THE LOCALIZATION OF ALBUMIN AND FIBRINOGEN IN HUMAN LIVER CELLS

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ABSTRACT

Human liver sections were stained with anti-human serum albumin and/or anti-human fibrin monomer fluorescent conjugates. Approximately 10 per cent of the hepatic cells stained specifically for human serum albumin, 1 per cent for fibrinogen, and 0.1 per cent for both. Approximately 18 per cent of the Kupffer cells stained specifically for human serum albumin and 33 per cent for fibrinogen. Staining of both cell types was mainly cytoplasmic, although albumin was found in the nuclei of some parenchymal cells, depending on the method of fixation. Cytoplasmic granules staining specifically for fibrinogen could be seen just inside the cell membrane facing the bile caniculi in many more parenchymal cells than the 1 per cent showing diffuse cytoplasmic staining. The technical difficulties involved in preparing fluorescent conjugates against these antigens and in the fixation of these antigens for immunofluorescent staining are discussed.

The synthesis of biological materials has of necessity been investigated in the cells, or cell fractions, from mixed populations derived from whole organs or parts of whole organs. Even when the population is rather homogeneous, as in the pancreas, the study of enzyme concentrations in various cell fractions prepared by centrifugation of homogenates does not provide information concerning the activity of individual cells in the population, or of individual granules or other organelles. Using labeled antibody, Marshall (1) has provided evidence that chymotrypsinogen, trypsinogen, and ribonuclease are present in most cells in the pancreas. In the case of the liver, Barnhart (2) has found, on the other hand, that prothrombin is detectable in only about 10 per cent of the hepatic cells of normal dogs.

Gitlin, Landing, and Whipple (3) first studied the distribution in many organs of several human plasma proteins, including albumin and fibrinogen. They reported that albumin was present in the liver in the hepatic cell nuclei, and in small amounts in the cytoplasm of hepatic cells in some instances. In contrast, large amounts were found in the sinusoids, and in the portal vessels and their adventitial connective tissue. Fibrinogen was present in the sinusoids and portal areas, but only traces could be found elsewhere. Although occasional hepatic cell nuclei displayed fluorescence, "little if any antigen was detected in the cytoplasm of hepatic cells." Humphrey (4) states that he and Gordon found that serum albumin was detectable by fluorescent antibody in the parenchymal cells of the liver, but only faintly. In the present report we will describe the detection of both human serum albumin and

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fibrinogen, in apparently high concentration in only a small number of the hepatic cells, thereby extending Barnhart's findings to man and to serum albumin and fibrinogen.

MATERIALS AND METHODS

The greatest difficulty encountered in the localization of the normal sites of synthesis or storage of antigenic materials by immunofluorescent methods is in the preparation of sufficiently pure antigen. Impurities contaminating the antigen will evoke antibodies during the preparation of the antiserum and will also be present in the tissue sections. This situation could lead to false conclusions.

Human serum albumin was crystallized three times by the method of Hughes (5). This was kindly supplied to us by Drs. S. Allerton and J. L. Oncley.

Human fibrin monomer was prepared by the method described by Donnelly et al. (6) for bovine fibrin monomer. The starting material was a 3 per cent solution of human fibrinogen in 1 molar sodium bromide, prepared according to the method of Blombäck and Blombäck (7) and kindly supplied by Mr. L. H. Larson of the Division of Biological Laboratories, Department of Public Health, Commonwealth of Massachusetts.[1] The conversion of fibrinogen to fibrin monomer was catalyzed by the use of bovine thrombin. Prothrombin was prepared according to Goldstein, et al. (8), and converted to thrombin by the method of Seegers, McClaughry, and Fahey (9). This preparation was kindly supplied by Dr. L. Pechet and Dr. B. Alexander. An initial attempt to use rabbit thrombin in order to avoid a foreign antigen failed because the method cited for the preparation of prothrombin was ineffective for rabbit prothrombin.

The antigens were finally precipitated with alum by a slight modification of the method of Proom (10): 5 rather than 9 ml of 10 per cent (w/v) aluminum potassium sulfate $(AlK(SO_4)_2 \cdot 12H_2O)$ was added for each 200 mg of protein. In the preparation of the fibrin monomer, care was taken to maintain the pH of the alum solution at 6.5 or below in order to prevent clotting of the fibrin monomer.

Four- to six-pound albino rabbits, caged individually, were injected with 40 mg of one of these two antigens intramuscularly. One month later, following an intravenous injection of 5 mg of 2-(benzhydryloxy)-N,N-dimethylethylamine hydrochloride (Benadryl), they received intravenously 10 mg of the appropriate antigen dissolved in saline. Five days thereafter a small trial bleeding was carried out and the serum was tested by the passive hemagglutination reaction of Stavitsky (11). Monthly intravenous

[1] Starting material, Cohn Fraction I, supplied by American Red Cross.

boosters of fluid antigen in 10-mg doses were administered until the passive hemagglutinin titer was above 300,000. The serum was then harvested.

The antialbumin serum obtained and employed in these experiments had an antibody nitrogen content of 600 γ per ml, and produced only one visible band in Ouchterlony agar diffusion plates against 100 mg per ml of purified human serum albumin and against undiluted normal human serum.

Anti-fibrin monomer rabbit serum had a titer of 1/650,000 in the passive hemagglutinin test. Quantitative precipitin determinations could not be made because conditions which prevented clotting (pH 6.3 or below) inhibited precipitin reactions. This anti-fibrin antibody solution was tested for purity by gel diffusion in Ouchterlony plates against human plasma, human serum, Cohn fraction I, fibrinogen prepared by the method of Blombäck and Blombäck, and fibrin monomer. A strong band of identity appeared between the antiserum and each of these antigen preparations, including the human sera. However, the band in serum was much weaker and could be made even fainter by carefully clotting the blood and separating the serum at 4 hours. This band probably represented the fibrin-antifibrin system. Three or four faint bands were also visible between the well containing the antiserum and the well containing the Blombäck-purified fibrinogen preparation. Since absorption with fibrin monomer of a sample of the conjugate subsequently prepared from this antiserum completely eliminated the staining of human liver sections, it seems unlikely that the antigen-antibody systems represented by the faint lines contributed to the staining of the sections.

Globulin fractions of each of the antisera were prepared by precipitating them three times with 18 per cent (w/v) anhydrous sodium sulfate solution at 37°C (12). The globulins were then conjugated by the method of Marshall, Evelund, and Smith (13) with fluorescein isothiocyanate (Baltimore Biological Laboratory, Inc.) or with rhodamine isothiocyanate (Baltimore Biologicals). With fluorescein isothiocyanate, the ratio of dye to protein was 1:40, and the reaction period was 6 hours at 4°C; with rhodamine isothiocyanate, a dye to protein ratio of 1:20 was used and a reaction period of 18 hours at 4°C. The conjugates were purified by fractionation on diethylaminoethyl cellulose columns as described by McDevitt et al. (14) with DEAE (Selectacel, Carl Schleicher and Schuell Co.). Fractions eluted at pH 6.4 with 0.05 or 0.10 M phosphate buffer were used for staining. Fluorescein isothiocyanate fractions were concentrated to their original volumes by dialysis against 20 per cent polyvinylpyrrolidone, or by precipitation with 18 per cent (w/v) sodium sulfate at 37°C. After sterilization by Seitz filtration, the fractions were stored at 4°C in sterile rubber-stoppered

bottles from which samples were withdrawn as usual with sterile syringe and needle.

Human liver specimens weighing approximately 0.5 grams were obtained by open biopsy from fifteen patients undergoing routine cholecystectomy. These patients ranged in age from 42 to 75 years. Part of each specimen was put in a test tube and frozen within 5 minutes after the biopsy by immersing the tube in an ethanol-dry ice mixture. The tubes were stored at $-14°C$ until frozen sections could be cut. The remainder of the specimen was cut into smaller pieces, each of which was placed in one of the various fixatives described in the section on Results.

Frozen sections were cut in a cryostat at $-20°C$ and the sections fixed on the slide by various methods to be described later.

Controls of specificity were carried out with conjugate from which specific antibody had been absorbed by the addition of antigen. In the albumin antialbumin system an amount of antigen calculated from the precipitin curve to be in slight antigen excess was employed. The same amount of antigen was used in the fibrinogen antifibrin monomer system since hemagglutination titers and optimum Ouchterlony plate concentrations were comparable, and, as indicated above, precipitin curves could not be obtained.

RESULTS

ALBUMIN: Immunofluorescent staining of human serum albumin was most satisfactory in sections that were cut from tissue blocks fixed in cold 95 per cent ethanol containing 1 per cent of glacial acetic acid by volume. The blocks were kept for 18 to 42 hours at 4°C in this solution and were then dehydrated, cleared, and embedded as described by Sainte-Marie (15).

Microtome sections were cut from such blocks at room temperature, floated on warm water, and mounted on clean slides without an adhesive. They were dried for 15 to 30 minutes in a warm room at 37°C. The paraffin was removed with xylene, and the sections were hydrated through successive ethyl alcohol baths (95 per cent, 80 per cent, 60 per cent, and 45 per cent C_2H_5OH) and finally washed in three changes of buffered saline (0.85 per cent sodium chloride with 0.05 M phosphate buffer, pH 7.0) for 5 minutes each.

Sections were stained with a fluorescein-labeled, antialbumin conjugate in a moist chamber at room temperature, for periods ranging from 20 to 120 minutes. They were then rinsed three times, washed in buffer for 10 minutes at room temperature, mounted in glycerol containing 10

per cent buffered saline, and examined under the Zeiss fluorescence microscope.

By this method it could be shown that only scattered cells, occurring singly or in groups throughout the lobule, contained albumin, and that the majority of these cells were found along the central veins. Fig. 1, though a Zenker-fixed section, illustrates this.
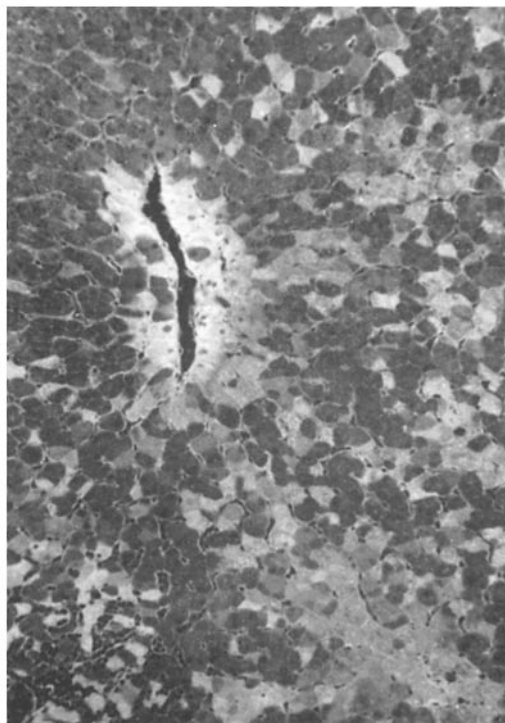

FIGURE 1 Albumin. The cells containing albumin are revealed by their green fluorescence, which appears white or gray. Paraffin section fixed with Zenker's solution, stained with fluorescein-labeled anti-human serum albumin conjugate. 54-year-old human female. $\times$ 170.

Higher magnification revealed that the fluorescence was confined to the cytoplasm of the hepatic cells (Fig. 2) and that the cytoplasm contained a finely woven network. The individual parenchymal cells stood out in sharp contrast against the dimly illuminated cells adjacent to them (Fig. 2). The positive cells seemed to fall into two classes with respect to the intensity of the fluorescence of their cytoplasm: about half were brightly fluorescent, the other half, weakly fluorescent. A surprisingly large number of albumin-positive cells were binucleate. Albumin could also be seen

in the cytoplasm of Kupffer cells, but not in their nuclei.

An estimate of the percentage of cells containing albumin was obtained by counting the number of fluorescent cells in three adjacent sections of the livers of five patients (250 to 750 cells), and then counting the total number of hepatic and Kupffer cells after staining the sections with hematoxylin-eosin (3000–5000 cells). Table I indicates the percentage of cells which contained albumin.

ALBUMIN WITH OTHER FIXATIVES: Of the many different fixation methods used in attempts to find an ideal one for serum albumin, Zenker's fixative with acetic acid was the only other satisfactory one. Cubes (4–6 mm sides) of freshly biopsied liver were dropped into Zenker's solution at 4°C and kept in it at 4°C for 4 hours. All subsequent

nuclei (Fig. 3). Cells which had no albumin in their cytoplasm did not manifest nuclear staining. The control sections, stained with antialbumin conjugate which had been adsorbed by human albumin, showed no fluorescence in either the cytoplasm or the nucleus.

FIBRINOGEN: Experiments with the fixation of fibrinogen demonstrated that its antigenicity was easily destroyed by techniques involving paraffin embedding. The most successful method for demonstrating it required the use of frozen sections. The frozen sections were fixed in 95 per cent ethanol containing 5 per cent glacial acetic acid by volume for 15 minutes at either 4°C or 25°C. They were then rinsed four or five times in cold staining buffer, and stained with anti-fibrin monomer conjugates for periods ranging from

TABLE I

Cell Populations which Stained with Fluorescent Anti-Human Serum Albumin Conjugates

Age and sex of patients	Fixative	Total parenchymal cells stained	Brightly stained	Weakly stained	Kupffer cells
		per cent	per cent	per cent	per cent
41 ♂	95 per cent ethanol with 1 per cent acetic acid	15	6	9	18
43 ♂	Zenker's solution	5	2	3	18
75 ♂	" "	10	5	5	24
59 ♂	" "	11	4	7	11
54 ♀	" "	10	5	5	21

steps in the fixation were carried out at room temperature. The blocks were placed in 95 per cent ethanol containing 0.2 per cent by volume of Lugol's solution for an hour, next in an aqueous solution of 0.25 per cent (w/v) sodium thiosulfate solution to remove the iodine, and then in distilled water for 1 hour. They were finally transferred to 70 per cent ethanol and were subsequently handled in the manner described above for blocks fixed in ethanol-acetic acid.

Sections cut from these blocks and stained for albumin presented a picture somewhat different from that of sections from alcohol-fixed material. The cells along the edge of the block were all uniformly stained to a depth of about 2 cells. This is interpreted as an artifact. The distribution of cells was similar to that described after alcohol fixation (Table I). There was, however, a new finding: many of the cells containing albumin in the cytoplasm also showed albumin in the

20 to 120 minutes. Some samples of the anti-fibrinogen solution were labeled with rhodamine isothiocyanate in addition to those labeled with fluorescein isothiocyanate. These sections were then washed, mounted, and examined as described for albumin.

Cells containing fibrinogen were less numerous than those containing albumin. They were scattered singly or, occasionally, in pairs throughout the liver lobule, with somewhat more in the portal areas just beneath Glisson's capsule than in the central areas around the central vein. However, the majority of fibrinogen-containing cells lay scattered throughout the lobule between these two locations.

Individual cells stained for fibrinogen showed fluorescence throughout the cytoplasm in very small granules or network-like arrangements difficult to resolve under the light microscope (Fig. 4). The nuclei were empty. In addition to these

brightly stained cells in which the whole cytoplasm was involved, other hepatic cells contained small amounts of fluorescent granular material in the cytoplasm just beneath the cell membrane along the bile canaliculi (Fig. 5). This picture characterized the great majority of all hepatic parenchymal cells in contrast to the very few which contained large amounts of fibrinogen and the relatively few which contained albumin. There was no analogous appearance of small albumin granules.

Approximately one quarter of the population of brightly stained cells containing large amounts of fibrinogen were binucleate. Table II indicates that these cells constitute from 0.5 to 1 per cent of the parenchymal cells in the livers investigated (six patients). The total population counted ranged from 5 to 10 thousand cells per patient, among which 250 to 750 bright fluorescent cells were found. Fibrinogen was also found in 17 to 45 per cent of the estimated total population of Kupffer cells based on counts in the hemotoxylin-eosin–stained sections.

STAINING FOR BOTH ANTIGENS IN THE SAME SECTION: Since the ideal fixation conditions varied considerably for the detection of the two antigens, a compromise procedure was developed: 1 per cent acetic acid in 95 per cent ethanol followed by paraffin embedding. It was found that the sections were best stained by exposing them first to rhodamine-labeled anti-fibrinogen, and then to fluorescein-labeled anti-albumin. The first reagent was left on for 30 minutes, the second, for 2 hours. For reasons which we do not understand, mixing the two reagents and staining for both substances at the same time failed to stain as well for either antigen.

Under these conditions, cells stained for fibrinogen and for albumin were readily identifiable and appeared in the expected numbers and locations. An occasional cell was found which stained for both fibrinogen and albumin, although in most instances only one or the other substance was found in a cell. Only about one cell in a thousand contained both proteins. In other words, approximately 1 in every 10 cells containing substantial amounts of fibrinogen also contained some albumin; of every 100 cells containing albumin, only one contained fibrinogen as well.

CROSS-REACTIONS BETWEEN FLUORESCENT ANTI-HUMAN SERUM ALBUMIN AND ANTI-HUMAN FIBRINOGEN CONJUGATES WITH LIVER CELLS OF OTHER SPECIES:

As a matter of interest and also as a control, our results with human liver sections were compared with the results of similar experiments on sections prepared from the livers of monkey, mouse, guinea pig, rat, and rabbit (Table III). For the determination of albumin, tissue blocks were fixed in 95 per cent ethanol containing 1 per cent by volume of glacial acetic acid and embedded in paraffin; those to be examined with the anti-fibrinogen conjugate were quick frozen, cut frozen, and fixed with 95 per cent ethanol containing 5 per cent glacial acetic acid. It is interesting to note that both monkey and mouse livers contained albumin which cross-reacted with anti-human albumin. The distribution of albumin-containing cells was similar to that found in human liver, but the number of such cells was fewer than in human liver. The significance of the positive staining in Kupffer cells and in blood in the liver sinusoids in the guinea pig and the rat is unknown.

Mouse liver did not cross-react with anti-human fibrinogen although, as might be expected, monkey liver did. The number of cells stained was fewer, but the distribution was similar to that of human liver fibrinogen. Here, too, the Kupffer cells and sinusoids of the guinea pig and the rat liver were stained for unknown reasons.

It is reassuring to note that no element of the rabbit liver stained with either of these conjugates, a result to be expected with conjugates prepared from rabbit serum, provided no non-specific reactions occurred.

It may be remarked in connection with these experiments that the use of mouse liver powder to absorb conjugates prepared against human serum albumin could remove the specific antibody and diminish or abolish specific staining. It is conceivable that this accounts for some of the failures of the past.

DISCUSSION

The rapid increase in knowledge of cellular function which has come from analyses of the chemical composition of cells and subcellular fractions is now being supplemented by specific cytochemical information on single cells. But the temptation is still strong to consider similar cells equivalent. Particularly in the liver, where the parenchymal cells appear so similar when examined by conventional morphological methods, it has been difficult to resist the assumption that each hepatic cell is the functional equivalent of every other. However, recently Wilson pointed out that
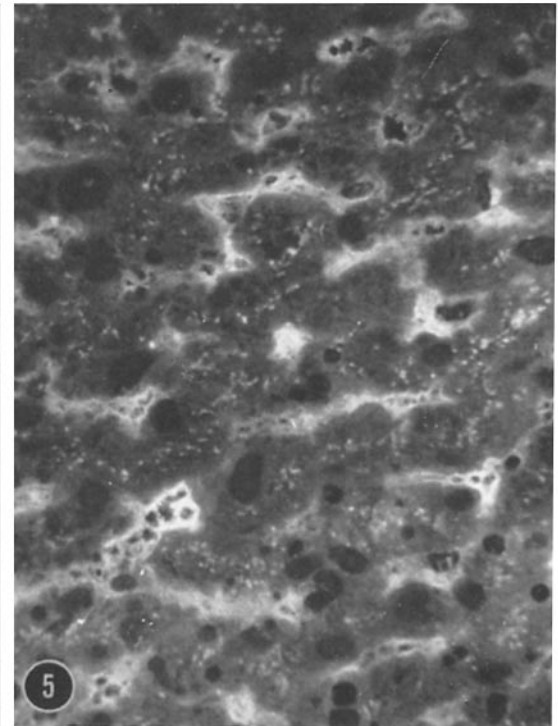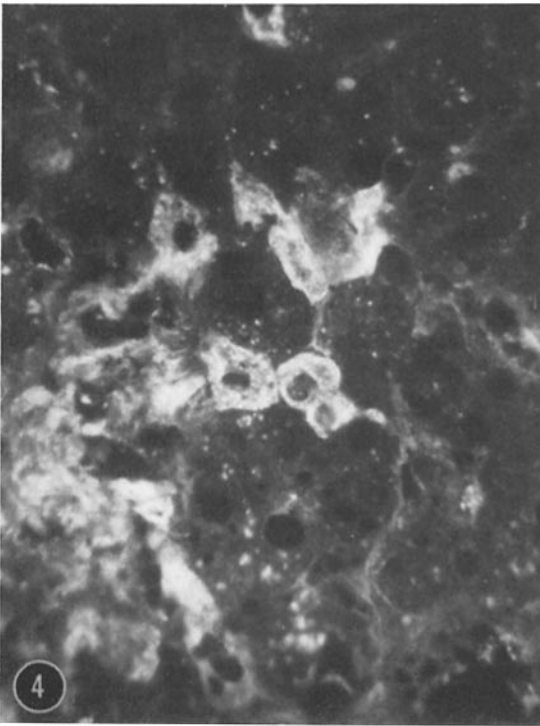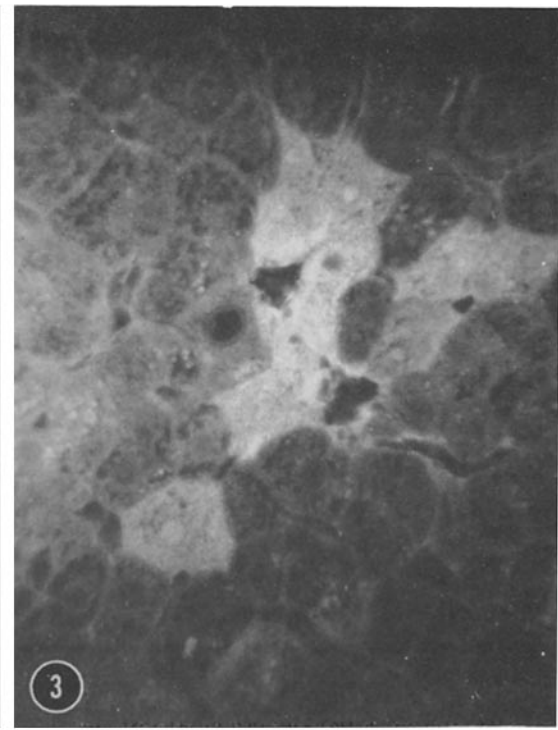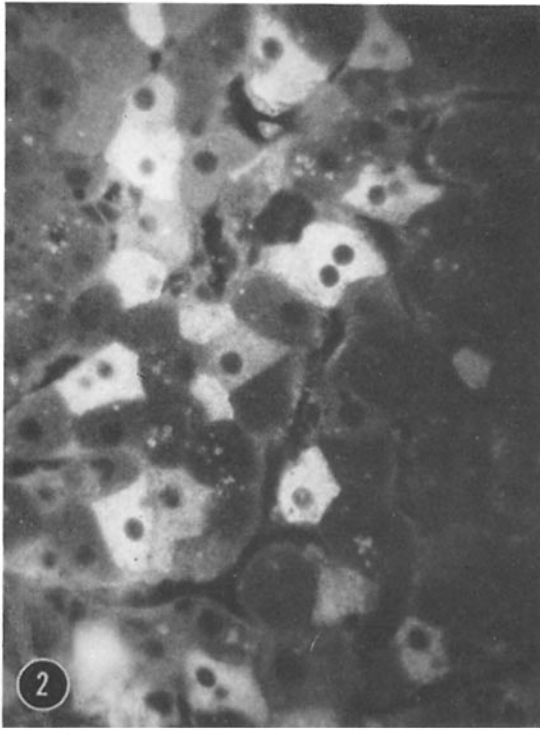
TABLE II

Cell Populations which Stained with Anti-Human Fibrinogen Conjugates

Age and sex of patients	Fixative	Type of section and staining	Total cell population stained	Intralobular distribution	Cells surrounding central vein	Peri-Glisson's capsule cells	Kupffer cells
			per cent	per cent	per cent	per cent	per cent
43 ♂	95 per cent ethanol with 1 per cent acetic acid	Paraffin, fluorescein	0.6	0.3	0.1	0.2	36
"	" "	Paraffin, rhodamine	0.6	0.2	0.1	0.3	17
"	95 per cent ethanol with 5 per cent acetic acid	Frozen, fluorescein	1.5	0.8	0.1	0.6	45
59 ♂	" "	" "	0.9	0.5	0.1	0.3	39
65 ♀	" "	" "	1.2	0.5	0.1	0.6	23
75 ♂	" "	" "	0.5	0.3	0.1	0.1	41

TABLE III

Staining Results with Liver from Other Species

	Stained with rabbit anti-human serum albumin conjugate			Stained with rabbit anti-human fibrinogen conjugate			
	Parenchymal cells	Kupffer cells	Sinusoid	Parenchymal cells	Fine granules in cell	Kupffer cells	Sinusoid
Monkey	+	+	+	+	+	+	+
Mouse	+	+	+	−	−	−	−
Guinea pig	−	+	+	−	−	++	++
Rat	−	+	+	−	−	+	+
Rabbit	−	−	−	−	−	−	−

FIGURE 2 Albumin. Shows brightly fluorescent cytoplasm, and the frequency of pairing of cells containing albumin. The small fluorescent granules are not green, but white or pink fluorescing material present in the liver. Paraffin section fixed with 1 per cent acetic acid and 95 per cent ethanol, stained with fluorescein-labeled anti-human serum albumin conjugate fraction. 43-year-old human female. × 650.

FIGURE 3 Albumin. Higher power to demonstrate presence of albumin in hepatic nuclei. Same section as Fig. 1. × 650.

FIGURE 4 Cells containing fibrinogen. Many areas contained fewer cells. Frozen section fixed with 5 per cent acetic acid and 95 per cent ethanol, stained with rhodamine-labeled anti-human fibrinogen conjugate, 75-year-old human male. × 650.

FIGURE 5 Fibrinogen in Kupffer cells, and in small granules in hepatic cell cytoplasm along the bile canaliculi. Frozen section fixed with 5 per cent acetic acid and 95 per cent alcohol, stained with fluorescein-labeled anti-human fibrinogen conjugate. 64-year-old human male. × 650.

"While all liver cells are presumably potentially alike, they may not all be doing the same things at the same time, and the same cell may not be doing the same things all the time. Whether or not it can perform many of its varied functions simultaneously is an important but unanswered question" (16). In this connection, it is interesting to note that Marshall (1) reported that, in the pancreas, ribonuclease and deoxyribonuclease were sometimes found in the cytoplasm in different sets of cells.

Clear evidence that hepatic cells contain different amounts of a protein synthesized by the liver was presented by Barnhart (2) in 1960 in studies of prothrombin. She employed solutions of antibody prepared in the rabbit against highly purified bovine prothrombin. Such antibodies, rendered fluorescent by coupling with rhodamine or fluorescein isothiocyanates, reacted with hepatic cells to reveal their prothrombin content. Only a fraction of the parenchymal cells fluoresced, indicating that most liver cells contained no prothrombin demonstrable by this method. Specific absorption with prothrombin of a sample of a fluorescent antiprothrombin solution removed its power to react with any hepatic cells. The distribution of the positive cells was uneven, varying from lobule to lobule; probably for this reason, she refrained from making an estimate of the number of cells engaged in the synthesis or storage of prothrombin. In subsequent experiments in the dog, preliminary accounts of which are available (17, 18), Barnhart and Anderson have reported that Dicoumarol (coumarin) "blocked the synthesis" of prothrombin, an interpretation based presumably on a diminished number of stained cells. Within 3 hours of the administration of vitamin K, prothrombin was demonstrable in all hepatic cells but only later did the prothrombin level in the blood stream rise, indicating a delay in its release.

Our studies indicate that albumin was present in only about 10 to 15 per cent of hepatic cells, and fibrinogen in only 1 per cent. The experiments carried out to determine whether these two products of the liver cell are ever present concurrently were blunted by the compromise necessary in the choice of fixative, which was suboptimal for fibrinogen. Even so, an occasional cell staining both green and orange was encountered, indicating the presence of both proteins.

Previous difficulties in the search for albumin (3, 4) and fibrinogen (3) in the liver were evidently due to the rapid loss of antigenicity, as well as the difficulty of fixing albumin. Loss of antibody during mouse liver adsorption of anti-human serum albumin conjugates is another potential source of difficulty. Even under the conditions described here, it is possible that some antigen escaped detection since the results obtained differ, depending on whether the tissue is fixed in Zenker's solution or acid-alcohol.

The finding of albumin in hepatic nuclei confirms earlier reports on sections prepared, unfixed, with the cold microtome (3), and the occasional observation of antibody (19) and of foreign protein (20, 21) in the nucleus, in frozen sections. To further investigate the significance of these findings it would be desirable to study the penetration of the nucleus by cytoplasmic proteins, native or foreign, in sections fixed by techniques unlikely to produce the artifacts encountered in frozen sections.

The synthesis and release of liver proteins appear to be under firm control, regulated, perhaps, by the blood levels of the specific proteins. The results of Barnhart and Anderson (17, 18) indicate that the number of cells containing prothrombin can increase dramatically in response to demand, from almost none to practically all. Similarly, our data show that in the steady, normal state only a small proportion of hepatic cells contain albumin and fibrinogen. The synthesis of albumin appears to be an all-or-none enterprise. However, the fact that tiny granules containing fibrinogen are present in many liver cells suggests that a small quantity of both fibrinogen and albumin may always be present, but that the albumin is more difficult to detect because of greater solubility.

Additional investigations on the distribution of specific proteins in the liver cells of animals subjected to conditions which alter the blood level of these proteins would be of considerable interest, and might help us understand the factors which control protein synthesis in the complex hepatic cell.

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