

Immunocytochemical Identification of Phenylalanine Hydroxylase and Albumin in Cultured Hepatoma Cells and Isolated Rat Hepatocytes

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ABSTRACT Rhodamine-conjugated antibodies specific for phenylalanine hydroxylase and serum albumin were employed as cytochemical probes to identify these two proteins in H4 hepatoma cells and in isolated rat hepatocytes. Each fluorescent antibody stained the cells specifically and in a distinctive manner. In both cell types, albumin staining was discretely localized in cytoplasmic "bundles," whereas, phenylalanine hydroxylase staining was diffuse and cytoplasmic and in H4 cultures varied somewhat from cell to cell. Evidence from cultures of REB15 cells, a strain derived by cloning H4 cells in tyrosine-free medium, suggested that the staining variability of H4 cells could reflect a variability in phenylalanine hydroxylase content. Hydrocortisone-treated H4 and REB15 cultures contain increased amounts of phenylalanine hydroxylase; and all cells in the culture appear to be induced by the hormone.

Evidence was presented to show that the albumin visualized within the isolated hepatocytes had been synthesized by these cells, and, furthermore, that quantitatively nearly all intracellular albumin in the isolated rat hepatocytes appeared to be entrained in the secretion pathway (analogous data already exist for H4 cells [Baker, R. E., and R. Shiman. 1979. *J. Biol. Chem.* 254: 9633-9639]). By scoring specific fluorescence, 86 and 98% of the H4 cells and 89 and 98% of the isolated hepatocytes were found to contain phenylalanine hydroxylase and albumin, respectively. Therefore, almost all cells in each population appeared to synthesize both proteins. An implication of these findings is that in rat virtually all liver parenchymal cells must synthesize both phenylalanine hydroxylase and albumin.

Serum albumin synthesis and phenylalanine hydroxylase expression are differentiated functions of normal mammalian liver. Both functions are also expressed by the Reuber hepatoma (rat) cell line H4 (1). Recently, we developed a new method for measuring protein turnover and used it to determine the half-life of phenylalanine hydroxylase in H4 cultures (2). Because the method, which we hoped also to apply in studies with perfused rat liver, involved the use of albumin as a reference protein, the question immediately arose as to whether or not albumin and phenylalanine hydroxylase were simultaneously synthesized within the same cells. Several studies (3-7) indicate that only a minority of liver parenchymal cells contain and, by implication, synthesize albumin. To our knowledge, there is no existing information regarding the distribution of phenylalanine hydroxylase among hepatic cells either in vivo or in culture. To answer the cosynthesis question,

we employed immunocytochemical methods to identify albumin and phenylalanine hydroxylase in H4 cultures and also in freshly isolated rat hepatocytes. Our results, reported here, are of quite general interest, because they supply information at the cellular level relating to the expression of specialized functions by differentiated tissue and by a permanent cell line derived from that tissue.

MATERIALS AND METHODS

Cell Culture and Hepatocyte Isolation

We used the Reuber hepatoma cell line H4 (1) and REB15, a strain of H4 derived in this laboratory. H4 cells were grown in monolayer culture with a modification (8) of Medium S-77 containing two times glutamine, 15 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), pH 7.4, and 10% fetal calf serum, but containing no antibiotics. Detailed cell culture conditions have been described (9).

REB15 was derived as follows: H4 cells were selected for three passages in culture medium lacking tyrosine. (Fetal calf serum used in the preparation of tyrosine-free S-77 was exhaustively dialyzed against PO₄-buffered saline¹ [PBS]). Surviving cells were plated sparsely in tyrosine-free medium and, after 2 wk, colonies were picked using small stainless steel cylinders (10). One such clone, designated REB15, was propagated in the manner described for H4 but using tyrosine-free S-77.

Isolated hepatocytes were prepared from livers of normal, fed rats by a modification of the collagenase perfusion method of Berry and Friend (11). Livers were initially perfused for 15 min in a flow-through mode with Ca⁺⁺-free Krebs-Henseleit bicarbonate buffer (12), pH 7.4, containing 27 mM glucose, 5 mM monosodium glutamate, 5 mM sodium pyruvate, and 10% (vol/vol) washed bovine erythrocytes. The perfusion system was then switched to a recirculating mode and 35 mg of collagenase (CLS II, lot 47D275, Worthington Biochemicals, Freehold, N. J.) was added to the remaining 100 ml of buffer. After 30 min of digestion the liver was gently passed through a stainless-steel sieve (E-C Collector, 10-mesh; E-C Apparatus Corp., Cambridge, Mass.) and any large pieces of undigested tissue were removed by filtration through a single layer of surgical gauze. Hepatocytes were collected and washed by three cycles of low-speed centrifugation and resuspended as previously described (13) with the same buffer that was used in the perfusion, but lacking erythrocytes, and supplemented to contain 2.4 mM Ca⁺⁺, 3% bovine serum albumin (Fraction V; Miles Laboratories, Miles Research Products, Elkhart, Ind.), and amino acids at 7.5 times the normal rat plasma level (14). After the final wash, cells were resuspended in 20 vol of buffer and either processed immediately for immunofluorescent staining or incubated in 250-ml polycarbonate Erlenmeyer flasks (40 ml suspension per flask) at 37°C in a shaking water bath (rotary motion, 150 rpm). All perfusion, wash, and incubation buffers were maintained at 37°C and equilibrated with an oxygen-carbon dioxide mixture (95:5%).

Antibodies

Purified rat serum albumin (15) and rat liver phenylalanine hydroxylase (16) were used to immunize New Zealand White rabbits. IgG was purified from the resulting antisera by caprylic acid precipitation (17). Rabbit anti-rat IgG and rhodamine-conjugated goat anti-rabbit IgG antibodies were purchased from N. L. Cappel Laboratories Inc., Cochranville, Pa. Before use, the anti-rat IgG antibody was absorbed against rat serum albumin and bovine serum albumin. These and all other antibody absorptions were performed by passing the antibody solution (in PBS) over a bed of Sepharose 4B to which the appropriate pure protein antigen had been coupled (18).

For direct immunofluorescence, anti-phenylalanine hydroxylase and anti-albumin IgG fractions were conjugated with tetramethylrhodamine isothiocyanate (TRITC; Research Organics, Inc., Cleveland, Ohio) following the procedure of Kearney and Lawton (19). These antibodies were shown to be monospecific by crossed immunoelectrophoresis (20, 21) against crude rat liver extract and rat serum, respectively.

Both the tissue-culture and hepatocyte incubation media contained bovine serum albumin. No cross-reactivity between our anti-rat albumin antibodies and bovine serum albumin could be detected by quantitative immunoelectrophoretic analyses. Also, in both the direct and indirect immunofluorescence staining procedures, anti-rat albumin antibodies absorbed with bovine serum albumin produced results identical to those obtained with unabsorbed antibodies.

Immunofluorescence

CELL PLATING: Hepatoma cells suspended in normal culture medium were plated onto 15-mm-round glass cover slips (Corning Glass Works, Science Products Div., Corning, N. Y.) in 24-well tissue culture plates (Falcon Labware, Div. of Becton & Dickinson, Cockeysville, Md.). After 48 h of incubation under normal growth conditions (cell number increases about twofold), the cells were processed for immunofluorescence. Isolated hepatocytes were diluted into tissue-culture medium and plated onto collagen-coated cover slips in 24-well plates, and the plates were incubated at 37°C for 40 min to allow the cells to attach. At this time, nearly all cells had attached and they were fixed for immunofluorescence.

DIRECT IMMUNOFLUORESCENCE: Except where stated otherwise, all manipulations were carried out at room temperature, and coverslips were handled in alumina staining racks (Arthur H. Thomas Co., Philadelphia, Pa.). Cover slips containing cells were rinsed with PBS and the cells were fixed for 30–45 min in PBS containing 3% formaldehyde. After a PBS rinse, the cover slips were incubated for 10 min in PBS containing 0.1 M glycine to quench remaining aldehyde moieties (22). The cover slips were again rinsed in PBS, air-dried briefly, placed in acetone at –20° for 4 min, and rehydrated in PBS.

¹ After Dulbecco (43).

The fixed, permeabilized cells were incubated with normal rabbit serum (diluted 1:3 in PBS) for 30 min at 37°C, rinsed with PBS, and then incubated with TRITC-IgG for 30 min at 37°C. These incubations were performed by inverting the cover slips over a drop (60–70 μ l) of the desired reagent in the bottom of a 24-well plate, which was then covered and placed in a humidified incubator at 37°C. The TRITC-IgGs were diluted into PBS containing 8% ovalbumin (23); actual dilutions are given in the figure legends. After the final incubation, the cover slips were washed for 5 min in each of three changes of PBS, rinsed in H₂O, and mounted on microscope slides cell-side-down using Elanol (24).

Control reagents for the direct immunofluorescence staining reactions were prepared from the TRITC-conjugated antibodies by absorbing their corresponding antigens. TRITC-anti-rat albumin and TRITC-anti-phenylalanine hydroxylase IgG fractions were passed, respectively, over columns of rat albumin-Sepharose or phenylalanine hydroxylase-Sepharose. In each case, antibodies were bound to the absorbant as evidenced by red coloration at the top of the columns. The unbound TRITC-IgG fractions, which in each case contained ~80% of the original TRITC-IgG (measured by OD₂₈₀), were used as controls to evaluate the specificity of staining by the corresponding unabsorbed reagents. When used for this purpose, the dilution employed was calculated so as to give an IgG concentration identical to that of the corresponding unabsorbed reagent.

INDIRECT IMMUNOFLUORESCENCE: Cells were fixed on cover slips and permeabilized exactly as described above for the direct immunofluorescence staining procedure. After the acetone treatment, the cover slips were incubated for 30 min at 37°C with the "first" antibody, either anti-rat IgG serum (4 mg antibody protein/ml), anti-rat albumin (IgG fraction, 6 mg IgG/ml), or normal rabbit serum. The antibodies were diluted 1:100 into PBS containing 8% ovalbumin and 1% Triton X-100 (25). Following this incubation, the cover slips were washed for 5 min in PBS containing 0.3% Triton X-100 followed by two 5-min washes in PBS (25). The cover slips were then incubated for 30 min at 37°C with TRITC-anti-rabbit IgG diluted 1:50 in PBS–8% ovalbumin–1% Triton. After washing as before, the cover slips were rinsed in H₂O and mounted cell-side-down in Elvanol.

MICROSCOPY: Microscopy was performed with a Leitz Ortholux II microscope equipped with a 75-W xenon light source, a Ploem-type vertical illuminator, and bright field and phase optics. TRITC fluorescence was observed with a BP530-560 excitation filter, an RKP580 dichroic mirror, and an LP580 suppression filter. Black and white photographs were obtained using Kodak Tri-X Pan film (Eastman Kodak Co., Rochester, N. Y.).

Quantitation of Albumin in Cell Extracts and Incubation Medium

1-ml aliquots of hepatocyte suspensions were removed from the incubation flasks at various times and put on ice. Cell pellets were obtained by centrifugation at 160 g for 2 min at 4°C. The medium was removed and saved. The cells were washed twice with cold PBS, resuspended in 0.5 ml of cold PBS containing 1% (wt/vol) sodium deoxycholate and Triton X-100, and sonicated for three 5-s bursts with a probe-type sonicator. (No measurable rat albumin is lost from the cells during the PBS wash procedure.) The lysates were centrifuged at 27,000 g for 30 min at 4°C to obtain clear cell extracts. Albumin in the medium samples and cell extracts was quantified by rocket immunoelectrophoresis performed essentially as described by Laurell (21, 26), except that the electrophoresis buffer consisted of 0.08 M Tris, 0.024 M Tricine (N-tris[hydroxymethyl]methylglycine), 0.3 mM calcium lactate, pH 8.6. Purified rat serum albumin was used as the standard (2). ³H-labeled albumin was isolated from the medium samples by immunoaffinity chromatography (2). ¹⁴C-labeled media proteins were added to the samples as internal recovery standards and [³H]albumin radioactivity was determined after sodium dodecyl sulfate polyacrylamide gel electrophoresis (2).

RESULTS

Phenylalanine Hydroxylase and Albumin in Cultured Hepatoma Cells

TRITC-conjugated antibodies were used to identify phenylalanine hydroxylase and albumin in H4 hepatoma cells. Each antibody stained its respective antigen specifically and in a characteristically distinct manner. Typical results are shown in Fig. 1. TRITC-anti-phenylalanine hydroxylase gives rise to a generalized cytoplasmic fluorescence, and the dark, unstained cell nucleus is discernible within the brightly fluorescent cytoplasm (Fig. 1*b*). In nearly all cells, TRITC-anti-albumin fluorescence is discretely localized in cytoplasmic "bundles," with

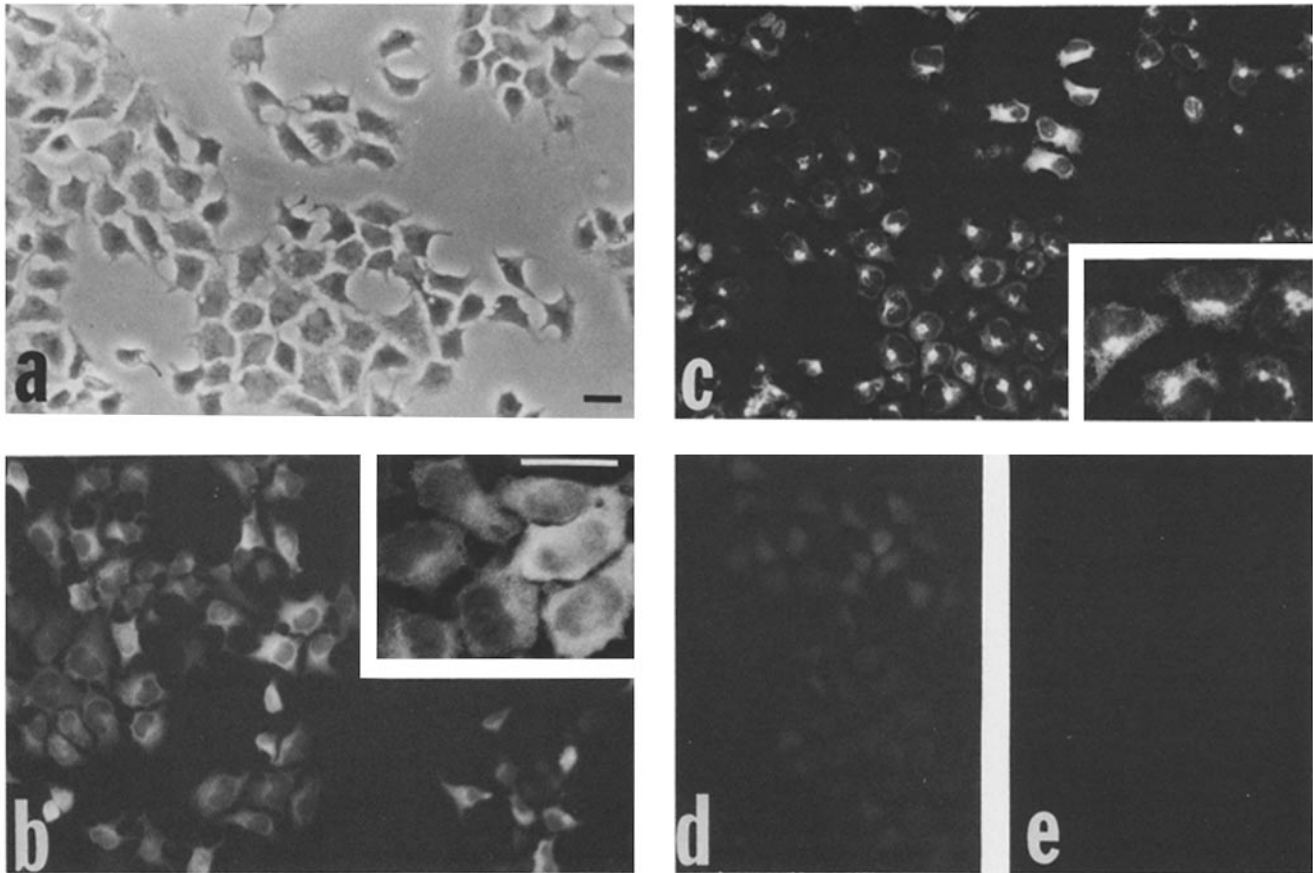


FIGURE 1 Direct immunofluorescence staining of phenylalanine hydroxylase and albumin in H4 cells. The staining procedure is described in Materials and Methods. *a* is a phase-contrast micrograph of H4 cells. *b–e* are fluorescence micrographs obtained using 4-min exposure times. *b*, staining by TRITC-anti-phenylalanine hydroxylase (0.6 mg IgG/ml). *c*, staining by TRITC-anti-albumin (0.3 mg IgG/ml), same field as *a*. *d*, control for phenylalanine hydroxylase staining. *e*, control for albumin staining. Bars, 25 μm . $\times 200$; insets, $\times 500$.

the majority of staining associated with one relatively large, juxtannuclear structure per cell (Fig. 1 *c*). The discrete localization of the albumin staining as well as the fact that this staining is abolished if acetone treatment is omitted during the staining procedure (data not shown) indicates that the albumin we are visualizing is located intracellularly. It must be noted that our previous characterization of albumin secretion by H4 cells (2) has shown that all albumin extractable from the cells can be accounted for as being newly synthesized and involved in the process of secretion.

The specificity of the staining reactions was demonstrated using control preparations of the TRITC-conjugated antibodies which had been absorbed with their corresponding pure antigens. The fluorescence of cells stained with these control antibodies is low and is uniform over the cells (Fig. 1 *d* and *e*). Thus, specific fluorescence can be scored by two criteria: the intensity of staining (as compared with controls) and the pattern of staining (i.e., the ability to distinguish a cell nucleus in the case of phenylalanine hydroxylase staining or bright “bundles” in the case of albumin staining). When scored independently by one of the authors (R. Baker) and two other unbiased observers, on the average $86 \pm 10\%$ of the cells were judged as being phenylalanine hydroxylase-positive and $98 \pm 1\%$ as being albumin-positive. (A total of six different fields with 100–200 cells/field viewed at $\times 200$ magnification were scored for each antigen.) Therefore $>80\%$ of the H4 cells must synthesize both phenylalanine hydroxylase and albumin.

An apparent cell-to-cell variability of fluorescence intensity is observed among TRITC-anti-phenylalanine hydroxylase-stained H4 cells (Fig. 1 *b*). An attempt to assess the significance of this variability was made by studying REB15 cells, a strain of H4 cells derived by cloning in tyrosine-free medium. As seen in Fig. 2 *a*, REB15 cells tend to be more flattened than H4 cells; and at low population density, they actively aggregate into clusters, forming more cell-cell contacts than H4 cells (Fig. 1 *a*). (It is important to recognize in all these experiments that only about a twofold increase in cell number occurs in the time period between cell plating, as a single-cell suspension, and fixation. As a result, neighboring cells, even in the clusters of REB15 cells, do not generally represent daughter cells.) Of interest in this context is the fact that, by and large, REB15 cells stained with the fluorescent anti-phenylalanine hydroxylase antibody present a cytoplasmic fluorescence with relatively less cell-to-cell variability in intensity than the H4 cells, suggesting that the variability observed among H4 cells could reflect a variability in the phenylalanine hydroxylase content of the H4 cells.²

Hydrocortisone is known to induce a four- to sixfold increase in both phenylalanine hydroxylase protein (2, 27) and phen-

² The REB15 population is also uniform with respect to albumin synthesis. Virtually all REB15 cells are specifically stained by the TRITC-anti-albumin antibody and the pattern of albumin staining is very similar to that of H4 cells (data not shown).

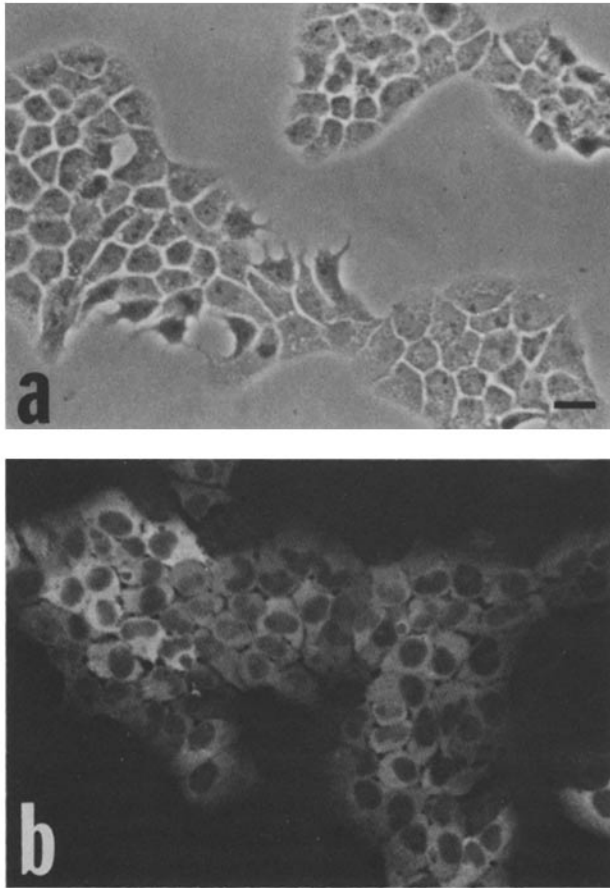


FIGURE 2 Direct immunofluorescence staining of phenylalanine hydroxylase in REB15 cells. The staining procedure is described in Materials and Methods. *a*, phase-contrast micrograph of REB15 cells. *b*, fluorescence micrograph (4-min exposure time) showing staining by TRITC-anti-phenylalanine hydroxylase (0.6 mg IgG/ml). Fluorescence of REB15 control preparations was no brighter than the H4 controls (Fig. 1 *d*). Bar, 25 μ m. \times 200.

phenylalanine hydroxylase activity (27, 28) in H4 cell cultures. Fig. 3 *a* shows that a hydrocortisone-treated H4 population is stained more intensely with TRITC-anti-phenylalanine hydroxylase than is an untreated population (Fig. 3 *b*), i.e., the average cell in the hydrocortisone-treated culture is brighter than the average cell in the untreated culture. Because the treated population displays at least as much variability in staining intensity as the control population, one can conclude that the hormone does not selectively induce only those cells with low enzyme levels; instead, it appears to act on all cells in the population. As would be expected, all cells in the REB15 population also show an increase in phenylalanine hydroxylase content after treatment with hydrocortisone (Fig. 3 *c* and *d*).

Phenylalanine Hydroxylase and Albumin in Isolated Hepatocytes

The immunocytochemical procedures we used for staining cultured hepatoma cells were easily adapted for similar studies with freshly-isolated rat hepatocytes. Fig. 4 shows the results obtained from a typical hepatocyte preparation; equivalent results were obtained in several experiments. TRITC-anti-phenylalanine hydroxylase gives rise to a generalized cytoplasmic fluorescence very similar to that observed for the hepatoma cells; unstained nuclei are clearly demarcated (Fig. 4 *b*).

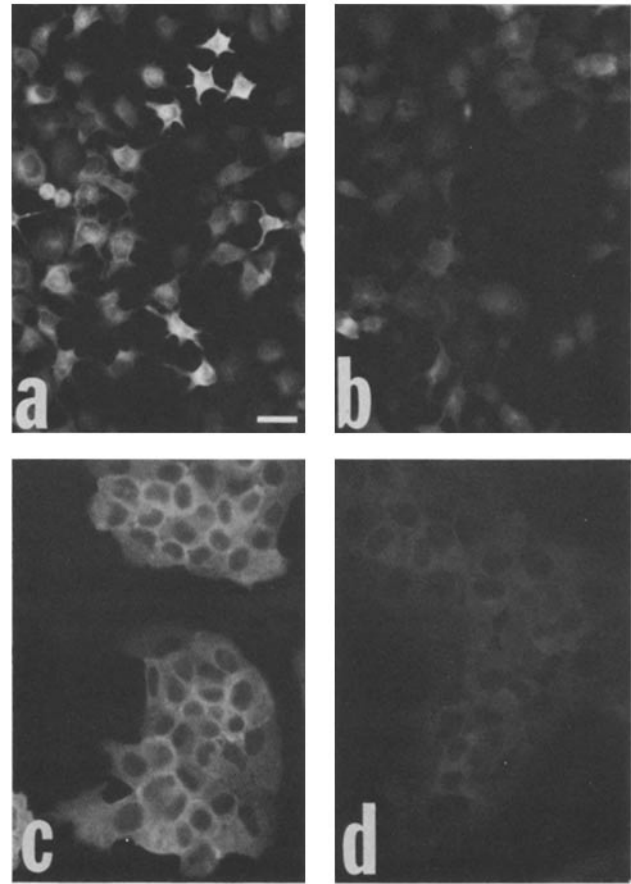


FIGURE 3 Hydrocortisone induction of phenylalanine hydroxylase in H4 and REB15 cultures. Cells were plated on cover slips and grown for 48 h in medium either containing *a* or *c* or not containing (*b* or *d*) 10^{-6} M hydrocortisone. *a* and *b*, fluorescence micrographs (2.5-min exposure times) of H4 cultures stained with TRITC-anti-phenylalanine hydroxylase (0.6 mg IgG/ml). *c* and *d*, fluorescence micrographs (4-min exposure times) of REB15 cultures stained with TRITC-anti-phenylalanine hydroxylase (0.2 mg IgG/ml). The staining procedure is described in Materials and Methods. Bar, 25 μ m. \times 200.

TRITC-anti-albumin fluorescence is localized in brightly-stained "bundles" (Fig. 4 *c*); but, different from the staining pattern seen in the hepatoma cells, hepatocytes contain several small, discrete regions of staining which often appear to encircle the nucleus (Fig. 4 *c* and *Inset*). The specificity of our staining reactions is again demonstrated by the fact that the fluorescence of cells stained with control antibodies is very weak and is uniform (Fig. 4 *d* and *e*). When micrographs were scored (in the same way as described above for the H4 cells) for albumin- and phenylalanine hydroxylase-specific fluorescence, $98 \pm 2\%$ and $89 \pm 2\%$ of the hepatocytes were judged as containing albumin and phenylalanine hydroxylase, respectively, indicating that nearly all cells in our hepatocyte population contain both proteins.

The experiments described above were performed with hepatocytes that had been allowed to attach to collagen-coated cover slips. To ensure that the attachment process did not select a special population of cells, a control experiment was performed in which hepatocytes still in suspension were fixed and specifically stained for albumin using an indirect immunofluorescence procedure. The stained cells were mounted directly and examined by fluorescence microscopy. As observed before, virtually all hepatocytes were found to contain albumin. This

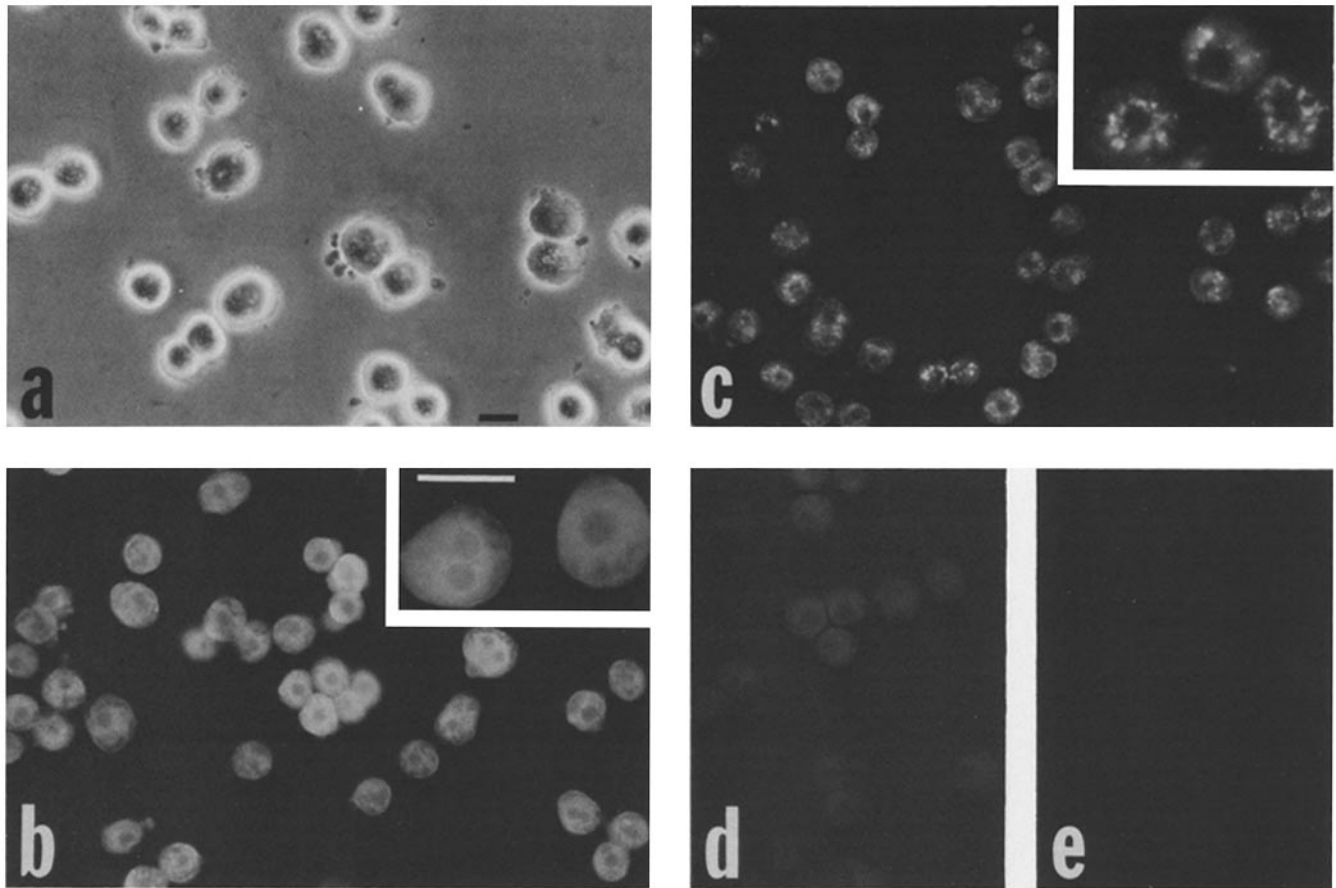


FIGURE 4 Direct immunofluorescence staining of phenylalanine hydroxylase and albumin in isolated hepatocytes. The staining procedure is described in Materials and Methods. *a* is a phase-contrast micrograph of the isolated hepatocytes. *b–e* are fluorescence micrographs; exposure times were 2 min for *c* and *e*, 1.5 min for *b* and *d*. *b*, staining by TRITC-anti-phenylalanine hydroxylase (0.15 mg IgG/ml). *c*, staining by TRITC-anti-albumin (0.08 mg IgG/ml). *d*, control for phenylalanine hydroxylase staining. *e*, control for albumin staining. Bars, 25 μm . $\times 200$, insets, $\times 500$.

result excludes the possibility that attachment to collagen selected a hepatocyte population enriched in albumin-containing cells.

Another control experiment was conducted to eliminate the possibility that the albumin we visualized in isolated hepatocytes had been derived *in vivo* from the circulating blood either by nonspecific binding to the hepatocytes or through fluid endocytosis. Nonspecific uptake was monitored using rat IgG, a major plasma protein, but one not synthesized by liver (29). Fig. 5*a* shows that when hepatocytes are stained for IgG as soon as possible after isolation, a weak but detectable specific fluorescence is observed. This IgG staining is localized to many small, spherical structures dispersed throughout the hepatocyte cytoplasm and is different in appearance from the staining patterns of either albumin or phenylalanine hydroxylase. After a 4-h incubation of the hepatocytes *in vitro*, no detectable IgG remains in the cells (Fig. 5*b*); in contrast, if the hepatocytes are stained for albumin after 4 h (Fig. 6), the results are virtually identical to those obtained without incubation. These findings lead us to conclude that any albumin remaining in the isolated hepatocytes as a result of *in vivo* binding or endocytosis does not contribute significantly to the albumin staining which we observe.

Albumin Secretion by Isolated Hepatocytes

Fig. 7 presents the results of an experiment designed to determine the albumin secretion time, the albumin secretion

rate, and the amount of intracellular albumin of isolated hepatocytes. The secretion time was determined by suspending cells in medium containing [^3H]leucine and measuring ^3H -labeled albumin in the medium during subsequent incubation. Fig. 7*a* shows that ^3H -labeled albumin begins to accumulate in the medium at a constant rate after ~ 27 min. This lag presumably represents the transit of newly synthesized albumin through the smooth endoplasmic reticulum and Golgi apparatus of the cell before its eventual secretion at the cell membrane (30, 31).

In the same experiment, samples of the hepatocyte suspension were removed to determine total secreted albumin and intracellular albumin. Fig. 7*b* shows that the hepatocytes secrete albumin continuously, and after 20 min the secretion rate remains constant at 0.25 mg albumin/ml of packed cells/h. During the initial 20–30 min of incubation, the secretion rate increases; this lag, which is observed consistently, probably reflects the cells' adjustment to their new physiological environment. Significantly, during this interval of increasing secretion rate, the amount of albumin inside the cells is also increasing (Fig. 7*b*). After 20 min, when the secretion rate is constant, the amount of intracellular albumin also remains constant at ~ 0.13 mg/ml of packed cells. This amount corresponds to ~ 31 min of secretion, a value in close agreement with the 27 min time period which elapses before the appearance in the medium of newly synthesized molecules (Fig. 7*a*). (In a separate experiment (data not shown), secreted and intracellular albumin

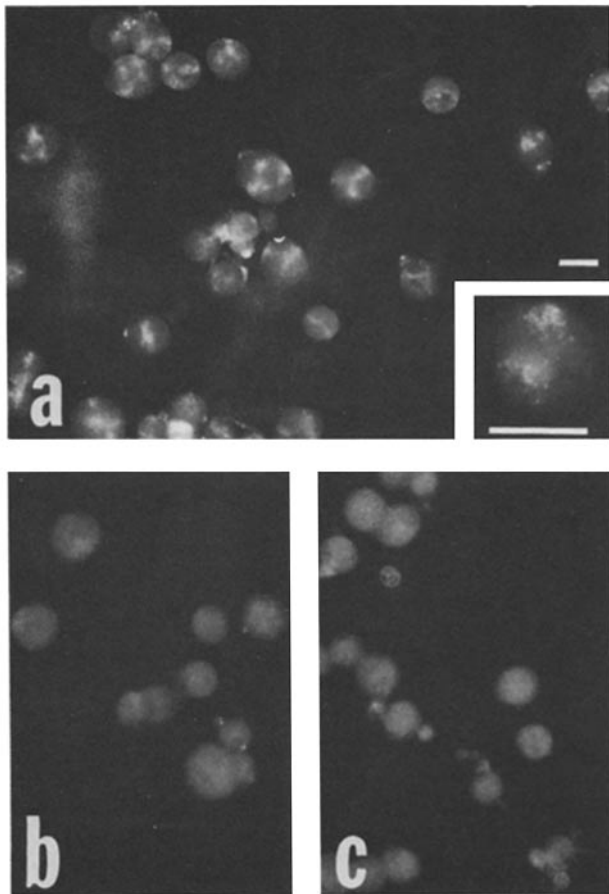


FIGURE 5 Indirect immunofluorescence staining of IgG in isolated hepatocytes. *a*–*c* are fluorescence micrographs obtained using 2-min exposure times. The staining procedure is described in Materials and Methods. Hepatocytes were prepared and stained for IgG either immediately upon isolation (*a*) or following a 4-h incubation in vitro (*b*). *c* shows hepatocytes fixed along with those in *a*, but stained using normal rabbit serum as the “first” antibody. Bars, 25 μm . $\times 200$; inset, $\times 500$.

were measured during a 4-h incubation; the secretion rate, constant after 30 min, was 0.35 mg/ml of packed cells/h, and the intracellular albumin was 0.18 mg/ml of packed cells, representing 32 min of secretion.)

The combined data of Fig. 7 *a* and *b* indicate that within experimental error nearly all intracellular albumin in the isolated hepatocytes can be accounted for as being newly synthesized and in the process of being secreted. These results provide convincing support for the conclusion drawn previously from the IgG immunofluorescence that any albumin present in the degradative compartments of the isolated hepatocytes is quantitatively insignificant by comparison to the albumin in the synthetic and secretory compartments. The fact that we identify albumin immunohistochemically in 98% of the hepatocytes must be interpreted to mean that 98% of the hepatocytes are actively synthesizing albumin. Furthermore, because at least 89% of the hepatocytes also contain phenylalanine hydroxylase, nearly all hepatocytes in our preparations must synthesize both of these proteins.

DISCUSSION

Using specific antibodies as cytochemical reagents, we have identified albumin and phenylalanine hydroxylase in cultured

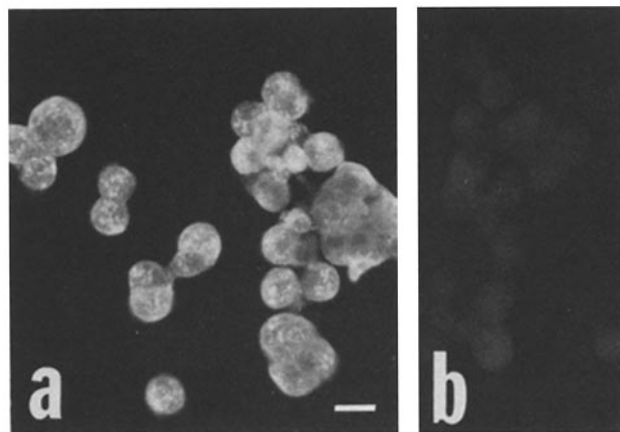


FIGURE 6 Indirect immunofluorescence staining of albumin in isolated hepatocytes incubated for 4-h in vitro. The staining procedure is described in Materials and Methods. *a* and *b* are fluorescence micrographs obtained using 1-min exposure times. *a*, albumin staining. *b*, control staining obtained when normal rabbit serum is used as the “first” antibody in the staining procedure. Bar, 25 μm . $\times 200$.

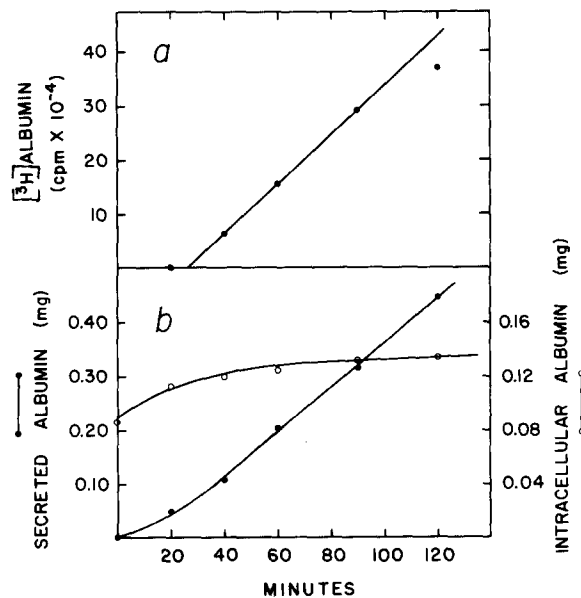


FIGURE 7 Albumin secretion by isolated hepatocytes. 1 ml of packed hepatocytes was suspended in 20 ml of S-77 medium containing 40 $\mu\text{Ci/ml}$ [³H]leucine (New England Nuclear, Boston, Mass.) and incubated at 37°C. At the indicated times, 1-ml aliquots of the suspension were removed for the measurement of total intracellular albumin, total secreted albumin, and secreted [³H]albumin. Details are given in Materials and Methods. Results are expressed per ml of packed cells.

hepatoma cells and in isolated hepatocytes. Subcellular fractionation studies have shown that in liver phenylalanine hydroxylase is a soluble, cytoplasmic component (32), whereas albumin is predominantly found associated with the endoplasmic reticulum and little is found free in the cytoplasm (33). Our immunofluorescence results are completely consistent with these observations. In both the hepatocytes and hepatoma cells, staining by the TRITC-anti-phenylalanine hydroxylase antibody is clearly cytoplasmic; nuclei are unstained. In contrast, TRITC-anti-albumin staining is discretely localized and little generalized staining is evident. Because nearly all intracellular albumin in H4 cells (2) or in isolated hepatocytes is entrained in the secretory pathway, the subcellular structures stained by

the fluorescent albumin antibody must be the organelles involved with albumin synthesis and secretion, i.e. the endoplasmic reticulum and Golgi apparatus (30, 31).

The H4 cell line was clonally derived (1), but phenotypic variability among cloned cells does occur (34, 35), and it would not be totally unexpected to find that albumin and phenylalanine hydroxylase synthesis occur only in given subpopulations of H4 cells. Significantly, however, our results show that at least 98% of H4 cells contain albumin and 86% contain phenylalanine hydroxylase, implying that almost every cell in the H4 population synthesizes both proteins. Qualitatively, then, nearly all H4 cells display a constant phenotype with respect to these two differentiated liver functions. Hydrocortisone, which is known to induce phenylalanine hydroxylase in H4 (27, 28) and REB15³ cultures, appears to induce all cells in the cultures.

Judging from fluorescent staining intensity, an average REB15 cell appears to be brighter than an average H4 cell, implying more phenylalanine hydroxylase per cell in the REB15 than in the H4 cell population. Consistent with this, in quantitative experiments, based on the recovery of immunoprecipitable radioactivity after growth in [³H]leucine-containing medium, REB15 cultures were found to contain an average of 1.8 times more phenylalanine hydroxylase protein per cell than H4 cultures.³

Combined histochemical and biochemical studies have shown that several liver enzymes, e.g., glucose-6-phosphatase, glucose-6-phosphate dehydrogenase, and glycogen synthase, are distributed heterogeneously within the liver parenchyma (36, 37) and that this heterogeneity is preserved in preparations of isolated, single hepatocytes (37). Our immunocytochemical results clearly show that phenylalanine hydroxylase is distributed almost uniformly among isolated hepatocytes. We have assayed phenylalanine hydroxylase enzyme activity in extracts of the isolated hepatocytes and found that they contain at least 80% of the activity found in liver (results expressed either as activity per gram of tissue or as specific activity).⁴ It is unlikely, therefore, that an existing population of phenylalanine hydroxylase-negative cells is being either lost or "turned-on" during our isolation procedure. The implication of our findings is that nearly all liver cells contain phenylalanine hydroxylase. It must be noted that our immunocytochemical method detects the presence of enzyme antigen, not the presence of enzyme activity. This distinction may be relevant with respect to phenylalanine hydroxylase in liver in view of the recent finding that this enzyme can exist in an inactive form (38).

Previous immunohistochemical studies aimed at identifying albumin-containing cells in mammalian liver (human [6, 7]; pig [39]; and rat [3-5, 40]) have produced conflicting results: the majority (3-7) indicated that only 10-50% of hepatocytes contain and, by implication, synthesize albumin; a minority of the studies, one in newborn pigs (39) and the other in rats (40), reported that nearly all hepatocytes do contain albumin. All of these investigations involved immunocytochemical localization of albumin-containing cells in fixed, histologic sections of liver. Although the reasons for the discrepancies in the results are not clear, several points should be noted: in two cases (5, 40), the authors themselves warned of technical problems encountered in tissue and section processing that could markedly affect results; in a review, Schreiber et al. (41) pointed out that

albumin is soluble in the acid-alcohol fixative employed in almost all these studies; and, finally, in no case were serial sections examined, which, given the intracellular localization of albumin, would probably be required to obtain an accurate count of the albumin containing cells.

In our investigation, albumin was identified in isolated rat hepatocytes. For this purpose, the isolated cells offer several advantages over tissue sections: (a) the hepatocytes are obtained free of blood, and rapid, efficient washing is possible; (b) the formaldehyde fixation is rapid; (c) microscopic examination of an entire unsectioned hepatocyte is possible; (d) albumin is stained as bright, easily-distinguishable "bundles" and can be scored unambiguously; and (e) two important control experiments could be performed that could not be easily undertaken either in histologic sections or in vivo; in one, we were able to show, using IgG as a control, that the albumin detected immunocytochemically did not appear to have arisen from fluid endocytosis in vivo but had to have arisen from biosynthesis within the hepatocyte; and, in the other, we found that the measured amount of albumin in the hepatocytes almost quantitatively could be accounted for as newly synthesized albumin in the process of being secreted.

Previous characterization (13) has shown that our isolated hepatocyte suspensions mimic liver with respect to albumin secretion: isolated hepatocytes secrete albumin continuously and at a rate which, when normalized with respect to DNA content, is ~90% of the rate measured for normal perfused liver;⁵ the albumin secretion time for isolated hepatocytes is similar to that of perfused liver (13) or in vivo (42); and, most significantly, the relative rates of albumin synthesis, i.e., albumin synthesis expressed as a fraction of total protein synthesis, are identical in isolated hepatocytes, perfused liver, and liver in vivo (13). Therefore, it is extremely unlikely that the isolation procedure either selects for albumin-synthesizing hepatocytes or induces albumin synthesis in hepatocytes which previously had not been engaged in albumin production. Because our immunocytochemical results demonstrate that 98% of the isolated hepatocytes synthesize albumin, we must conclude that at least in rat nearly all liver parenchymal cells actively synthesize albumin.

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