In Vitro Synthesis and Secretion of Albumin by Morris Hepatomas 5123C and 7800

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ABSTRACT

Morris hepatomas 5123C and 7800 were incubated in vitro with radioactive L-leucine in Krebs-Ringer-bicarbonate-saline, and the incorporation of radioactivity into albumin precursors, serum albumin, and trichloroacetic acid-precipitable proteins was measured and compared to that which occurs in normal rat liver slices. At the end of a 10-min pulse incubation with radioactive L-leucine, Morris hepatoma 7800 incorporated 0.25% of the total protein radioactivity into an albumin fraction while Morris hepatoma 5123C incorporated 0.5% and rat liver slices showed a much larger proportion of incorporation (4.8%) into albumin. The pulse-labeled tissues were further incubated with nonradioactive L-leucine for different periods of time, and they all released radioactive proteins into the incubation medium. Only 5% of the total protein radioactivity released into the medium by Morris hepatoma 7800 and 7.8% of that released by Morris hepatoma 5123C were incorporated into albumin while, in contrast, rat liver slices released 46% of its protein radioactivity as albumin. Both hepatomas were capable of releasing 80% of the pulse-labeled albumin into the medium at the end of a 75-mm chase period. Analyses of the nascent albumin within the tissues of Morris hepatomas 7800 and 5123C and in rat liver slices showed that, at the end of a 10-min pulse incubation, the intracellular albumin fraction could be identified as a precursor of serum albumin (proalbumin) and that, as the chase incubation proceeded, proalbumin was converted intracellularly to another form of albumin which was chromatographically distinct from serum albumin. The radioactive albumin which was released into the medium, however, closely resembled serum albumin. Like normal liver, Morris hepatomas 5123C and 7800 are able to segregate nascent proalbumin in the microsomal cell fraction, and only a small amount (8%) was found in the soluble cytoplasmic fraction. Also like normal liver, a messenger RNA fraction isolated from Morris hepatoma 7800 can be translated by an in vitro protein-synthesizing system into a larger albumin precursor containing L-leucine in positions 7, 8, 9, and 10. These studies indicate that Morris hepatomas 5123C and 7800, while synthesizing smaller amounts of albumin than does normal liver are, however, capable of normal processing of precursor into serum albumin and appear to secrete serum albumin into the incubation medium.

INTRODUCTION

Albumin, in normal rat liver, is assembled on polysomes attached to the membranes of the ER and is transported in stepwise fashion to the lumen of the rough ER, the smooth ER, and the Golgi where it is concentrated and packaged within secretory vesicles. These vesicles then move to the plasmalemma at the hepatic blood front, fuse with it, and empty nascent albumin into the space of Disse (14). During this intracellular pathway, albumin is thought to undergo several modifications. For instance, recent studies indicate that, when albumin mRNA is translated by a cell-free protein-synthesizing system, the protein product contains an octadecapeptide extension at the amino terminus (26, 27, 30). This suggests that albumin, like other secretory proteins (1), is first synthesized as a larger molecule (preproalbumin) with a “signal” hydrophobic extension at its amino-terminal end and that during vectorial discharge into the ER this hydrophobic peptide is enzymatically cleaved to yield proalbumin, which then contains a basic hexapeptide extension at the amino terminus (16, 21). This precursor protein exists in the ER and is converted into albumin in the Golgi cell fractions, just prior to its secretion into the blood (4, 7, 18).

The above mechanism for the biosynthesis and intracellular transport of albumin may, however, be different in some Morris hepatomas, since early studies indicated that Morris hepatomas 5123C and 9121 do not secrete albumin into the blood stream of eviscerated rats (23, 24); this idea was substantiated by Ueno and Ono (30), who further indicated that, in contrast to normal liver, Morris hepatoma 5123C synthesizes albumin on free rather than on membrane-attached polysomes. Later, McLaughlin and Pitot (13) showed that, in contrast to normal rat liver, in Morris hepatomas 5123C and 7800, the nascent albumin peptide chains could be detected antigenically on free polysomes but not on membrane-bound polysomes. These data support the hypothesis that these Morris hepatomas, which are thought not to secrete albumin, may contain a deficient mechanism for the attachment of albumin-synthesizing polypeptides to the membrane of the rough ER, which therefore precludes the vectorial discharge of albumin into the lumen of the rough ER and its subsequent secretion. Following this rationale, Strauss et al. (26) demonstrated that mRNA isolated from rat hepatoma 5123C, like that obtained from normal rat liver, directs the in vitro synthesis of preproalbumin; they therefore suggested that, since this tissue is thought not to secrete albumin, some component...
other than the “signal" extension to nascent albumin is defective in this hepatoma. These arguments are, however, valid only if these Morris hepatomas do, in fact, in contrast to normal liver, synthesize albumin on free polysomes, are therefore incapable of vectorial transfer of nascent albumin to the lumen of the rough ER, and therefore do not secrete albumin.

Recent information casts some doubt on the inability of the Morris hepatomas to secrete albumin. It has been demonstrated by Schreiber et al. (25) that single-cell suspensions of Morris hepatoma 5123C, prepared by treatment with collagenase and hyaluronidase, are capable of transferring nascent proteins to the incubation medium. This is in contrast to the apparent inability of these cells to secrete albumin in vivo (23). Also, it has been demonstrated that Morris hepatoma 5123C, both in vivo and in cell suspensions, is capable of producing precursor albumin (proalbumin) and that the single-cell preparation is capable of converting it to serum albumin (5, 24).

Because of these conflicting results between in vivo and single-cell suspension studies and because Morris hepatomas 5123C and 7800 are thought to synthesize albumin aberrantly on free rather than on membrane-attached polysomes, leading to a postulation that these cells may have a pathological condition involving polysome-membrane interactions, we have reinvestigated the biosynthesis and secretion of albumin in Morris hepatoma 5123C and 7800, emphasizing its intracellular processing and its in vitro secretion.

MATERIALS AND METHODS

Animals and Hepatomas. The hepatomas were obtained from Dr. H. P. Morris and were carried on the hind legs of male Buffalo rats. Hepatoma 5123C was in its 125th generation and hepatoma 7800 was the 110th generation when the studies were started, and both hepatomas were transplanted twice during the course of this study.

Preparation of Tissues for in Vitro Incubation. The tumors were removed, quickly placed in cold KRBS, and dissected to remove connective and necrotic tissues. The dissected hepatomas were, in the early experiments, sliced with a hand Stadie-Riggs microtome, but since these tissues are fragile and difficult to slice, in subsequent experiments a cell preparation was obtained by scraping the tissues with a spatula to remove the cells from adhering muscle and then gently mincing them with scissors. Both methods yielded preparations with similar capacities to incorporate radioactive L-leucine into TCA-precipitable protein and into "albumin." The tissues thus obtained were suspended in cold KRBS and washed free of accompanying blood by centrifuging in an International PRJ centrifuge at 2°, for a short time, just until the rotor reached a speed of 1000 rpm; it was then stopped by applying the brake. This washing procedure was performed 3 times. The washed tissues were then used for in vitro incubations.

Pulse-labeling and Chase Incubation. The tissues to be pulse-labeled were incubated at 37° for 10 min in KRBS gassed with 95% O2-5% CO2 and containing 0.21 M glucose and L-[4,5,3H]leucine (30 μCi/ml). About 10 ml of incubation medium were used for each 0.5 g of hepatoma. Following this “pulse" incubation, the tissues were recovered by centrifugation and washed 3 times with 0.7% L-leucine in 0.154 M NaCl to remove the radioactive leucine. The washed tissues were then "chased" for varying periods of time with nonradioactive KRBS containing 0.21 M glucose and 2 mM L-leucine. At the end of each chase period, both the tissues and the incubation medium were recovered by centrifugation and analyzed for protein content and incorporation of radioactivity into TCA-precipitable protein and into albumin. Before analyses were performed on the tissues, they were further washed 3 times with cold 0.154 M NaCl to remove any contamination with radioactive proteins released into the chase medium.

Isolation of Albumin and Proalbumin from Incubated Tissues and from the Medium. To measure the presence of these radioactive proteins in incubated tissues, the cells were homogenized in 0.154 M NaCl containing 0.01 M Tris-HCl, pH 7.4. In order to release proteins contained within membranous vesicles, 0.5% sodium deoxycholate was added. The material thus obtained was washed 3 times with cold 0.154 M NaCl and then treated with cold 5% TCA to precipitate albumin in order to precipitate the albumin in this fraction (17). The radioactive albumins in the immunoprecipitate were then obtained by a modification of the methods described by Judah and Nicholls (9) and Judah et al. (8). The precipitate was then centrifuged at 50,000 rpm in a Beckman 50 Ti rotor for 1 hr, and the resulting supernatant fraction was treated with carrier rat serum albumin and a slight excess of rabbit antiserum to rat serum albumin in order to precipitate the albumin in this fraction (17). The radioactive albumins in the immunoprecipitate were then obtained by a modification of the methods described by Judah and Nicholls (9) and Judah et al. (8). The precipitate was washed 3 times with cold 0.154 M NaCl and then treated with cold 5% TCA. The TCA precipitate was washed 3 times with cold 5% TCA and was then extracted 3 times for 30 min each time with 1% TCA in 95% ethanol. The ethanolic extraction was dialyzed overnight against 0.1 M Tris-HCl, pH 7.8. The dialyzed material was then centrifuged to remove any insoluble material and cochromatographed together with 40 mg of rat serum albumin on DEAE-cellulose columns as previously described (18). The first radioactive material which eluted prior to rat serum albumin is termed "proalbumin," and the second radioactive material which eluted later than proalbumin but prior to rat serum albumin is termed "hepatic albumin." Radioactive albumin released into the incubation medium was isolated in a fashion similar to that described above. The medium was also treated with 0.5% sodium deoxycholate, and the albumin was immunoprecipitated, alcohol extracted, and chromatographed on DEAE-cellulose. The radioactive albumin thus obtained cochromatographed with rat serum albumin.

In some experiments, the radioactivity in the dialyzed alcohol extract was taken as the total albumin radioactivity since it was shown that all of the measurable radioactivity that was placed on the DEAE-cellulose column eluted as either proalbumin, hepatic albumin, or serum albumin. In the experiments in which proalbumin, hepatic albumin, and serum albumin were isolated and then further separated by DEAE-cellulose chromatography, a proteolytic inhibitor was added to the in vitro incubation and to all other solutions during the isolation procedures. The inhibitor used was 10-4 M N-tosyl-L-lysylchloromethylketone HCI (6).

Cell Fractionation. The hepatomas were homogenized in 5 volumes of 0.25 M sucrose with 6 strokes of a tight Dounce glass homogenizer, and the homogenate was...
strained through 2 layers of cheesecloth. Some unbroken cells remained after this treatment, and most of them were removed by centrifugation at 1000 rpm in an International PRJ centrifuge for 2 min. The supernatant fraction which still contained some unbroken but damaged cells, as determined by light microscopy, was taken as the homogenate fraction. A nuclear fraction was obtained by centrifugation at 480 × g for 10 min in a Sorvall SS34 rotor. The nuclear fraction, which contained the remainder of unbroken cells, was washed twice by resuspension in 0.25 M sucrose and centrifuged at 480 × g for 5 min each time. A mitochondrial cell fraction was obtained by centrifugation at 15,900 × g for 10 min in the Sorvall SS34 rotor, and the mitochondrial pellet was washed twice by resuspension in 0.25 M sucrose and recentrifuged. The postmitochondrial supernatant fraction was then centrifuged in a Spinco 50 Ti rotor at 105,000 × g for 1 hr to obtain the microsomal cell fraction as a pellet and the soluble cytoplasmic cell fraction as the supernatant fraction.

Preparation of a mRNA Fraction and Its in Vitro Translation by a Protein-synthesizing System. RNA was extracted from hepatoma 7800 by phenol-chloroform (10), and a polyadenylate-rich mRNA fraction was then obtained by chromatography on oligodeoxythymidylate II cellulose columns (11). A wheat germ cell-free protein-synthesizing system was prepared as previously described using L-[4,5-3H]leucine as an amino acid precursor (31). The radioactive albumin produced was obtained by immunoprecipitation using rabbit antiserum to rat serum albumin (17). The precipitate was washed by centrifugation at 10,000 × g for 15 min through a layer of 1 M sucrose, 10 mM Tris-HCl buffer (pH 7.4), 0.154 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 10 mM L-leucine (20). The resulting pellet was further washed 3 times with 0.154 M NaCl, and then dried in a vacuum before being dissolved at 45° for 1 hr in 0.1 M sodium phosphate (pH 7.2), 1% SDS, 6 M urea, and 1% 2-mercaptoethanol. This material was then electrophoresed on 7.5% polyacrylamide gels, using 0.1 M sodium phosphate (pH 7.2) with 0.1% SDS as the buffer. The radioactive activity which coelectrophoresed with albumin was eluted by cutting that portion of the gel, placing the cut portion on another tube, and eluting the radioactivity by further electrophoresis. This eluted material was then used for amino acid sequencing to determine the locations of L-[3H]leucine in the 16 amino-terminal portions of the molecule (31).

Other Procedures. Lactate dehydrogenase was assayed as described in the Worthington Manual, Worthington Biochemical Corp., Freehold, N. J.; glucose-6-phosphatase was assayed by the method of Swanson (29); and succinic dehydrogenase was assayed as described by Bonner (2).

Protein was determined by the method of Lowry et al. (12) using bovine serum albumin as a standard.

The nonradioactive albumin used as a marker in DEAE-cellulose chromatography was prepared from 50 ml of rat serum, obtained from Pel-Freeze Biologicals, Inc., Rogers, Ark., by precipitation with 5% TCA, extraction with 1% TCA in 95% ethanol as described above, and chromatography on Sephadex G-100.

To determine the radioactivity in TCA-precipitated proteins, the washed precipitates were dissolved in 1 ml of Soluene, and then 0.1 ml of glacial acetic acid and 10 ml of toluene scintillation phosphor were added prior to counting in a liquid scintillation spectrometer. When determining radioactivity in proteins eluted from DEAE-cellulose column, 1-ml samples were added to 10 ml of Aquasol.

Amino acid sequencing was performed in a Beckman model 890C automatic sequencer as previously described (31).

Sources of Material. Radioactive amino acids were purchased from Amersham/Searle Corp., Arlington Heights, Ill.; DEAE-cellulose (DE52) from Whatman Corp., Clifton, N. J.; Soluene from Packard Instruments Corp., Downers Grove, Ill.; Aquasol from New England Nuclear, Boston, Mass.; and N-tosyl-L-lysylchioromethylketone HCl from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Time Course of Release of Radioactive Albumin and TCA-precipitable Proteins into the Incubation Medium after a 10-Min Pulse Incubation with L-[3H]Leucine. In order to determine the optimal time for pulse-labeling of nascent secretory proteins, the tissues were incubated at 37° in KRBS containing 0.21 M glucose and L-[3H]leucine for 30 min; at 5-min intervals, the TCA-precipitable radioactivity in the tissues and that released in the incubation medium were determined. The tissues incorporated radioactive activity at a linear rate into TCA-precipitable protein for 30 min, but little TCA-precipitable protein radioactivity was seen in the incubation medium until 15 min of incubation. Since appreciable labeling of tissue protein occurs within the first 10 to 15 min of incubation with little release of protein radioactivity into the medium, subsequent experiments were performed by pulse-labeling the tissues for 10 min with L-[3H]leucine. The cells were then recovered by centrifugation, washed in the presence of nonradioactive L-leucine, and then chased by a second incubation at 37° in KRBS containing 2 mM L-leucine. At various times of the chase incubation up to 75 min, the tissues and incubation media were recovered and assayed to determine radioactivity incorporated into total TCA-precipitable protein; into total albumin; and into prealbumin, hepatic albumin, and serum albumin.

When Morris hepatoma 7800 was pulse-labeled with L-[3H]leucine and chased for periods of 15 to 75 min, radioactive TCA-precipitable proteins appeared in the chase incubation medium at 15 min and continued to be released for 45 min. The total albumin radioactivity released into the medium was very low and was negligible for the first 15 min of the chase period, but then a small amount of radioactive albumin (about 4.5 to 5% of the total TCA-precipitable protein radioactivity) appeared at 30 to 75 min (Chart 1A).

The radioactivity in TCA-precipitable proteins and in total albumin remaining in the tissue during the chase incubation was also determined. At the end of the 10-min pulse period, 0.24% of the radioactivity in total TCA-precipitable protein in the tissue was determined to be albumin. During the 75-min chase incubation, the radioactivities in both albumin and TCA-precipitable protein diminished but that into albumin dropped at a rate faster than that of the radioactivity of total TCA-precipitable proteins. At the end of 75 min, the
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of the chase incubation (and remaining constant for 75 min), the intracellular radioactivity had dropped to 5,000 cpm/mg of protein, indicating good chase conditions.

Morris hepatoma 5123C behaved much like hepatoma 7800, with some notable exceptions. The release of radioactive TCA-precipitable proteins into the medium appeared at an earlier time during the chase incubation with over 50% of the radioactivity in TCA-precipitable protein being released by 15 min of the chase period. Albumin released into the medium was (as in hepatoma 7800) only a small fraction of the total protein radioactivity. In hepatoma 5123C, about 7.8% of the radioactivity released after 75 min was found to be associated with albumin (Chart 2A).

As with 7800, the level of radioactive albumin remaining in the tissues dropped during the chase period, with a radioactivity in albumin accounted for only 0.06% of the total protein radioactivity remaining in the tissues (Chart 1B).

As a measure of the efficiency of the chase incubation, the intracellular levels of TCA-soluble radioactivity were measured at the end of the 10-min pulse period and during various times of the chase incubation. At the end of the 10-min pulse period, the intracellular radioactivity of L-[3H]leucine was 43,000 cpm/mg of tissue protein; at 15 min

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Chart 1. The rate of release and retention of radioactive proteins by Morris hepatoma 7800 incubated in vitro. Hepatoma 7800 tissues were prepared for in vitro incubation as described in "Materials and Methods." Washed tissues (0.5 g) were pulse-labeled for 10 min at 37°C with L-[4,5-3H]leucine (30 μCi/ml) in 10 ml of KRBS plus 0.21 M glucose. Following this pulse period, the tissues were recovered by centrifugation and washed 3 times with 0.7% L-leucine in 0.154 M NaCl. The washed tissues were then further incubated at 37°C in a 10-ml chase medium of KRBS with 0.21 M glucose and 2 mM L-leucine for varying periods of time. At the end of each chase period, the tissues and the medium were recovered by centrifugation and were analyzed for radioactivity in total albumin and in TCA-precipitable (ppt) protein. The tissues were washed 3 times with cold 0.154 M NaCl before these analyses were performed. A, secreted proteins; B, intracellular proteins.

Chart 2. In vitro release of radioactive albumin and total proteins by Morris hepatoma 5123C tissues. The tissues were pulse-labeled for 10 min with L-[3H]leucine for varying periods of time as described in Chart 1. The radioactivities in both the intracellular and the secreted albumin and in TCA-precipitable (ppt) proteins were determined as described in "Materials and Methods." A, secreted proteins; B, intracellular proteins.
sharp decline in the first 30 min of chase incubation (Chart 2B). The radioactivity in total TCA-precipitable proteins showed a small decrease and did not decline as sharply as did the albumin radioactivity. At the end of the pulse period, the albumin radioactivity accounted for 0.6% of the total protein radioactivity; at the end of the 75-min chase, it had dropped to 0.2%.

Following the pulse incubation, as with hepatoma 7800, the intracellular levels of free L-[3H]leucine dropped precipitously from 33,000 to 2,000 cpm/mg tissue protein at the end of a 10-min chase.

These data indicate that Morris hepatomas 7800 and 5123C both synthesize albumin, that the levels of radioactive albumin within the tissue drop during a chase incubation, and that there is a concomitant appearance of low levels of radioactive albumin in the incubation medium.

In comparison with Morris hepatomas 7800 and 5123C, normal rat liver slices also showed release of radioactive TCA-precipitable protein and albumin into the medium during a 75-min chase period, but in marked contrast to the hepatomas, the radioactive albumin released accounted for a major part (46%) of the radioactivity (Chart 3A).

Of the protein radioactivity retained in the liver slices, albumin at the end of the 10-min pulse accounted for 4.8% of the total protein radioactivity, and it dropped to about 2.75% at the end of 75 min of chase incubation. The intracellular albumin radioactivity appeared to drop less sharply in liver slices than in the hepatomas, and there was only a slight decrease in the radioactivity of total TCA-precipitable protein during the chase incubation (Chart 3B). As in the hepatomas, the intracellular levels of L-[3H]leucine were shown to drop nearly 8-fold in the interval following the 10-min pulse and the 15-min chase period, again showing that the chase incubation conditions effectively removed the bulk of the intracellular radioactive leucine. In the experiment reported in Chart 3, the rat liver slices were obtained from the liver of a rat bearing a 7800 hepatoma in its hind legs, but similar results are obtained with liver from non-tumor-bearing rats.

These experiments indicate that Morris hepatomas 7800 and 5123C appear to synthesize less albumin as a percentage of total hepatic protein than does normal liver and that they therefore release less albumin into the medium.

Isolation and Identification of Radioactive Proalbumin and Albumin. Immunoprecipitation of radioactive albumin from liver tissues with the use of rabbit antiserum to rat serum albumin is capable of precipitating both precursor albumin and serum albumin. These 2 types of albumin may be recovered from the immunoprecipitate by treatment with cold 5% TCA, followed by ethanolic extraction, and then separated by DEAE-cellulose chromatography, since proalbumin contains a basic hexapeptide extension attached to the amino-terminal portion of serum albumin, which allows it to be eluted prior to serum albumin (8, 9, 18). Proalbumin contains arginine at the amino-terminal position, which contrasts with the amino-terminal glutamic acid of serum albumin (16, 21), and this difference may also be used to distinguish between these 2 proteins.

The radioactive albumins in the incubated tissues of hepatomas 5123C and 7800 obtained at the end of a 10-min pulse incubation, and also at various times of chase incubation, were analyzed in this fashion. At the end of a 10-min pulse incubation, all of the radioactive protein which was immunoprecipitated with anti-rat serum albumin eluted on DEAE-cellulose chromatography as a single peak prior to the elution of added serum albumin, suggesting that most of the radioactive albumin is proalbumin. In the first 15 min of chase incubation, a pattern very similar to that seen in pulse-labeled tissues was obtained. However, as the time of chase incubation proceeded, samples taken at 30 and 45 min and 60 to 75 min showed the presence of 2 radioactive albumins. At 30 and 45 min, the major radioactive protein still eluted as proalbumin, but there appeared another albumin species which eluted later than proalbumin but
slightly ahead of added nonradioactive carrier serum albumin (Chart 4C). Earlier studies by Peters and Reed (15) and also from our laboratory (18) indicate that the second peak closely resembles serum albumin in amino-terminal sequence but that it behaves differently on DEAE-cellulose chromatography. The chemical difference between this compound and serum albumin is not yet defined, but in order to differentiate between them we term this albumin hepatic albumin. At 60 to 75 min of chase incubation, there was a smaller proportion of proalbumin, but more radioactive hepatic albumin was present (Chart 4D). Similar results were obtained for hepatomas 7800 and 5123C.

Very similar patterns are obtained when the intracellular radioactive albumins from liver slices are assayed for proalbumin and albumin. During the 10-min pulse, a sharp proalbumin peak is obtained. At 15 min of chase incubation, the majority of radioactive albumin is proalbumin; but at 45 and 75 min, a small amount of radioactive hepatic albumin appears (Chart 5). In the experiment using normal liver slices, the separation between proalbumin and hepatic albumin is better defined than in the experiments with hepatoma 5123C (compare Charts 4 and 5).

In contrast to the radioactive albumin retained within the tissues, that which is released into the medium cochromatographs identically on DEAE-cellulose with added nonradioactive serum albumin. In Chart 6 is shown the elution profile of radioactive albumin released into the medium by hepatoma 7800 after it had been pulse-labeled for 10 min.

Chart 4. DEAE-cellulose chromatography of radioactive intracellular albumin synthesized by Morris hepatoma 5123C. Morris hepatoma 5123C was minced, washed, and pulse-labeled for 10 min with \( ^{3}H \)leucine and chased with nonradioactive L-leucine for various times as described in Chart 1. The intracellular albumin was isolated by immunoprecipitation and alcohol extraction and was then chromatographed on DEAE-cellulose together with 50 mg of rat serum albumin, as described in "Materials and Methods." The DEAE-cellulose chromatography was performed at room temperature on 1-x 50-cm columns. After the application of the samples, the column was washed with 50 ml of 0.1 M Tris-HCl (pH 7.8), and then the proteins were eluted with a 500-ml linear gradient of 0.1 M to 0.3 M Tris-HCl (pH 7.8). Fractions of 3 ml were collected and assayed for radioactivity and absorbance at 280 nm. All of the measurable radioactivity and absorbance at 280 nm eluted in the fractions shown above with no indication of radioactivity or protein in preceding fractions. Elution pattern of the intracellular albumin (A) at the end of a 10-min pulse and (B) after 15-min chase. C, 2 pooled samples taken at 30- and 45-min chase periods; D, 2 pooled samples taken at 60 and 75 min. It was necessary to pool these samples from late chase periods in order to obtain sufficient radioactive albumin for the assays.

with \( ^{3}H \)leucine and then chase-incubated for 75 min. Both the radioactivity and the absorbance of 280 nm, which is due to the carrier serum albumin, coincide, indicating that the radioactive albumin released into the media is probably serum albumin but that it differs chromatographically from the radioactive albumins retained within the tissues.

Chart 5. DEAE-cellulose chromatography of radioactive intracellular albumin synthesized by rat liver slices. The liver of a rat bearing a Morris hepatoma 7800 was removed, rinsed, sliced with a hand microtome, incubated for 10 min with \( ^{3}H \)leucine and chased with nonradioactive L-leucine for various times as described in Chart 3. The intracellular radioactive albumin was isolated by immunoprecipitation and alcohol extraction and chromatographed on DEAE-cellulose as described in Chart 4. A, elution pattern of radioactive albumin released into the medium by Morris hepatoma 7800 incubated in vitro. Morris hepatoma 7800 was pulse-labeled for 10 min with \( ^{3}H \)leucine and then chased for 75 min with 0.2 mM L-leucine as described in Chart 1. The radioactive albumin released into the medium was isolated by immunoprecipitation and alcohol extraction and coelectrophoresed on DEAE-cellulose with 50 mg of rat serum albumin as described in Charts 4 and 5. ---, radioactive activity; ---, protein activity; -----, A_260 of added serum albumin.

Chart 6. DEAE-cellulose chromatography of radioactive albumin released into the medium by Morris hepatoma 7800 incubated in vitro. Morris hepatoma 7800 was pulse-labeled for 10 min with \( ^{3}H \)leucine and then chased for 75 min with 0.2 mM L-leucine as described in Chart 1. The radioactive albumin released into the medium was isolated by immunoprecipitation and alcohol extraction and coelectrophoresed on DEAE-cellulose with 50 mg of rat serum albumin as described in Charts 4 and 5. ---, radioactive activity; -----, A_260.
To identify further the radioactive protein retained in the tissues which elutes prior to serum albumin and which, because of its immunoprecipitation and chromatographic behavior we have assumed is proalbumin, we performed SDS-polyacrylamide gel electrophoresis on this compound and compared it with that of rat serum albumin. We also analyzed the amino-terminal moieties of the protein to determine whether it contained arginine, since this is characteristic of the hexapeptide extension which occurs in proalbumin.

To determine whether proalbumin coelectrophoresed with rat serum albumin on SDS-polyacrylamide gel electrophoresis, hepatoma 5123C was incubated with L-[3H]leucine for 15 min at 37°, and the radioactive albumin in the tissue was isolated by immunoprecipitation, alcohol extraction, and DEAE-cellulose chromatography as described above. The proalbumin peak was dialyzed against water, lyophilized, and dissolved in 0.1 M phosphate buffer (pH 7.2), 6 M urea, 1% SDS, and 1% mercaptoethanol. The radioactive material was then coelectrophoresed together with rat serum albumin on 7.5% polyacrylamide gels. The gels were stained with Coomassie blue, cut into 0.5-mm slices, and counted for radioactivity. A single radioactive area occurred on the gels, and it comigrated with serum albumin (not shown).

Rat proalbumin is known to be a protein with a hexapeptide extension to serum albumin with the following aminoterminal sequence: Arg-Gly-Val-Phe—Arg-Arg—serum albumin. Therefore, to identify further the proalbumin peak, hepatoma 7800 was incubated for 20 min with both L-[14C]leucine and L-[3H]arginine. The proalbumin was isolated as before by immunoprecipitation, alcohol extraction, and DEAE-cellulose chromatography. The DEAE-cellulose pattern of radioactive material and that of added rat serum albumin are shown in Chart 7A. The central portions of the radioactive peak were then collected and subjected to NH2-terminal sequence analysis. Radioactivity due to L-[3H]arginine was, as expected if the protein is proalbumin, found at the amino-terminal position and also in positions 5 and 6. No significant radioactivity due to L-[14C]leucine was noted in the 10 amino-terminal residues. This is to be expected since proalbumin does not contain leucine in those positions (Chart 7B). The failure to obtain L-leucine radioactivity in those positions also indicates that the tissues do not contain preproalbumin since preproalbumin has L-leucine in positions 7, 8, 9, and 10.

Measurement of the Amount of Pulse-labeled Albumin Which Is Released Into the Chase Medium. The experiments described above indicate that Morris hepatomas 7800 and 5123C synthesize albumin but that they produce smaller amounts of albumin in relation to total TCA-precipitable protein than does normal liver. However, the experiments also indicate that some radioactive albumin is released into the chase medium. It has been proposed that these tissues are not capable of albumin secretion, and thus the albumin released into the medium may merely be a small percentage of the total albumin synthesized by the hepatoma. Furthermore, this released albumin may be due to cell rupture rather than to secretion. To quantitate the percentage of pulse-labeled albumin which is released into the medium, hepatomas 5123C and 7800 were pulse-labeled for 10 min with radioactive L-leucine, and the amount of total radioactive albumin produced by the hepatomas in that time period was measured. The hepatomas were then chased for 75 min, a time at which all of the radioactive albumin has been released into the medium (Charts 1 and 2). Both the radioactive albumin released into the medium and that retained by the tissues were measured.

In Morris hepatoma 7800, 81% of the radioactive albumin produced during the pulse period was released into the medium during the chase period, and only 19% was retained by the tissues. The recoveries of radioactive albumin were close to 100% (when compared to those tissues which were pulse-labeled for 10 min but not chased), indicating that little degradation of intracellular albumin had occurred.
during the incubation period. The results from an experiment with hepatoma 7800 are shown in Table 1. Similar results (not shown) were obtained with hepatoma 5123C.

As mentioned above, the release of radioactive albumin into the chase medium may be due not to secretion but rather to lysis or rupture of the hepatoma during the chase incubation. Therefore, to obtain a measure of damage to the cells during incubation, the soluble intracellular enzyme lactate dehydrogenase was measured in hepatomas before the initial 10-min period, after the 10-min pulse incubation, before the start of the chase incubation, and at the end of 75 min of chase incubation. Lactate dehydrogenase activity released into the medium during the initial 10-min pulse period and during the 75-min chase period was also measured. These studies showed that some lactate dehydrogenase activity was released into the medium both during the initial 10-min pulse-labeling and during the 75-min chase incubation. During the 10-min pulse incubation, 18.5% of the enzyme activity was released into the medium (Table 2). This indicates that some cells are losing soluble enzymes during this period, but it should be noted that previous experiments indicated that there was no radioactive albumin or TCA-precipitable protein released into the medium during this stage. In the chase incubation period, which lasts 75 min, 35.5% of the remaining lactate dehydrogenase was released and 64.5% was retained within the tissues. By contrast, during this chase period, 81% of the pulse-labeled radioactive albumin was secreted into the medium, and only 19% was retained (Table 1). Thus, some of the radioactive albumin released into the medium may be due to cell rupture or lysis since 35.5% of lactate dehydrogenase was released during this period, but lysis does not account for the 81% of radioactive albumin which is released to the medium during this same period, indicating that a substantial portion of the radioactive albumin in the medium is indeed secreted.

Intracellular Location of Nascent Albumin. It has been determined that hepatomas 7800 and 5123C, in contrast to normal liver, contain nascent, antigenically active albumin on free polysomes rather than on membrane-attached polysomes (13). This together with the purported failure of these tissues to secrete albumin suggests a defective cellular mechanism for the attachment of polysomes to the ER membrane and for the vectorial discharge of albumin into the rough ER. If this were true, it would be expected that nascent albumin released from free polysomes will reside not in the lumen of the rough ER but rather in the soluble cytoplasmic fraction. To determine if this is true, hepatoma 7800 tissues were incubated with radioactive L-leucine for 15 min, washed with 0.154 M NaCl to remove radioactivity in the incubation medium, and then homogenized in 0.25 M sucrose and fractionated into various cell fractions. Total radioactive albumin was then isolated from the homogenate and from each of the cell fractions. In the various cell fractions, 62% of the radioactive albumin obtained from the homogenate was recovered. Of the recovered radioactive albumin, only 8% was in the soluble cytoplasmic fraction with most of the albumin located in particulate organelles (Table 3). The majority, 57.5%, of the radioactive albumin was in the microsomal cell fractions, although significant amounts were also present in the mitochondrial and nuclear cell fractions. Similar results were obtained at 5 and 10 min of incubation. The distribution of marker enzymes, such as glucose-6-phosphatase for the ER and succinic dehydrogenase for mitochondria, indicated some cross-contamination between nuclear, microsomal, and mitochondrial fractions (Table 3), possibly explaining the presence of radioactive albumin in the mitochondrial and nuclear fractions. The fact that the majority of radioactive albumin is associated with microsomal and other particulate cell fractions does not rule out the possibility that albumin is synthesized on free polysomes in these hepatomas, since the microsomal cell fraction contains both free and membrane-bound polysomes. Therefore, in order to determine whether the radioactive albumin was associated with free polysomes,
was associated with membrane-bound polysomes, or was contained within the cisternae of the microsomal vesicles, the microsomal cell fraction, obtained as described in Table 3, was further fractionated by placing it over a 2.0 M sucrose cushion and centrifuging it at 105,000 × g for 16 hr to recover the free polysomes as a pellet and to obtain the polysomes attached to microsomal vesicles at the top of the 2.0 M sucrose layer. The membrane-attached polysomes were then treated with 0.5% sodium deoxycholate to solubilize the microsomal membrane and to release the nascent radioactive polypeptides contained within the microsomal vesicles. The amount of radioactive albumin in the various microsomal subfractions was then determined. The large majority (72%) of radioactive albumin present in the microsomal cell fraction was found in the deoxycholate-soluble fraction which derived from membrane-attached polysomes. There was no detectable radioactive albumin at 15 min of incubation in either the free polysomes or the polysomes obtained by deoxycholate treatment of membrane-attached polysomes. The recovery of total TCA-precipitable protein radioactivity in the various microsomal subfractions was 78%. This experiment indicates that at 15 min most of the nascent albumin is sequestered within the microsomal vesicles and has already left the polysomal site of synthesis. At shorter times, 2 to 3 min, when the majority of nascent proteins are still on the polysomes, we could not obtain sufficient radioactive albumin still attached to the polysomes to determine which class of hepatoma polysomes synthesizes albumin.

It is, therefore, still possible that in these hepatomas albumin is synthesized by free polysomes and not by membrane-attached polysomes and that nascent albumin is released directly into the cytoplasm and is not vectorially discharged into the lumen of the endoplasmic reticulum, but that during homogenization much of the cytoplasmic albumin is trapped inside membrane vesicles. However, studies with exocrine pancreas by Scheele et al. (22) suggest that this is an unlikely possibility. The results of this experiment show that most of the nascent albumins are segregated within the ER and are similar to those obtained in the liver, again suggesting that the hepatomas, while producing less albumin than liver, are capable of normal intracellular segregation and secretion of albumin.

In Vitro Synthesis of Preproalbumin. A mRNA fraction isolated from rat liver has been shown to be capable of directing the in vitro synthesis of a larger albumin precursor, preproalbumin (27, 28, 31). Further to characterize the synthesis and secretion of albumin by these hepatomas and to compare it with that of normal rat liver, similar experiments were performed with mRNA isolated from Morris hepatoma 7800.

Isolation of a mRNA fraction, its translation with a mRNA-dependent wheat germ protein-synthesizing system in the presence of L-[3H]leucine, followed by immunoprecipitation of the translated albumin yielded 3 radioactive products when assayed by electrophoresis on 7.5% SDS-polyacrylamide gels (Chart 8A). One of the radioactive bands comigrated with serum albumin, and the other 2 appeared to have lower molecular weights and may be prematurely released translation products of albumin mRNA. The radioactive protein which comigrated with serum albumin was eluted from the polyacrylamide gel and analyzed for the presence of L-[3H]leucine by automatic sequence analysis of 17 amino-terminal residues of the polypeptide. Rat preproalbumin obtained from liver is known to contain leucine in positions 7, 8, 9, and 10 (27, 28, 31), and the material which immunoprecipitated with antialbumin and which coelectrophoresed with serum albumin also yielded radioactive L-leucine in sequence cycles 7, 8, 9, and 10 (Chart 8B). This indicates that hepatoma 7800, like normal liver, contains mRNA capable of producing a protein which resembles preproalbumin.

DISCUSSION

These data show that, in vitro, Morris hepatomas 5123C and 7800 synthesize less albumin as a percentage of total TCA-precipitable protein than does normal liver but that like normal hepatocytes they are capable of intracellular processing of nascent albumin from larger precursor forms (preproalbumin and proalbumin) to its final form, serum albumin. Furthermore, these hepatomas appear to be able to secrete, within a period of 1 hr, a large percentage (80%) of the radioactive albumin which is pulse-labeled for 10 min. They are also capable of segregating the bulk of nascent albumin within the microsomal cell fraction with only a small amount (which may be due to organelle rupture) found in the soluble cytoplasmic fraction. Thus, in these respects, the hepatomas closely resemble normal rat liver.

In marked contrast to the in vitro situation is the apparent inability of hepatoma 5123C, carried in the hind legs of rats, to secrete albumin into the bloodstream (5, 24). This may be due: (a) to a real failure of in vivo hepatomas to secrete albumin, caused by a defect in the secretory mechanisms which may be overcome or lost during the preparation of single-cell suspensions and hepatoma slices; (b) to the presence of an unknown inhibitory mechanism in tumor-
small amounts of albumin which may have gone undetected in the in vivo experiments. The tissues are not treated enzymatically and they prove capable of both synthesis and secretion of albumin. It should be noted that our preparation, which is akin to a liver slice, probably contains a mixture of cells, only some of which are hepatomas. Since, however, it is believed that only hepatic cells produce albumin, this does not affect the measurement of the percentage of albumin which is secreted, nor does it affect the experiments which deal with the nature of intracellular and secreted albums. Since, however, cells other than hepatocytes also incorporate radioactive L-leucine into TCA-precipitatable protein, it does affect the measurement of the amount of albumin synthesized, expressed as a percentage of total TCA-precipitatable proteins. Because of this possibility, the values given for albumin synthesized by these hepatomas may not be accurate.

Also, since the incubated tissues may contain cells which are not hepatomas, this may also explain the relatively high percentage of lactic dehydrogenase released during incubation. In the 10-min pulse period, 18.5% of lactic dehydrogenase was released into the medium, but during that time there was little or no albumin secreted, indicating that radioactive albumin is segregated differently within the cells than is lactic dehydrogenase. This may be due partly to the fact that most of the radioactive albumin is located on the microsomal cell fraction (Table 3), while lactic dehydrogenase is a soluble enzyme. It is also possible that cells other than hepatomas in these tissue preparations may be more labile than cells in the hepatomas and will more easily release lactic dehydrogenase into the medium during incubation.

Evidence from 2 laboratories indicates that hepatomas 5123C and 7800 preferentially synthesize albumin on free polysomes rather than on membrane-bound polysomes as is the case for normal rat liver (13, 30). This finding is in agreement with the suggestion that these hepatomas are incapable of albumin secretion, since in normal liver which resembles albumin on membrane-attached polysomes the newly synthesized albumin is quickly segregated in the rough ER by its vectorial discharge from membrane-attached polysomes. This process of vectorial discharge places the nascent albumin in the correct cellular location to follow a secretory path to the outside of the cell (14). If, however, albumin is assembled on free polysomes, it may be expected that, because of some aberrant mechanism in these hepatomas, the polysomes will not become attached to the ER and that they will therefore release nascent albumin into the soluble cell fraction rather than into the lumen of the ER. This appears not to be the case, since most of the nascent albumin, even at early time points such as 5, 10, and 15 min, is located in the microsomal fraction and not in the soluble cytoplasmic fraction (Table 3). The fact that these hepatomas also contain a mRNA which appears to be capable of producing a precursor albumin which resembles preproalbumin in that it contains...
L-leucine in positions 7, 8, 9, and 10 (Chart 8) also suggests that these cells do not lack the ability to produce the "signal" extension which leads the secretory protein to the ER membrane. However, since the signal portion of pre-proalbumin of these hepatomas has not yet been sequenced, we do not know whether it differs from that produced by normal liver.

Not explained, however, is why albumin appears to be synthesized by free polysomes rather than by membrane-attached polysomes. Attempts by us to reproduce the in vitro experiments of Uenoyama and Ono (30) did not yield conclusive results because of the low amounts of radioactive albumin produced by these hepatomas. Immunoprecipitation of the radioactive translated products, using rabbit antiserum to rat serum albumin, without further purification of the radioactive albumin by alcohol extraction followed by either DEAE-cellulose chromatography or SDS-urea-polyacrylamide gel electrophoresis does not yield a useful measure of albumin synthesis, since free polysomes appear to synthesize an unidentified radioactive protein which coprecipitates with antialbumin but which does not appear to behave like albumin on further purification.

The studies of McLaughlin and Pitot (13) show that these hepatomas differ from liver in that a nascent protein which reacts antigenically with iodinated Fab fragment of antialbumin is present on free polysomes of hepatomas but by contrast is predominantly present on membrane-attached polysomes of normal liver. At present, the information available does not explain why these hepatomas should differ from normal rat liver in this respect. These hepatomas are able to produce the proper precursor albumins, are capable of processing them to the final product, and appear to be able to secrete albumin into the medium; thus they must be regarded as having a secretory pathway for albumin closely resembling that of normal hepatocytes. Therefore, this difference in the polysomal location of nascent albumin in hepatomas and normal hepatocytes may be of interest in the elucidation of the mechanism of polysome-membrane interaction.

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REFERENCES


In Vitro Synthesis and Secretion of Albumin by Morris Hepatomas 5123C and 7800
