The Dynamics of α-Fetoprotein and Albumin Synthesis in Human and Rat Liver During Normal Ontogeny

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The dynamics of synthesis of α-fetoprotein (AFP) and albumin have been studied in rat and man by immunocytochemical localization of each protein in the liver and its quantitation in serum at different periods of normal intrauterine and extrauterine life. The proteins are present only in the cytoplasm of hepatocytes. During physiologic development, an inverse relationship exists between the serum concentrations of the two proteins, the latter for each appearing to be directly related to the number of hepatocytes synthesizing it. AFP-containing cells are randomly distributed, while albumin-containing cells are more uniformly spread out. Both, however, are often preferentially located around venous channels. In the case of AFP-containing cells, the last to disappear with advancing postnatal age are seen around the hepatic veins. When both proteins are present in serum, several hepatocytes seem to synthesize the two simultaneously, though others contain only one of them. It is unlikely that in the physiologic state different populations of hepatocytes are assigned to synthesize AFP and albumin separately. (Am J Pathol 86:359–374, 1977)

AMONG THE MULTITUDE OF FUNCTIONS the mammalian liver performs, a major one is the synthesis of certain categories of proteins for secretion to blood. Albumin and α-fetoprotein (AFP) are important members of this class of proteins. Under physiologic conditions, serum concentrations of the two show reciprocal variations during different phases of prenatal and postnatal life.[1,2] Albumin is present through most part of life at relatively high concentrations, rising from lower values in the fetus to a high plateau in the adult. Levels of AFP on the other hand are high only in the embryo and fetus, quickly dropping to extremely low values soon after birth.[1-7] An increase may again occur in the postnatal period in the event of development of certain hepatic disorders associated with neoplastic or restorative proliferation of liver cells.[5,8-13] The two proteins, though immunologically distinct, show similarities in several of their physicochemical properties,[14-16] and available evidence indicates that both are synthesized either almost exclusively or mainly in the liver,[17] predominantly by the hepatocyte.[18-20] Unlike that of albumin, the precise physiologic function of AFP is unknown. Recently, using tissue culture systems the phases of cell generation cycle during which these proteins are

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synthesized and secreted have been identified.[21-22] Also, tumorous liver cells growing in culture appear to produce AFP and albumin separately.[21] However, the relative biology of synthesis and storage of the two proteins in the *in vivo* physiologic state remain essentially unexplored. For example, it remains to be ascertained whether at any given time different liver cells are assigned the task of producing albumin and AFP or whether the same cells produce the two simultaneously or at different times. It is also unknown whether the variation in serum level of each protein seen at different periods in life is due to recruitment of varying number of hepatocytes producing it or merely to alterations in the amount synthesized by an otherwise fixed number of preassigned cells.

We have earlier reported adequate and reliable localization of AFP in the hepatocytes by immunoperoxidase staining performed on specially prepared paraffin sections of the liver.[19] The present study was designed to identify and compare with the use of this immunohistochemical technique and also by immunofluorescence, the populations of hepatocytes that synthesize AFP and albumin during normal physiologic growth and development. Valid comparisons between staining of cellular constituents in identical cells by application of immunochemical and other procedures on consecutive serial paraffin sections of liver have recently been demonstrated for AFP [18,19] and hepatitis B antigenic components.[23,24]

Materials and Methods

Liver and blood collected from normal healthy rats and humans at various periods of intrauterine and extrauterine life were studied.

Collection and Processing of Livers

Rat

Sprague-Dawley rats outbred in our animal house were used. Three to five livers were examined at each age period of study. Livers from fetuses were collected at 7, 12, 15, 18, and 21 days of gestation. In the postnatal period, livers were obtained from newborn pups between 0 and 12 hours of birth and from young and adult animals at ages of 4, 7, 14, 18, 21, 28, 35, 60, and 120 days.

All animals, 35 days and older, were sacrificed by exsanguination following light ether anesthesia. Younger rats and fetuses were decapitated.

Human

Livers from fetuses delivered at 16, 22, 28, and 40 weeks of gestation by hysterotomy or by induction were obtained from the abortion clinic of our hospital. Postnatal liver specimens were obtained by needle or wedge biopsies from infants, 1- and 4-week-old children, and from adults who underwent abdominal surgery for nonhepatic lesions. Three to five livers were examined for each age period.

All tissues were cut into small pieces not larger than 1 × 2 × 3 mm and were immediately fixed in cold acetic alcohol and processed for paraffin embedding as described

by us earlier.[19] Paraffin blocks were stored at 4 C and generally sectioned within few hours of preparation.

Collection of Blood

Along with the liver, blood was collected in each case for detection and estimation of the levels of albumin and AFP in serum. In the rat, blood from fetuses, newborns, and young pups up to 28 days was collected by decapitation, while in the older animals cardiac puncture was carried out after opening the chest under anesthesia. In fetuses, newborns, and 4-day-old pups, each sample represented pooled serum from 3 to 5 animals. In older rats, blood was separately collected from each animal.

Blood from human fetuses and newborns was collected from the cord and in the adult, venipuncture was performed. At each time period three to six samples were examined.

Detection and Quantitation of AFP and Albumin

Detection

Initial testing was done by diffusion, in 1% Noble agar (Difco), of appropriately diluted serum (1:100 to 1:1000 for albumin and neat to 1:25 for AFP), against the respective antirat or antihuman rabbit antiserum. In our laboratory the sensitivity of this technique is down to a concentration of approximately 4/μg/ml protein. All samples were positive for albumin. In the case of AFP, samples which were negative by this test were put up for counterimmunoelectrophoresis (CIEP) in 1% agarose (Sigma chemical) against specific antisera, according to the method of Alpert *et al.*[25] The sensitivity of this technique in our hands is down to 250 ng/ml of AFP.

Quantitation

Laurell's rocket technique of electroimmunodiffusion [26] was carried out with suitably diluted test sera, using 1% agarose containing 2.5 to 3% specific antiserum. The following standards were used: a) human cord serum with known concentration of AFP and b) purified rat AFP, both supplied by the International Agency for Research on Cancer; c) pure human albumin obtained from National Blood Transfusion Centre, Paris; d) rat albumin purified in our laboratory from pooled normal adult rat serum by initial precipitation with half saturated ammonium sulfate and subsequent column chromatography of the supernatant in Sephadex 200.[27] The third peak (elution volume 32 to 38 ml) tested for a high protein content by spectrophotometry at 280 nm wave length was concentrated, after lyophilization. In immunoelectrophoresis with rabbit antiserum against normal adult rat serum (prepared in our laboratory), this preparation (reconstituted to 1 ml) gave a single precipitation arc in the mobility region of albumin.

Antisera

Methods of preparing monospecific antisera against human and rat AFP as well as against horseradish peroxidase (anti-HRP) have been described by us earlier.[19] Antisera against rat and human albumin were raised in rabbits using the same purified albumins described above as standards for the quantiation of serum proteins. The immunization schedules were identical to those described for anti-AFP sera. When adequate antibody titers were obtained, the antisera were adsorbed against small amounts of species-specific globulins. Monospecificity of all antisera was verified by immunodiffusion, immunoelectrophoresis, and counterimmunoelectrophoresis (CIEP). Standard antihuman and antirat AFP sera obtained from the International Agency for Research on Cancer and from Dr. Abelev were used in parallel as controls.

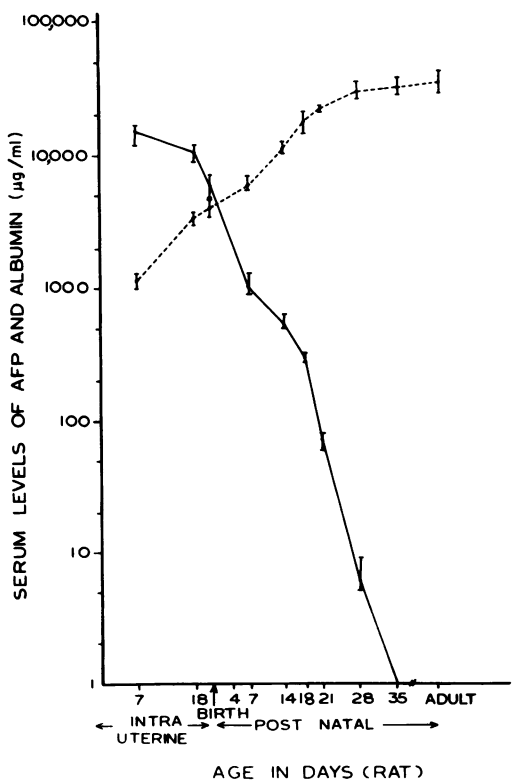
Anti-rabbit immunoglobulin (IgG) from goat, unconjugated (GARG) and conjugated

with FITC (FGARG), were obtained from Cappel Laboratories, Inc. Specificity of these was tested by gel diffusion and immunoelectrophoresis against our anti-AFP and antialbumin sera as well as against normal rabbit globulins.

Tissue Localization of AFP and Albumin

Paraffin blocks were sectioned at 3 μ. From each liver, serial sections picked up on clean glass slides were numbered 1 to 6. Sections 1 to 3 were used for immunofluorescence (IF) staining and Sections 4 to 6 for immunoperoxidase (IP) staining. Sections 1 and 4 were stained for AFP and Sections 2 and 5 for albumin. Sections 3 and 6 served as controls. Thus in each liver, localization of the two proteins could be compared in either of the immunostained preparation (Section 1 with 2 in IF and Section 4 with 5 in IP) while that of each protein could be compared in sections stained by IF and IP (Section 1 with 4 for AFP and Section 2 with 5 for albumin).

Immunostaining for each protein was carried out by using the respective monospecific antisera according to the techniques described for AFP by us.[19] Indirect IF was done by treating the sections with species-specific anti-AFP or antialbumin serum followed by FGARG. IP staining was carried out using in succession anti-AFP or anti-albumin serum, GARG, anti-HRP and horseradish peroxidase (Type II, Sigma Chemical) in the sandwich technique, followed by staining with diaminobenzidine (DAB). Sections treated with normal pooled rabbit serum served as controls. In addition, specificity of staining for each protein of the two species was tested by the following controls before the experiments and



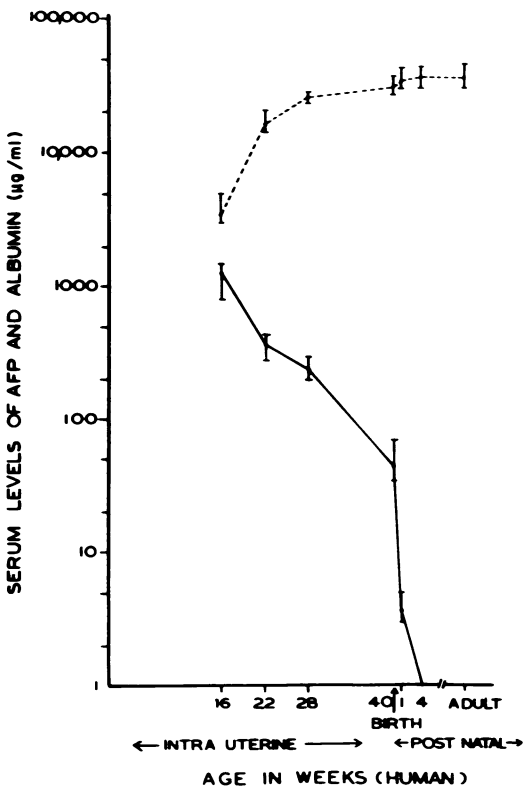
TEXT-FIGURE 1—Serum AFP (*solid line*) and albumin (*dotted line*) concentrations in the rat at different periods of intrauterine and extrauterine life. Bars at each age period represent mean and range of values.

also in randomly selected sections during the staining procedures: a) treatment with antiserum previously neutralized by corresponding protein; b) only HRP followed by histochemical staining (DAB); and c) only histochemical staining (DAB). All tissue sections stained for peroxidase were counterstained with methyl green. A Zeiss fluorescent microscope was used to study the IF preparations and IP-stained tissues were examined under light microscope.

Results

AFP and Albumin in Serum

Concentrations of the two proteins in the serum of rats and human at different phases of the intrauterine and postnatal life are depicted in Text-figures 1 and 2. It is clear that in both species the curves for serum levels of the two proteins show an inverse relationship. In the rat, the level of serum AFP steadily declines from 15,000 μg/ml on the seventh intrauterine day to less than 250 ng/ml at 35 postnatal days and afterwards. During this period serum albumin rises from 1,150 to 34,000 μg/ml (Text-figure 1). In the human also, serum AFP levels drop steadily from 1250



TEXT-FIGURE 2—AFP (*solid line*) and albumin (*dotted line*) concentrations in human serum at various periods of intrauterine and extrauterine life. Bars at each age period represent mean and range of values.

μg/ml at 16 weeks of intrauterine age to less than 250 ng/ml at 4 weeks of postnatal age; during this time albumin levels rise from 3500 to 38,000 μg/ml (Text-figure 2). Techniques used in this study could not detect concentrations of AFP below 250 ng/ml.

Localization of AFP and Albumin in Liver

The two proteins, albumin and AFP, whenever present could be localized by both IF and IP staining. In comparable sections, sites of localization of either protein were identical by the two techniques. However, as has been reported earlier,[19] the IP preparation offered distinct advantages over the IF one, particularly with regard to clarity in the morphology of localization and ease of comparison.

The descriptions for the localizations of the two proteins that follow are therefore based on observations made on the IP-stained tissues. Positive staining for protein was recorded when dark brown colored coarse or fine granules were observed throughout the entire cytoplasm or in a large part of it. The intensity of staining in individual cells seemed to vary from area to area and within one area as well. Accentuated intensity was frequent in perivascular areas (central or peripheral part of lobule). Attempts to quantitate the proportion of positively staining cells had to be abandoned because of the extremely random distribution of such cells in the lobule. Even when the stained cells were present mainly as narrow mantles around vascular territories, the distribution and number of such cells in any given tissue section were unpredictable. Evaluation of a very large number of sections from different parts of the liver, which may have lent some statistical significance to counts of positive cells, was considered impractical and not strictly essential for the present study.

AFP

The protein was localized in the cytoplasm of hepatocytes only, whether of the adult or the small fetal type. Kupffer cells and other cells did not stain. Perivascular location of positive cells was generally conspicuous but random. Coagulated serum in the larger veins and sometimes in the perivenous tissue in livers of very young fetuses also stained for the protein.

In the rat, AFP-containing cells were maximally seen at 12 days of fetal age and comprised of a major part (approximately four-fifths) of the hepatocyte population. This number progressively declined through 15 and 18 days of fetal age till at birth such cells amounted to less than half the total number of hepatocytes (Figures 1A and 2A). In the 15-day fetus a distinct concentration of positive cells around the central vein of the

lobule and other hepatic venous tributaries first became obvious. Simultaneously, such cells in the rest of the lobule became fewer and were seen as small or large clusters. In subsequent periods, AFP-containing cells diminished in all parts of the lobule, but particularly so in areas away from the central vein. At and soon after birth, besides being present as cuffs around several but not all central veins, stained cells were often scattered singly or in small groups in the rest of the lobule as well (Figure 2A). Their number gradually fell with advancing fetal age. Four days after birth, AFP-positive cells were mainly present as two to four layers around the central veins though a few were also scattered in the lobule (Figure 3A). At 7 days a thinner mantle of cells was stained in the central area and only an occasional one in the lobule (Figure 4A). A week later the former cells were still fewer and no intralobular localization was observed. At 18 days, perivascular cells were further reduced and at 21 days, only occasional positive cells were observed at this location (Figure 5A). By 28 days after birth AFP was localized only in a stray hepatocyte, and later none were observed.

In the human liver the distribution, approximate number, and staining intensity of AFP-containing cells were closely similar to those in the rat. A large number of hepatocytes showed positive staining at the 16th week of fetal life. Their number gradually fell with advancing fetal age (Figure 6A). No AFP could be demonstrated in liver cells at 1 week after birth and later.

Albumin

As for AFP, the pattern of distribution, number, and intensity of staining of albumin-containing hepatocytes was similar in the rat and human. Positive cells were more diffusely and uniformly distributed in the lobule than the AFP-containing ones at comparable periods (Figures 1B, 2B, 3B, 4B, 5B, and 6B), though perivascular accentuation was still obvious. Unlike AFP, an accentuation of albumin localization was seen predominantly around portal tracts as well as around hepatic veins (Figures 5B, 6B, and 7A). The number of albumin-containing cells increased with advancing fetal age until sometime before birth, at 40 weeks in human and 21 days in rat fetuses, almost all hepatocytes stained positively for the protein (Figure 5B). At this age and later, the location of stained cells was generally diffuse, though in some animals, periportal cells were more intensely stained. In others, a "skipped" appearance was noted in the sense that cells not staining for albumin intervened between stained ones (Figure 7B).

AFP *vis-á-vis* Albumin

Consecutive serial sections, one stained for AFP and the other for albumin, were compared to see if staining for the two proteins occurred in the same cell and site. Both in the rat and the human, whenever livers showed AFP as well as albumin, several hepatocytes appeared to contain the two proteins simultaneously (Figures 6A and B, 8A and B). This was apparent not only in cells around the vascular territories but also in individual ones elsewhere in the lobule. There were, however, cells which stained for only one of the two proteins (Figures 8A and B), those with albumin alone by far outnumbering the ones with AFP only. In earlier periods of fetal life (the 12th and 15th days of fetal age in rat), several cells with AFP did not contain albumin. At other periods, however, cells that showed AFP also frequently stained for albumin (Figures 2A, 2B, 4A, 4B, 6A, 6B, 8A, and 8B).

Discussion

In conformity with previous reports,[1-9] the AFP levels in our rat and human sera registered progressive drops during fetal development. By the relatively insensitive technique used by us (detecting down to approximately 250 ng/ml of protein), no AFP was found in the serum of rats at and after 35 days of birth and in human serum very soon after birth. The progressive changes in the level of serum albumin showed an inverse relationship to that of AFP in both rat and man. Such phenomena have been recorded by others as well.[1,2]

The specificity and reliability of localization of AFP by the immunoperoxidase technique has been shown earlier.[19,20]

Intracellular demonstration of albumin by the peroxidase technique has been reported in human livers by Feldman and co-workers.[28] Immunospecific demonstration of the two proteins inside the cytoplasm of hepatocytes suggests synthesis of these proteins by the cells. Strict monospecificity of the antisera used in our study was established by various immunochemical techniques. Also, the fact that our anti-AFP sera did not contain antibodies against albumin was shown by absence of staining in adult livers. Similarly, the antialbumin sera did not have anti-AFP antibodies. The possibility of nonspecific adsorption of the proteins from the serum on to the cells has been convincingly ruled out by the studies of Engelhardt and co-workers.[18] Moreover, *in vivo* studies on human albumin [28] and rat AFP [29] using immunoelectron microscopy and *in vitro* radioisotope studies on rat AFP [30] have demonstrated that these proteins are indeed secreted by the ribosomes attached to the rough endoplasmic reticulum. Differences in intensity of staining of individual cells observed

in the present study as well as in some others [28] may indicate varying amounts of protein in the cell.

The random distribution of AFP-containing cells at any given age has been stressed by us in previous reports.[19,31] This was confirmed in the present study, and the importance of this finding in obviating adequate quantitative assessment has already been indicated. The preferential localization of the protein around vascular channels, particularly hepatic veins has been repeatedly shown by immunostaining methods.[18-20,31-33] The reasons for this selective localization and in case of AFP, its significance in the face of a rapid reduction in the number of positive cells elsewhere in the lobule seen in the postnatal period are, however, unknown.

α-Fetoprotein and albumin were seen only inside hepatocytes. Hamashima and co-workers [34] reported positive fluorescence for albumin in Kupffer cells as well. However, like Feldman and his colleagues [28] and Lane,[35] who did not find Kupffer cell localization of albumin on peroxidase histochemistry of human livers and immunofluorescence of rat liver, respectively, we too failed to observe positive staining for this protein in the Kupffer cells, either in the human or in the rat. Localization of both the proteins has been seen only in liver cells of adult or fetal type. Some workers [36,37] suggest that AFP is produced by the small hepatocyte and by the so-called oval cell—a transitional form between the precursor cell and the fetal hepatocyte. Though the oval cell origin of AFP may be true in situations where such cells develop, as in experimental carcinogenesis, none of our rat or human livers showed them among cells that stained for the protein. Similarly, in normal ontogeny of the mouse only cells recognizable as hepatocytes show immunofluorescence for AFP.[38]

Many hepatocytes staining for the proteins were seen scattered as individual cells. This seems to indicate that neither is there a strictly zonal distribution of protein synthesizing cells in hepatic lobules nor is the synthesis of each protein necessarily the function of a particular clone of cells represented by close proximity to one another.

In case of each protein the serum concentration is directly proportional to the number of hepatocytes staining for this protein. Thus both in the human and the rat, with increase in age, serum AFP concentration as well as AFP-positive cells decrease, while serum albumin levels and albumin-positive cells increase. It is obvious that variation in the level of each of these proteins in the serum is related to the number of cells synthesizing the protein rather than to the amount synthesized by a relatively constant number of cells. However, the present study does not entirely rule out the possibility of variations in the quantity of protein synthesized by individ-

uals among a changing population of cells. Intensity of staining varied among cells, but the direct relationship of serum level of either protein to the number of cells staining positively for it was more striking. When serum AFP went below the level detectable by the technique used in this study, no AFP-containing cells were demonstrable. Identical findings have been reported in mice using immunofluorescence for AFP.[38] It is well known that a very small amount of this protein continues to be secreted throughout adult life.[4,5,7] The number of cells secreting this small amount of protein would, however, be so few and the level of synthesis in each cell so low that it would be unlikely to identify such hepatocytes in random sections.

An important finding in the present study is the simultaneous synthesis of the two proteins by the same liver cells. Such cells were frequently observed, though many hepatocytes containing AFP or albumin alone were also present. Similar comparisons on staining of consecutive paraffin sections have been demonstrated earlier for various intracellular material.[18,19,23,24] The two proteins are similar in their physical and chemical properties [14-16] and it is possible that the same cell may be assigned to synthesize the two proteins either at the same or different times.

It appears then, that in the normal liver separate clones of hepatocytes are not assigned the tasks of synthesizing albumin and AFP. In a few cases of human hepatoma studied by us in a similar way, both AFP and albumin were observed in several tumor cells.[39] Rat hepatoma cells grown in synchronous cultures, on the other hand seem to show different clones engaged separately in the synthesis of AFP and albumin.[21] This may, however, be a phenomenon peculiar only to autonomous neoplastic cells. Several unpredictable abnormalities in intracellular enzymes are now described in aflatoxin B–induced liver tumors in animals.[40] Some of our recent studies [39] and those of Pihko and Rouslahti,[13] on reappearance of AFP in adult mouse liver, suggest that following hepatic injury increased secretion of the protein into serum takes place hours before the cell enters mitosis. This would indicate that liver cells which are normally engaged in synthesizing albumin alone start producing AFP in addition to or in place of albumin, even before undergoing mitotic division. Studies on culture of fetal hepatocytes suggest that albumin is synthesized throughout the cell cycle whereas AFP is synthesized prior to S phase and released prior to M phase. These hepatocytes, even when arrested in the late G_1 phase, can synthesize and secrete AFP into the medium. Hepatoma cells growing in culture, on the other hand, may show a different pattern of synthesis of these proteins. AFP is synthesized from G_1, through S to G_2 phase, while albumin synthesis may be seen only in the S and early G_1 phase.[21]

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[*Illustrations follow*]

Legends for Figures

All tissue sections are stained by immunoperoxidase-methyl green. Except in Figure 7, **A** and **B** represent consecutive sections stained for AFP and albumin, respectively.

Figure 1—Liver of 15-day-old rat fetus. Note that albumin-containing cells (**B**) are already more numerous than the AFP-containing ones (**A**). For both proteins, variations in staining intensity of cells are seen (*arrows*) (× 180)

Figure 2—Liver of newborn rat (6 hours). AFP-containing cells (**A**) are randomly scattered with some aggregation around vascular territories. Albumin-containing cells are much more diffusely distributed (**B**). (× 65)

Figure 3—Liver of 4-day-old rat. AFP-containing cells (**A**) are fewer than before and are chiefly located around veins (*arrowheads*) though several are still scattered in the lobule. Albumin staining is more widespread and intense (**B**); an area packed with positive cells (*arrow*) is relatively free of AFP in the corresponding section (compare with **A**). (× 65)

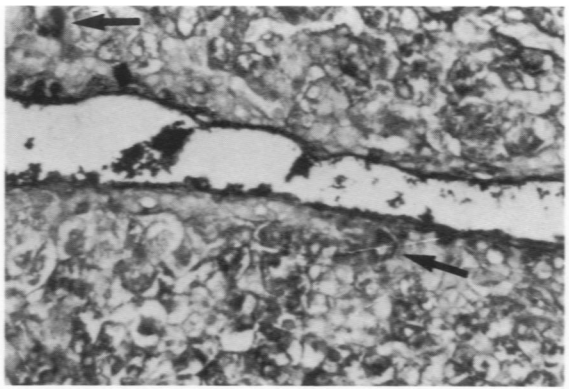
Figure 4—Liver of 7-day-old rat. Only a narrow cuff of AFP-positive cells is seen around a hepatic vein tributary (**A**). Albumin is present in these cells as well as in many others. (× 65)

Figure 5—Liver of 21-day-old rat. AFP-containing cells have almost completely disappeared (**A**). Albumin staining is diffuse with distinct accentuation around many portal (*thin arrows*) as well as hepatic veins (*thick arrow*). (× 65)

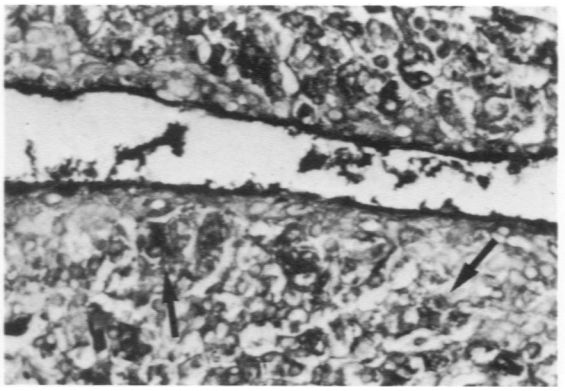
Figure 6—Liver of a 22-week-old human fetus. AFP-containing cells are seen (**A**) in perivascular as well as random intralobular areas (*thin arrow*). Many more cells show albumin (**B**). Some cells contain albumin but not AFP (*thick arrows*) while others contain both proteins (*curved arrows*). (× 350)

Figure 7—Albumin in the liver of an adult rat. Note perivascular as well as random distribution (**A**) and differences in intensity of staining. Lightly staining or nonstaining cells are seen in between heavily stained ones (**B**), imparting a "skipped" appearance. (**A**, × 65; **B**, × 400)

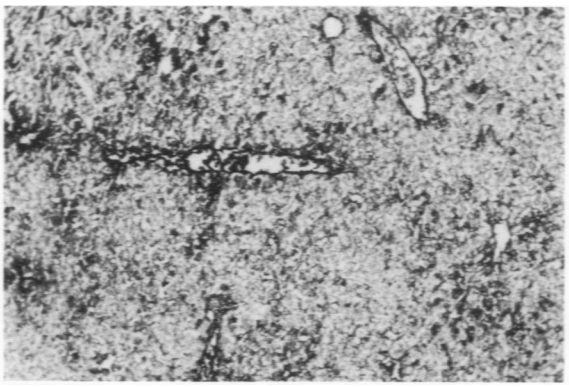
Figure 8—In this liver from a newborn rat (12 hours) several of the AFP containing cells (**A**) also contain albumin (**B**). Some hepatocytes contain AFP but not albumin (*straight arrows*), while others stain for albumin only (*curved arrows*). (× 220)



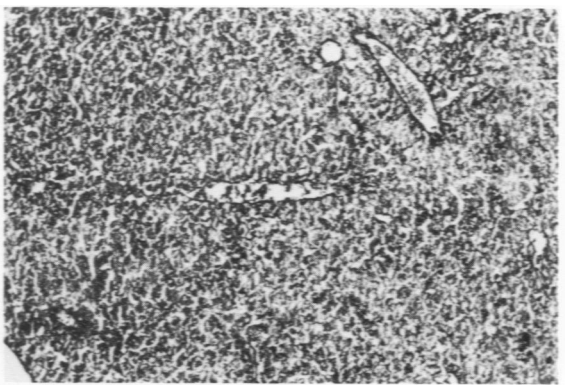
1A



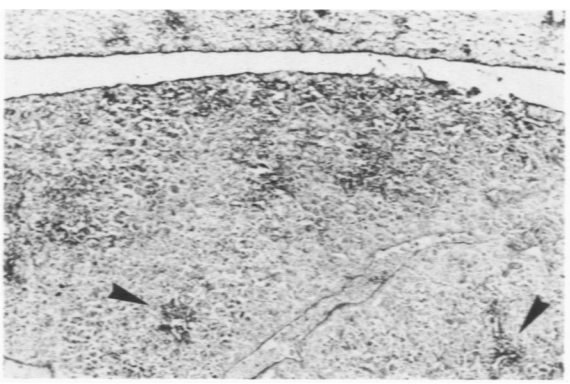
1B



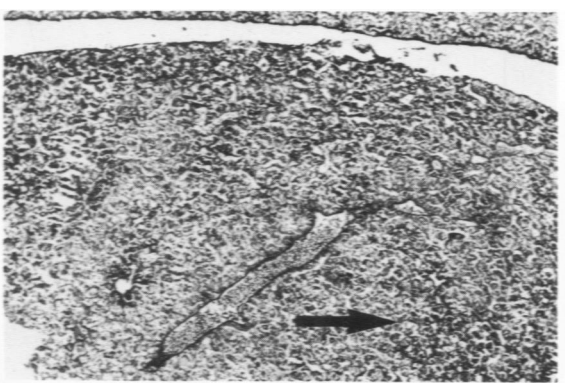
2A



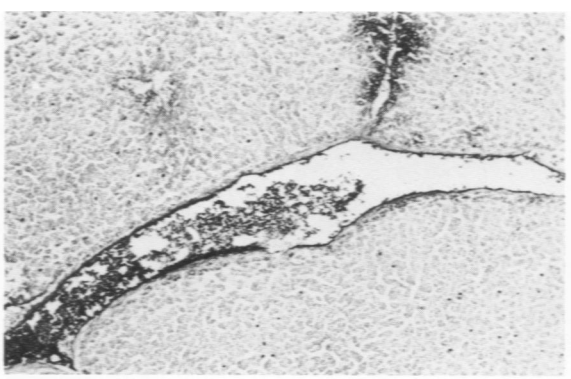
2B



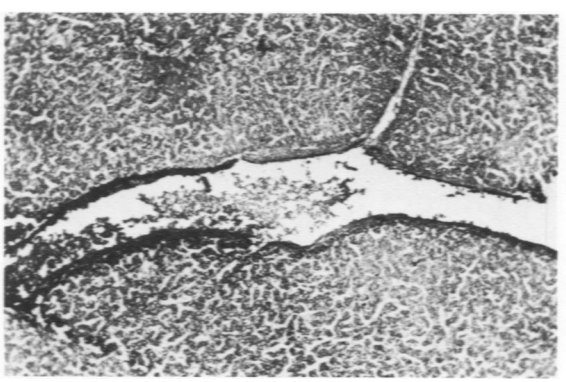
3A



3B

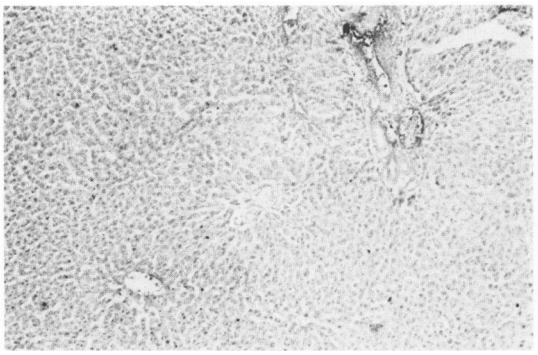


4A

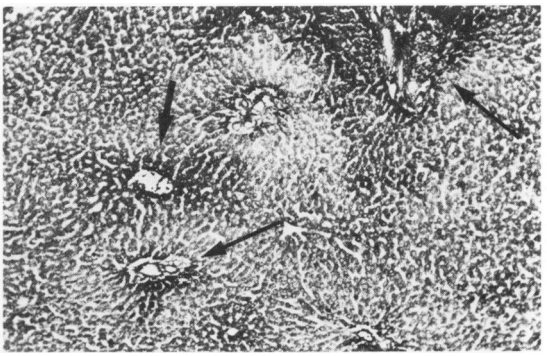


4B

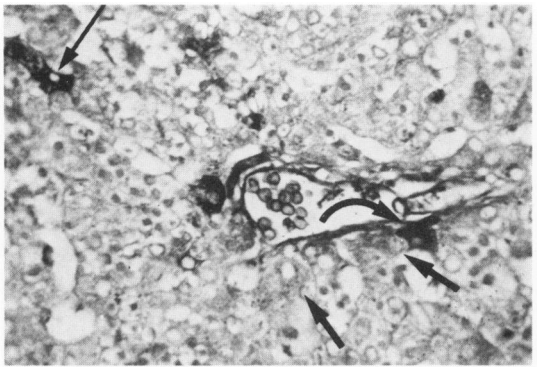
5A



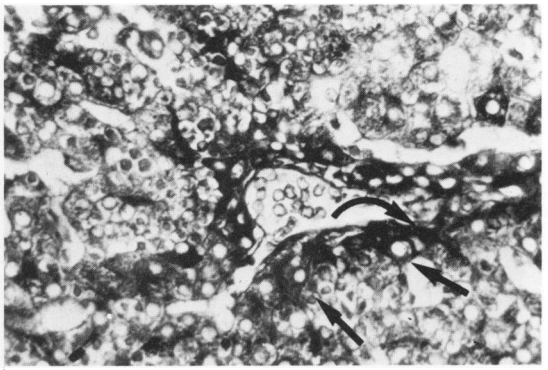
5B



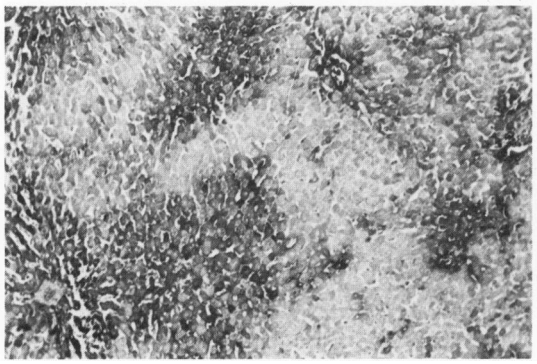
6A



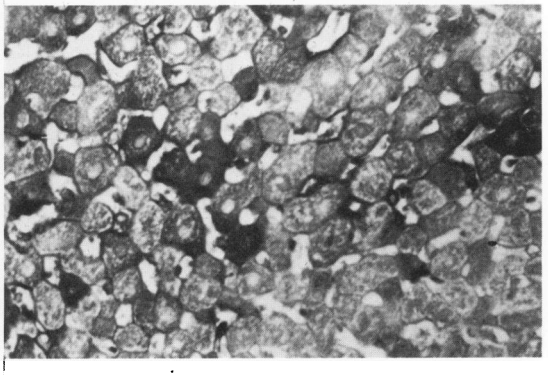
6B



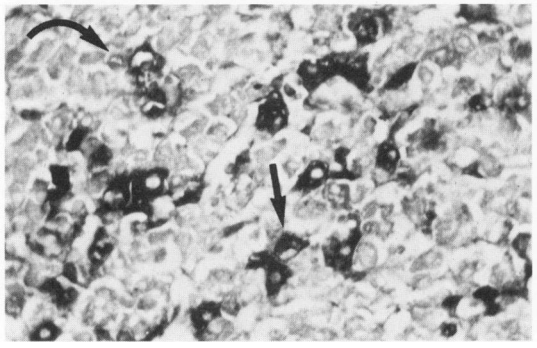
7A



7B



8A



8B