml}$ in the presence or absence of $7.8 \times 10^{-8} \text{ M}$ hydrocortisone-21-sodium succinate as indicated below: (●) adrenalectomized rat, no glucocorticoid added; (○) adrenalectomized rat, glucocorticoid added at zero time; (□) adrenalectomized rat, glucocorticoid added after 9.5 h incubation; (△) normal rat, glucocorticoid added at zero time. 1 U is defined as $1 \mu\text{mol}$ hydroxyphenyl pyruvate produced/min at 37°C .

cient cycloheximide to inhibit protein synthesis by 95% (Table I). This is in agreement with previous observations that protein turnover is generally independent of protein synthesis (15, 46).

Also of interest in the pattern of amino acid utilization by the cell suspension are the very rapid loss of arginine from the medium and the large apparent increase in lysine (Fig. 4). It is our supposition that this unusual pattern is caused by arginase leaking into the medium from initially damaged cells. Arginine is converted rapidly to urea and ornithine by arginase; ornithine emerges with lysine on our amino acid analyzer. There is sufficient arginase activity in liver cells (45) to hydrolyze all of the arginine in the medium within seconds if the enzyme were exposed to its substrate. In light of this consideration, it is not surprising that all of the arginine disappears from the medium within 2 h.

Leucine is catabolized very slowly by the liver cells; nevertheless, there is a considerable amount

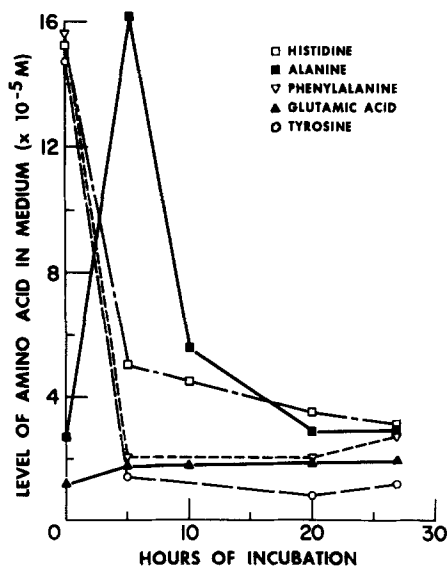


FIGURE 3 Utilization of amino acids by the hepatocyte suspension. Aliquots of the incubation medium were treated with 10% TCA to remove protein, then analyzed on a Jeol amino acid analyzer (courtesy of Dr. C. Y. Lai, Institute of Molecular Biology, Roche, Nutley, N. J.).

of conversion in 20 h. The extent of catabolism of leucine and the specific activity of the remaining [³H]leucine are shown in Fig. 5.

Protein Secretion

When the data on the incorporation of [³H]leucine into secreted protein is corrected for the changes in [³H]leucine specific activity, the rate of incorporation is found to be nearly constant during the 24-h incubation (Fig. 6). This calculation assumes that the leucine incorporated into protein is in equilibrium with leucine in the medium, an assumption supported by studies showing the equilibration of amino-acyl tRNA with extracellular amino acids (29, 51).

In view of the fact that protein synthesis and secretion continue to be linear throughout the incubation, it is of interest to examine the extent of intracellular proteolysis that occurs without disrupting the protein secretion process. Subtracting the original amount of leucine in the medium from the sum of the leucine in the medium at the end of the incubation, the leucine catabolized, and the leucine incorporated into protein gives the amount of leucine released by the cells and, therefore, an indication of the amount of intracellular proteolysis. It has been reported that the liver protein

contains 12% leucine by weight (47). By the Lowry method, we find that the cells contain 2.2 mg protein/10⁶ cells, in agreement with the data of Howard and Pesch (22). From these data, it appears that the cells can degrade 10–15% of their intracellular protein without substantially affecting protein secretion.

Intracellular Protein Synthesis

In contrast to the constant rate of synthesis of secreted protein, the rate of incorporation of [³H]leucine into intracellular protein is maximal

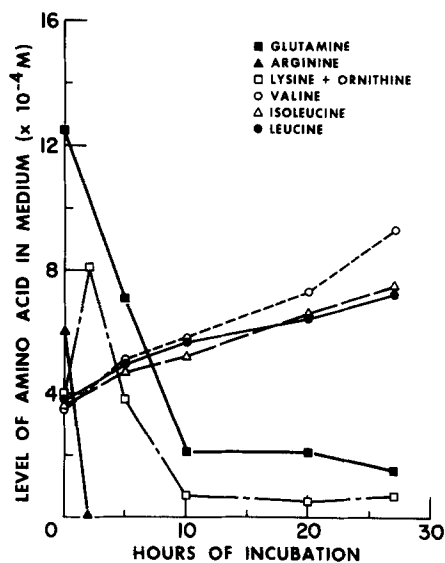


FIGURE 4 Utilization of amino acids by the hepatocyte suspension. (○—○) valine; (●—●) leucine; (△—△) isoleucine; (■—■) glutamine; (□—□) lysine + ornithine; (▲—▲) arginine.

TABLE I

Lack of Effect of Cycloheximide on Accumulation of Slowly Catabolized Amino Acids in Incubation Medium

Amino acid	Concentration of amino acid in medium ($\times 10^{-4}$ M)		
	Level of cycloheximide	0	0.1 mM
Leucine	9.58	8.23
Valine	11.2	8.01
Isoleucine	8.42	7.09

Cells were incubated for 20 h in the presence or absence of cycloheximide. The medium was then separated from the cells by centrifugation at 50 g for 4 min and analyzed for amino acids as described in Fig. 3.

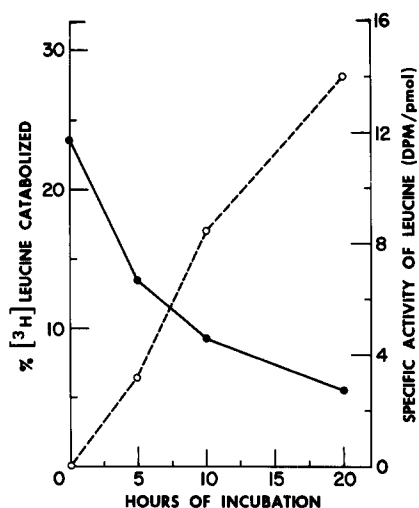


FIGURE 5 Catabolism and change in specific activity of [³H]leucine during incubation. 2 μ Ci of L-[4,5-³H]-leucine was incubated in 4 ml of F-13 medium containing 8×10^6 hepatocytes. The extent of catabolism of [³H]leucine was determined by measuring the fraction of radioactivity lyophilized from the acidified medium. Specific activities were calculated from leucine concentrations determined by amino acid analysis. (●) specific activity of leucine, DPM/pmol; (○) % [³H]leucine catabolized.

during the 1st h, then decreases throughout the remainder of the 24-h incubation (Fig. 7). We have not analyzed the incorporation pattern in detail; however, it is undoubtedly a composite of several processes. Intracellular proteins are synthesized and degraded at varying rates, and, in the steady state, the proteins most rapidly synthesized must also be most rapidly degraded. Thus, proteins that turn over rapidly tend to equilibrate rapidly with the leucine in the medium, and the total amount of [³H]leucine incorporated into them will at some time begin to decrease. The more slowly degraded proteins will continue to accumulate [³H]leucine but at a decreasing rate as the specific activity of the amino acid decreases. Furthermore, it is possible that the rate of intracellular protein synthesis is elevated initially because of cellular repair processes which replace proteins destroyed during the perfusion. It may be relevant that, whereas cells in normal medium tend to aggregate after 10 h of incubation, we find that cells treated with cycloheximide do not. It is possible that membrane proteins required for cell adhesion are being replaced in the first hours of incubation. This possibility is strengthened by the fact

that cells incubated in the presence of low levels of collagenase or elastase also do not aggregate although they survive and secrete fibrinogen quite well. In addition, M. Ernest (private communication) has found that actinomycin D also inhibits the aggregation process.

The Synthesis and Secretion of Albumin and Fibrinogen

Although considerable variation in the synthesis rates as a function of time occurs among different cell preparations, the average rate for both proteins is relatively constant throughout the 20-h incubation period, as is shown in Fig. 8. The initial high rate of albumin secretion reflects the secretion of albumin from the intracellular pool, as well as newly synthesized albumin.

As can be seen in Fig. 9, at zero time the cells contain approximately 88 pmol albumin/ 10^6 cells,

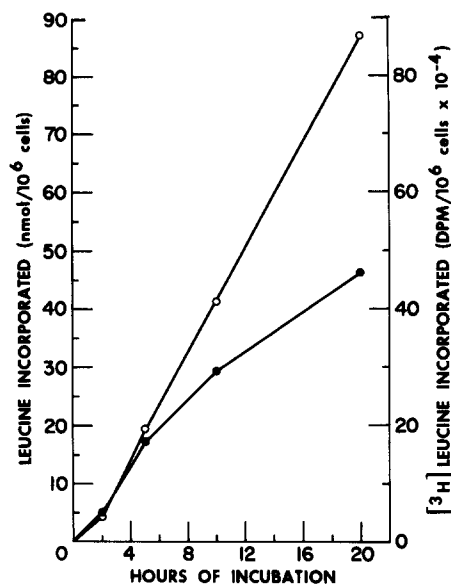


FIGURE 6 Secretion of newly synthesized protein by hepatocytes. Cells were incubated in the absence of hormones. At the indicated times, samples were withdrawn, and aliquots of the incubation medium were treated with TCA, and the radioactivity of the precipitate was determined as described in the text. To determine the amount of leucine incorporated into protein at each time-point, the specific activity of leucine was corrected for the increase in the amount of leucine in the medium due to catabolism of liver protein. (●) radioactivity as dpm incorporated into TCA-precipitable protein; (○) amount of leucine incorporated into TCA-precipitable protein (nmol/ 10^6 cells).

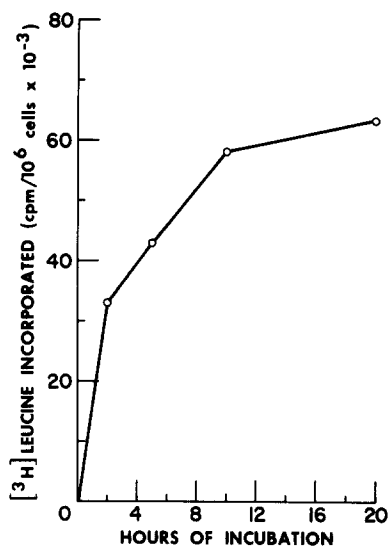


FIGURE 7 Incorporation of [³H]leucine into intracellular protein by hepatocytes. Cells were incubated as described in Fig. 6. At the indicated times, cells were separated from the medium by centrifugation at 50 g for 4 min, then solubilized, and the amount of [³H]leucine incorporated into protein was determined as described in the text. The curve shown is not corrected for changes in the specific activity of leucine due to protein catabolism.

or 40 pmol/mg cellular protein. This is exactly the amount of albumin found by Peters and Peters (42) in the particulate fraction from normal rat liver. They found negligible amounts of albumin in the soluble fraction. Unlike the intact animal, however, the cells lose their intracellular albumin as the incubation progresses (Fig. 9).

The rate of fibrinogen secretion is linear over the incubation period. A very small pool of fibrinogen was found in the isolated cells, and no accumulation of fibrinogen occurred inside the cells during incubation (a constant level of approximately 3 pmol fibrinogen/10⁶ cells was found throughout the incubation period). No pool of intracellular fibrinogen has been reported in the intact animal, so it seems reasonable to conclude that in both systems synthesis is very tightly coupled to secretion. It should be emphasized that these are the synthesis rates in the absence of any added hormones. In our early experiments (10), we observed an increase in the rate of fibrinogen synthesis after about 5 h of incubation and a further increase between 17 and 24 h. We felt at the time that the late increase might reflect a specific response of the cell to starvation, but further experiments, to be discussed presently, have led us

to conclude that the increase in synthesis rate was due to the stimulatory effect of cortisol.

Although the total amount of fibrinogen and albumin secreted into the medium increases linearly as the incubation progresses, because of the decrease in specific activity of the proteins, it seemed possible that extensive degradation had occurred. To test this, we incubated [¹⁴C]methylated fibrinogen and albumin for 24 h in the cell suspension and examined the extent of adsorption of labeled proteins to the Sepharose-bound antibodies. We observed no decrease in the amount of fibrinogen or albumin adsorbed to the antibody. If degradation occurs at all, it does not affect the results in this assay system.

Effect of Hormones on General Protein Synthesis and on the Synthesis and Secretion of Albumin and Fibrinogen

A study of hormonal control of plasma protein synthesis in isolated cells permits one to eliminate systemic effects, competing or mediating effects of other hormones, and animal-animal variations since controls can be run on cells from the same

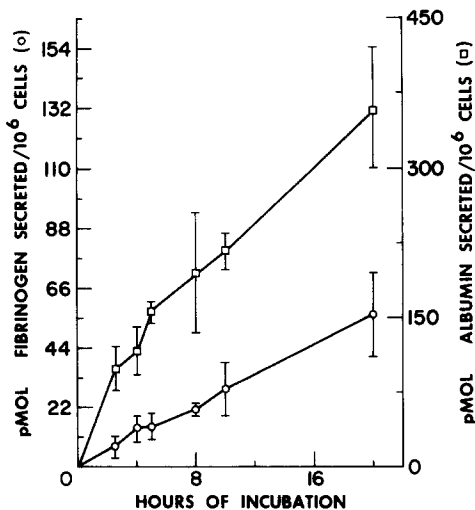


FIGURE 8 Secretion of fibrinogen and albumin by isolated rat hepatocytes. Cells were incubated at a density of 2×10^6 cells/ml. At the indicated times, samples were withdrawn, and aliquots of the medium frozen until assayed. Both proteins were quantitatively determined by triplicate solid-phase radioimmunoassays as described in the text. Each point on the curves is an average of cell preparations from seven different rats. The vertical line through each point represents the standard deviation for that time period.

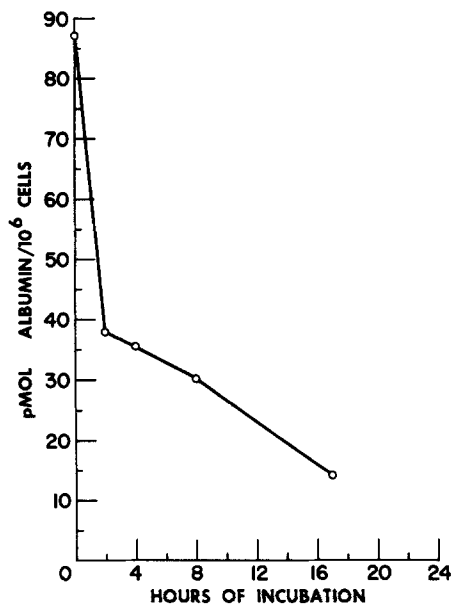


FIGURE 9 Amount of albumin remaining inside the cells. Cells were incubated in the absence of hormones, and samples were removed at the indicated times. Cells were separated from the medium by centrifugation and frozen until assayed. Before assay, cells were disrupted by solubilization in 0.05 M potassium phosphate pH 7.2, containing 0.3 M KCl, 2 mM EDTA, 0.5% TX-100, and 0.5% sodium DOC. The resulting suspension was centrifuged for 15 min at 10,000 g, and the supernates were assayed for albumin by the usual method. For these assays, the standard was also assayed with an appropriate amount of detergent added.

preparation. We have tested the response of isolated cells to the individual hormones insulin, cortisol, epinephrine, and to a mixture of cortisol and epinephrine. We have measured the effects of these hormones on protein synthesis and secretion as well as their specific effects on albumin and fibrinogen synthesis and secretion.

EFFECT OF INSULIN

In addition to its effects on carbohydrate metabolism, insulin stimulates general protein synthesis (54). Present evidence indicates that its effect occurs at the translational level of synthesis in muscle ribosomes (55), though the mechanism of action remains to be established. We have found that synthesis of both intracellular and secreted protein is rapidly stimulated in isolated cells incubated in the presence of insulin (Fig. 10). Wagle has reported (52) enhancement of [^{14}C]leucine incorporation into total protein in the presence of insu-

lin in a system of isolated liver cells, and finds the magnitude of the enhancement to be a function of the amount of glycogen remaining in the hepatocytes after the preparation process.

The synthesis of albumin is rapidly enhanced, as Fig. 10 shows, as is the synthesis of transferrin (9). Fibrinogen synthesis, however, is not significantly affected. The selectivity of enhancement is somewhat surprising, considering that the proposed site of insulin action in muscle is at the ribosome (56), but is in agreement with the work of Jeejeebhoy et al. (26), who added insulin in a mixture of other hormones to a suspension of isolated hepatocytes and observed no stimulation of fibrinogen synthe-

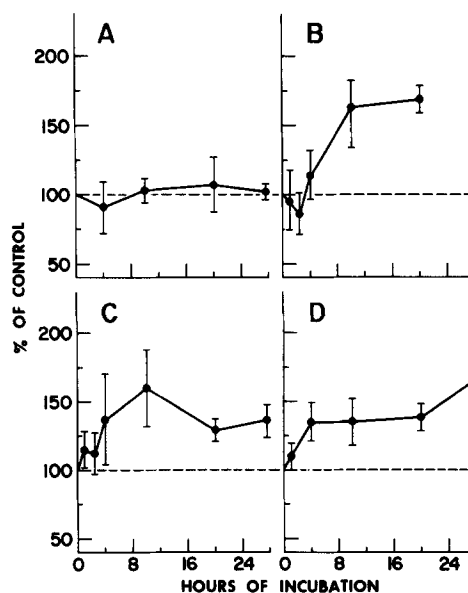


FIGURE 10 Effect of insulin on synthesis and secretion of protein by isolated hepatocytes. Liver cells isolated from normal rats were incubated with or without insulin (0.0002 U) for periods up to 24 h. At the indicated times, the cells were sedimented, and the amounts of fibrinogen and albumin in the medium were determined by radioimmunoassays. Protein secretion and intracellular protein were determined by measuring the amounts of [^3H]leucine incorporated into protein in the medium or in the cells. The results are expressed as ratios of the amount of synthesis observed in the presence of the hormone to the amount observed in the absence of the hormone. (A) secretion of fibrinogen; (B) secretion of albumin; (C) extracellular protein; (D) intracellular protein. In Figs. 10-13, the results for normal cells are averages of experiments on cells isolated from the livers of seven animals. The vertical lines indicate the maximum range in hormonal response observed.

sis. The site of action of insulin in the stimulation of protein synthesis in the liver may be somewhat different from that in muscle.

EFFECT OF CORTISOL

The effects of corticosteroids on plasma protein synthesis have been studied both *in vivo* and in the isolated, perfused liver, with somewhat conflicting results. Miller and John (38), found that, in their perfusion system corticosteroids were necessary for optimum synthesis of plasma proteins; however, others have demonstrated that cortisol depresses albumin synthesis (16).

CELLS FROM NORMAL RATS: In our original attempts to determine the effects of cortisol on protein synthesis in isolated hepatocytes, we used cells from normal rats. If the *in vitro* response of the cells to cortisol were to mirror the synthetic response found *in vivo*, we would expect to see a gradual stimulation of fibrinogen synthesis (24). The pattern we observed (Fig. 11 A) was a sharp early depression of synthesis followed by a gradual increase to slightly above control values. The response was highly variable, however, in the intermediate hours of incubation. Albumin synthesis (Fig. 11 B) was much less variable, being somewhat depressed throughout the incubation. Intracellular protein synthesis (Fig. 11 D) was first slightly depressed and then slightly stimulated during the incubation with cortisol, mirroring somewhat the complex patterns found by Kim and Kim (30) upon administration of glucocorticoid to the intact animal.

The variability of the data, especially the pattern of fibrinogen synthesis, was of some concern. Considering the apparent preinduction of TAT in normal cells (Fig. 2), it seemed possible that previous exposure to hormone was affecting the cellular secretion pattern. Kim and Kim have reported (30) that the response of the liver *in vivo* to glucocorticoids in terms of protein synthesis is complex and varies with time and extent of hormone dosage.

CELLS FROM ADRENALECTOMIZED RATS: The response pattern of cells from adrenalectomized rats was much less variable (Fig. 11). The major effect of cortisol on protein synthesis by cells from adrenalectomized animals was to dramatically enhance fibrinogen synthesis. After a lag period of several hours, fibrinogen synthesis in hormone-treated cells begins to exceed that in control cells so that by the end of the 20-h incubation period the hormone-treated cells have se-

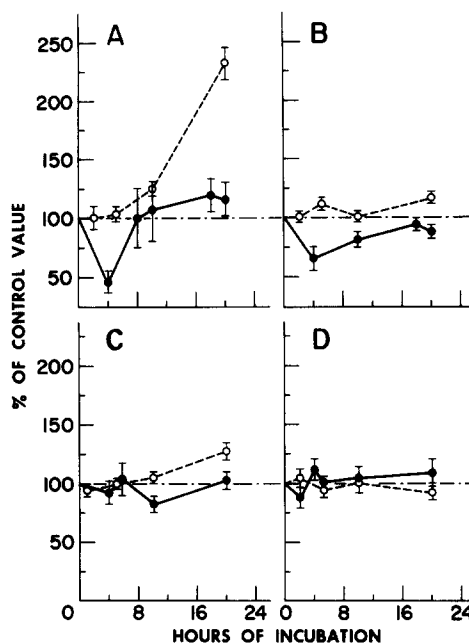


FIGURE 11 Effect of hydrocortisone-21-sodium succinate on protein synthesis and secretion. Liver cells isolated from either normal or adrenalectomized rats were incubated with or without hydrocortisone-21-sodium succinate (7.8×10^{-5} M) for periods up to 20 h. Samples were measured and results were expressed as in Fig. 10. (●) cells from normal rats; (○) cells from adrenalectomized rats. (A) fibrinogen secretion; (B) albumin secretion; (C) extracellular protein; (D) intracellular protein. In Figs. 11-13, the results for cells from adrenalectomized animals are averages of experiments on four animals. The vertical lines represent the maximum range in response observed.

creted nearly 2.5 times the amount of fibrinogen produced by control cells (Fig. 11 A). This secretion pattern resembles the response observed *in vivo* with intact animals (24). The fact that we observe similar results in cells from adrenalectomized animals indicates that cortisol stimulates fibrinogen synthesis by direct action upon parenchymal cells.

Intracellular protein synthesis (Fig. 11 D) was not markedly altered by incubation with cortisol, and the early depression and stimulation seen in normal cells was not observed in cells from adrenalectomized rats. Nor was albumin synthesis strongly affected by cortisol, although the hormone significantly depressed synthesis of albumin in cells from normal rats. It is difficult to compare these albumin synthesis rates with those obtained

in studies done in the intact animal because of the sensitivity of albumin synthesis to stimulation or depression as a result of amino acid supply. Since one of the functions of cortisol is the mobilization of amino acids from body tissue, one might expect a delayed enhancement of albumin synthesis in the intact animal, and this has been reported (25). Such a secondary effect is not possible in an isolated cell system incubated in a fixed medium.

EFFECT OF EPINEPHRINE

With adrenalin (Fig. 12) the responses of cells from normal rats were again highly variable. The only reproducible response obtained was depression of all forms of protein synthesis in the first 4 h of incubation. As the incubation progressed, general protein synthesis approached that observed in normal cells. Fibrinogen and albumin syntheses are so variable that the results in the later hours of incubation are inconclusive.

The effect of epinephrine on cells from adrenal-

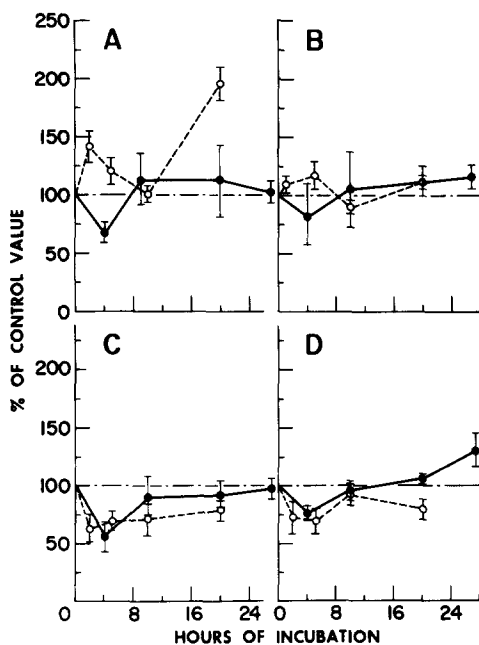


FIGURE 12 Effect of epinephrine on protein synthesis and secretion. Liver cells isolated from either normal or adrenalectomized rats were incubated with or without 2.5×10^{-4} M epinephrine bitartrate for periods up to 27 h. Samples were measured and results were expressed as in Fig. 10. (●) cells from normal rats; (○) cells from adrenalectomized rats. (A) fibrinogen secretion; (B) albumin secretion; (C) extracellular protein; (D) intracellular protein.

ectomized rats was somewhat different. Although general protein synthesis was again immediately depressed and continued to be depressed throughout the incubation, albumin and fibrinogen syntheses were clearly seen to be enhanced. Within 2 h of exposure of the cells to epinephrine, almost 1.5 times as much fibrinogen had appeared in the medium as in the system with no added hormone. This immediate enhancement steadily diminished until at 10 h no hormonal effect was apparent. Then, again, between 10 and 20 h, the hormone-treated cells secreted almost twice as much fibrinogen as control cells. An explanation for this biphasic effect is not readily available.

The enhancement of albumin synthesis, though also immediate, is slight and is again not sustained.

EFFECT OF THE COMBINATION OF CORTISOL AND EPINEPHRINE

The combination of cortisol and epinephrine affected cells from normal and adrenalectomized rats similarly for most of the parameters measured (Fig. 13). The combined hormones inhibited general protein synthesis, an effect similar to the addition of epinephrine alone. Albumin synthesis was strongly depressed; this response resembles the pattern seen for each hormone when added alone to cells from normal animals, again suggesting some hormonal influence on those cells before isolation. The depressive effect in cells from adrenalectomized rats was particularly interesting because neither hormone, when added alone to these cells, significantly depressed albumin synthesis. A cooperative effect is apparently necessary.

The effect of the combination of hormones on fibrinogen synthesis is identical for both types of cells in the delayed response but differs substantially in the early effect. Synthesis in the early hours is depressed in cells from normal animals, as is seen with each hormone alone. In contrast, early synthesis is enhanced in cells from adrenalectomized rats and follows the pattern produced with adrenalin alone. It would appear that some of the hormonal effects are cooperative, while others seem to be substantially independent.

The effects of the hormone combination are far less variable in cells from intact animals than the effect of either hormone added alone. This is true for all the parameters measured.

DISCUSSION

A number of laboratories have adopted the use of isolated hepatocyte suspensions to study specific

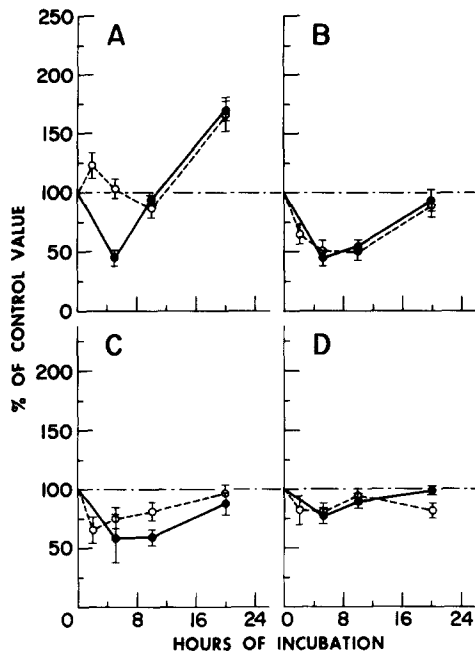


FIGURE 13 Effect of a combination of epinephrine and hydrocortisone-21-sodium succinate on protein synthesis and secretion. Liver cells isolated from either normal or adrenalectomized rats were incubated with or without 2.5×10^{-4} M epinephrine bitartrate plus 7.8×10^{-5} M hydrocortisone-21-sodium succinate for periods up to 20 h. Samples were measured and results were expressed as in Fig. 10. (●—●) cells from normal rats; (○—○) cells from adrenalectomized rats. (A) fibrinogen secretion; (B) albumin secretion; (C) extracellular protein; (D) intracellular protein.

effects of hormones on the metabolic processes of the liver. Most of these studies have been of short duration, and the functionality of the hepatocytes has been of concern for only a few hours.

Our interest is in the synthesis of plasma proteins by the liver and in the synthetic control exerted by various agents in regulating the circulating levels of some of these proteins. Many observations in vivo have shown that regulation in these processes is a fairly slow phenomenon, changes in specific protein levels in the blood taking several hours rather than several minutes to occur (25, 35).

For this reason, we have been concerned with the use of hepatocytes maintained in suspension over a period of many hours and with the necessity of knowing how the cellular environment is changing. The rapid utilization of amino acids in the medium led us to reduce the cell density from $4 \times$

10^6 cells/ml to 2×10^6 cells/ml. In some experiments, we have reduced the density still further to 1×10^6 cells/ml. Although the rate of utilization of amino acids decreased with the lower cell density, as one would expect, there was no significant change either in viability or in most of the parameters of protein synthesis. Albumin synthesis alone seems sensitive to nutritional depletion; at a cell density of 4×10^6 cells/ml, the rate of albumin synthesis frequently decreased sharply after 20 h of incubation although the rate of fibrinogen synthesis would continue undiminished (10). At the lower cell density of 2×10^6 cells/ml, a decrease in the rate of albumin synthesis was usually not observed. Replacement of the medium at 10 h by centrifuging and resuspending the cells in fresh medium also did not substantially affect their viability or rate of protein synthesis for the first 20 h.

It is interesting that protein secretion is maintained at a linear rate at 20 h under normal incubation conditions even though the level of amino acids remaining in the medium is low and the cells have catabolized a significant amount of their own protein. It would seem that protein secretion is a function maintained by the cells under conditions of considerable stress. On the other hand, we have occasionally prepared hepatocytes that appear viable and remain so, yet secrete very little protein. It is possible that some portion of the secretory apparatus is easily destroyed in the cell preparation procedure. Because protein secretion does seem to be a function maintained preferentially by the cells, the hepatocyte suspension is well suited for studying the effects of agents whose action is delayed for several hours.

It is known that in vivo injections of cortisol or epinephrine stimulate the synthesis of fibrinogen (2, 24, 31, 35, 39), and one or both of these hormones have been implicated in the stimulation caused by a variety of other agents (21, 34, 35). The major stimulation by both hormones seems to occur after a period of several hours, although the mechanism by which stimulation occurs is not known. It was of interest to see whether the same sort of synthesis pattern would occur in an isolated hepatocyte suspension, i.e., is the delay caused by a sequence of intracellular events or by the requirement for a mediator produced by some other tissue? Upon addition of cortisol to a suspension of hepatocytes, no effect at all is seen for about 5 h. Then, the rate of synthesis in hormone-treated cells begins to exceed that in control cells, but the maximum rate of synthesis is reached much later.

In contrast, the induction of TAT in the same cells achieves the maximum rate of synthesis in about 5 h.

Since cortisol is thought to induce TAT via synthesis of new messenger RNA (48, and references therein), which is a relatively slow metabolic change, it is interesting that the induction of fibrinogen by cortisol is even more delayed.

Addition of epinephrine to a suspension of hepatocytes, on the other hand, produces an immediate effect. Such an immediate increase in the output of a secreted protein, which probably requires approximately 20 min transit time in the secretion process, suggests that new message synthesis is not involved. This rapid increase is followed by a much slower but more substantial stimulation of fibrinogen synthesis. The delayed stimulation has been observed in vivo (35), but McKenzie et al. (36) failed to see any immediate increase after intravenous injection of epinephrine in dogs.

Of the several hormones tested in our system, the epinephrine effect seems to be the most easily lost. In several experiments in which the cells were not substantially viable after 24 h, no delayed enhancement of fibrinogen synthesis by epinephrine was seen even though the cells responded somewhat to cortisol. This selective lack of response to epinephrine may indicate that after 10 h the cells are no longer capable of responding, or that the membrane receptors are destroyed initially. In these experiments, the early time-points were not measured. Experiments are presently underway to resolve this question by measuring the effect of epinephrine on gluconeogenesis, an effect not dependent upon long-term viability of the cells. An additional problem is the possible presence of epinephrine in the fetal calf serum used for medium supplementation. In a single experiment, no enhancement by epinephrine was seen when the viability of the cells was normal for the 20-h incubation; the rate of fibrinogen synthesis for control cells, however, was quite high between 10 and 20 h, possibly indicating that the cells were stimulated initially.

The system of isolated cells at its present level of development as described above offers both advantages and disadvantages in the study of hormonal regulation of metabolic processes.

The system needs considerable refinement to be maximally useful. The presence of serum in the medium, especially when hormonal effects are being studied, presents uncertainty in the cellular environment. Other effects of the medium must

also be considered, e.g., amino acid depletion, as discussed above, or the possibility of bile acid accumulation if the cells are actively secreting. Whether liver cells in suspension actively secrete bile acids is still an open question. M. Bissell has found (private communication) that liver cells in primary culture will take up, conjugate, and secrete bile acids added exogenously, but no information on endogenous synthesis is yet available. If the cells are actively secreting bile acids, however, the accumulation of these acids in the medium has no apparent effect on the parameters measured in this work. When the cells are removed from the medium by centrifugation after 10 h and resuspended in fresh medium, the patterns of synthesis and secretion continue as before (L. J. Crane and D. L. Miller, unpublished observations).

Finally, if a hormone that acts by way of a membrane receptor is being investigated, disruption of membrane receptors during the cell preparation process can be a problem. These are problems in methodology, however, and should be resolvable with further investigation.

Even with the present limitations, the system seems to be a good model for the study of liver function. Each of the hormones tested affected the synthesis of the proteins of interest, particularly fibrinogen, in a manner resembling that seen in the intact animal, insofar as those effects have been characterized. These profiles provide immediate answers to considerations of other bodily processes in the individual hormonal responses. The fact that epinephrine or cortisol each produces a response resolves the question of the necessity for interaction between the two.

With this system it is now possible to ask more specific questions about the mechanisms of the hormonal responses, e.g., is the epinephrine response mediated by α - or β -receptors; is continuous exposure to hormone necessary to trigger the response; what is the lifetime of the hormones in this system; what subcellular steps take place before increased secretion of protein into the medium. These and other questions can be investigated with isolated cells.

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