REGULATION OF PROTEIN SYNTHESIS IN LIVER

(ONTOGENY AND REGULATION OF ALBUMIN AND ALPHA FETOPROTEIN SYNTHESIS IN LIVER, BRAIN AND SKIN)

SUMMARY

THESIS SUBMITTED FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY IN THE FACULTY OF SCIENCE OF ALIGARH MUSLIM UNIVERSITY, ALIGARH, 1981.

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Ontogeny of tissue specific protein markers provides useful experimental model for investigating regulation of gene expression during differentiation. Alpha fetoprotein and albumin are important specific differentiation markers and they bear a reciprocal relationship during ontogeny. The following lines summarize the results obtained regarding ontogeny of AFP and albumin in different tissues of rat as well as developmental profile of AFP in human brain.

AN IMPROVED PROCEDURE FOR ISOLATION OF AFP

A direct negative immunoabsorption procedure was developed to isolate homogeneous preparation of rat and human AFP from amniotic fluid. It requires no more than two steps: (a) removal of adult serum proteins present in amniotic fluid by a direct negative immunoabsorption (immunoprecipitation) with rabbit anti-normal adult rat or human serum IgG, and (b) removal of excess of immunoglobulins using DEAE-cellulose chromatography. Purified AFP was characterized by double immunodiffusion, immunoelectrophoresis, crossed immunoelectrophoresis, PAGE, SDS-PAGE, Con-A affinity chromatography, and estradiol binding. Rat AFP has $K_a = 1.4 \times 10^8 \text{ M}^{-1}$ for estradiol-17β with one binding site for estradiol per mole of AFP.

Isolation of homogeneous AFP by a direct negative immunoabsorption is a significant improvement over the time consuming, multistep procedures reported for AFP. In
addition, the present procedure did not require specific antiserum against AFP and employed very gentle isolating conditions. The procedure yielded 87% recovery of AFP.

**ONTGENY AND DISTRIBUTION OF AFP IN FETO-NEONATAL TISSUES OF RAT AND HUMAN FETAL BRAIN**

Most of the feto-neonatal tissues, except thymus, spleen and pancreas, of rat were found to contain elevated levels of AFP. High levels of AFP observed during fetal life in various tissues registered a decline with development. Ontogeny of AFP in these tissues was different from that observed in serum. AFP disappears after 7 d of postnatal life (P) in brain and adrenals; 14 P in lung, heart, stomach, intestinal and kidney; 21 P in liver and skin. Determination of heme concentration in these tissue extract revealed that contamination of AFP from fetal blood could not account for more than 5-10% of the observed levels of AFP in various feto-neonatal tissues of rat.

AFP present in various feto-neonatal tissues of rat was immunologically identical to serum AFP. AFP from serum, brain, skin and kidney also displayed a similar electrophoretic mobility on 10% PAGE and IEF. Estradiol binding assay of AFP in brain and skin gave an association constant, $K_a = 1.3 \times 10^8 \text{ M}^{-1}$ and $1.0 \times 10^8 \text{ M}^{-1}$. However, brain and skin cytosols from newborn rat were found to
differ in the relative amounts of Con A-non reactive AFP variant and contained 37% and 42% of this variant respectively. In view of these observations it appears that AFP in various feto-neonatal tissues of rat does not entirely represent fetal blood contamination and probably arises as a result of its independent synthesis in each tissue.

Studies on the distribution of AFP in feto-neonatal rat brain have revealed that it is distributed uniformly throughout the brain. Ontogeny of AFP in olfactory region, cerebrum, cerebellum and rest portions of the rat brain was similar to that in whole brain and it disappeared in these discrete regions after 7 d of postnatal development.

Presence of AFP was also demonstrated in human fetal brain. It was immunologically and electrophoretically identical to human fetal serum AFP. Developmental profile of AFP in human brain was distinct from that observed in serum compartment. It registered a peak level in brain at 20th week and declined rapidly to low levels after 24th week of gestation. It was absent (as measured by rocket assay) in the human fetal brain of subsequent ages. Unlike rodent AFP and like human AFP, it did not interact with estradiol. Brain AFP does not arise from fetal blood contamination since the blood contamination could account for traces of the AFP present in brain. Further ontogeny or AFP in brain is distinct from that observed in serum.
DEVELOPMENTAL CHANGES IN AFP AND ALBUMIN CONTENT IN SERUM OF RAT

Developmental profile of serum AFP exhibited an inverse relationship with age during postnatal life of rat. Serum AFP level declined after birth to 2.4% of the level observed in newborn rat, at 21st day of postnatal life. AFP could not be detected in sera of 28 d old rats by the present procedure. In contrast serum albumin elicited an increase by 37% during the same period. The albumin level rose in serum continuously to adult value of 37.5 mg ml\(^{-1}\) in 9 weeks old rat.

SYNTHESIS AND SECRETION OF AFP AND ALBUMIN BY ISOLATED HEPATOCYTES IN CULTURE: CORRELATION OF SERUM AFP AND ALBUMIN PROFILES WITH THE LIVER CONTENT AND THEIR RATE OF SYNTHESIS IN THE HEPATOCYTES DURING DEVELOPMENT

AFP and albumin are synthesized in the hepatocyte and secreted into the serum. Serum level of these proteins undergoes striking change during fetoneonatal development.

Hepatocytes prepared from rat liver at various stages of development (18, 20 E and 1, 7, 14, 21, 28, 35 P and adult) by collagenase digestion procedure were maintained in short term (24 hrs) culture. Rate of synthesis and secretion of AFP and albumin were determined by following the incorporation of \(^{14}\text{C}\text{-leucine}\) into immunoprecipitable AFP and albumin by the hepatocytes cultured in leucine-free medium. Hepatocytes from newborn rat synthesized AFP at a
very high rate accounting for about 85% of the total protein synthesis while albumin synthesis was only 9%. Rate of AFP synthesis and secretion declined with the maturation of the hepatocytes. Hepatocytes from 21 d old rat demonstrated only 3% of the rate observed in fetal hepatocyte from 18 E rat. No synthesis of AFP could be demonstrated in the hepatocytes prepared from 4 week old rats. Serum AFP level and its content in liver also reduced to 1.3% and 4% respectively in 3 week old rat when compared to the values observed in 18 E fetal rats. It appears that age related change in serum AFP level and its content in liver is governed by the rate of AFP synthesis and secretion by the developing hepatocytes.

Maturation of the hepatocyte was accompanied by a marked increase in the rate of albumin synthesis, secretion and its intracellular content. Ratios of the rates of albumin to AFP synthesis in the hepatocytes from 18 E fetus and 1, 7, 14, 21, 28 d old neonatal rats were 0.05, 0.10, 0.28, 3.5 and 48 respectively. It would thus appear that an increase in serum albumin level and its intrahepatic content is due to a proportionate increase in the rate of albumin synthesis and secretion in the developing rat liver.

It may be concluded that differentiation and maturation of the hepatocyte is accompanied by a sequential inactivation of AFP and activation of albumin gene expression.
DEMONSTRATION OF AFP AND ALBUMIN SYNTHESIS IN DEVELOPING RAT BRAIN AND SKIN: CORRELATION OF TISSUE CONTENT OF AFP AND ALBUMIN WITH THEIR RATE OF SYNTHESIS

Dissociated mammalian brain cells and skin explants in primary cultures offers a useful system to investigate the biochemical events accompanying differentiation and growth of these tissues. Dissociated brain cells and skin explant cultures from newborn rat were used to answer the following questions: Is estradiol binding protein in developing rat brain and skin AFP? If so, where does this protein originate from?

Our observations, derived from $^{14}$C-leucine incorporation into immunoprecipitable AFP and albumin by dissociated brain cells and skin explant maintained in short term cultures, have provided evidence for the synthesis of AFP and albumin in brain and skin of newborn rat. Cycloheximide inhibited the linear incorporation of $^{14}$C-leucine into immunoprecipitable AFP and albumin as a function of time by these cultures. It strongly suggests that $^{14}$C-labelled AFP and albumin observed in brain cells and skin lysates (synthesis) and in medium (secretion) are constantly synthesized and secreted by these cultures. In addition, the intracellular content of both AFP and albumin in brain cells did not change significantly during the whole culture period. Moreover, these cells elicited a linear
accumulation (secretion) of the quantifiable amounts of AFP and albumin into the medium. Total amounts of AFP and albumin secreted by brain cells were far in excess of their intracellular content at the initiation of the cultures. In view of these observations it was concluded that developing brain and skin represent an independent pool of AFP and albumin.

AFP and albumin secreted by newborn rat brain cells and skin explants cultures was immunologically, electrophoretically and with regards to molecular weights and estradiol binding properties similar to serum AFP and albumin. It may be concluded that genes for AFP and albumin in developing brain and for AFP in skin are also expressed these tissues.

Rates of synthesis of AFP and albumin during fetal-neonatal development of rat brain showed a continuous decline and their synthesis seems to be turned off after 7 d of postnatal life. High levels of these protein in fetal brain were due to their high rates of synthesis. Age related decrease in brain AFP and albumin reflected a proportionate decrease in the rates at which they are synthesized in this tissue.

Rate of synthesis of AFP in skin also decreased with increasing age and its synthesis is turned off after 14 d of postnatal life. A decrease in the skin AFP level can be accounted for by corresponding decline in the rate of its
HORMONAL CONTROL OF AFP AND ALBUMIN SYNTHESIS IN NEWBORN RAT LIVER, BRAIN AND SKIN

Effect of various hormones on the rate of AFP and albumin synthesis in the hepatocytes, brain cells and skin explant from newborn rats, was studied to gain some insight into ontogeny related regulation of synthesis of these proteins in rat tissues.

Dexamethasone was the most effective in inhibiting the rate of AFP synthesis and secretion in vitro cultures of hepatocytes (-45%), brain cells (-40%) and skin explant (-25%). It also caused an accelerated decrease in serum AFP level in vivo in newborn rats. dbcAMP also decreased the rate of synthesis and secretion of AFP in brain cells (-15%), hepatocytes (-7%) and skin explants (-4%). It also suppressed the serum AFP level in vivo in newborn rats supporting its in vitro effect.

Insulin exhibited a dual effect on the rate of AFP synthesis in various tissues. It increased AFP synthesis in hepatocytes (+21%) and demonstrated an opposite effect in brain cell (-15%) and skin explant (-4%). However, it did not affect serum AFP levels of newborn rats in vivo.

Neither did estradiol significantly affect the rate of AFP synthesis in various tissues in vitro nor did it change serum AFP level in vivo. The effects of dexamethasone, dbcAMP, insulin and estradiol on the rate of albumin
synthesis in vitro or its level in vivo were not significant. It appears that effects of these hormones are specific for AFP synthesis.

It appears that expression of AFP gene is a function of undifferentiated or less differentiated stem cells or embryonal cells. Different embryonal cell types with diverse ontogenic history do indeed synthesize AFP although the extent of synthesis is different. Thus expression of AFP and albumin genes during development offer a useful model for investigating molecular mechanism underlying tissue differentiation.
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who did all their best efforts in bringing me
all possible happiness.

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August 5, 1981.
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Scientist  
Biochem.Div.  

August 4, 1981

This is to certify that the work embodied in this thesis has been carried out by Shri Masarrat Ali under my supervision. He has fulfilled all the requirements for the degree of Doctor of Philosophy of Aligarh Muslim University, Aligarh, regarding the nature and period of investigational work. The work included in this thesis has not been submitted for any other degree, and unless otherwise stated, is all original.
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Cell differentiation during development is an expression of orderly and sequential gene regulation. Accumulation or loss of gene products, e.g. structural and functional proteins, prepares the tissue for the next stage of development resulting in well observed functional differentiation. Sometimes re-expression of certain fetal antigens during oncogenesis has proved invaluable in diagnosis of malignancy. Alpha-fetoprotein (AFP) is an important serum glycoprotein of this category. It is synthesized by the cells derived from endoderm-like yolk sac and liver, early in fetal development. AFP and albumin are good differentiation markers. They bear a reciprocal relationship during ontogeny. Probably this reason has prompted some investigators interested in its function to call it as 'fetal albumin'.

Ontogeny of AFP and albumin were investigated in different tissues of rat and human brain and the following leads were obtained.

1. A simple method based upon immunoabsorption of proteins other than AFP from amniotic fluid followed by DEAE-cellulose chromatography was developed for the isolation of rat and human alpha fetoprotein. This procedure gives high yield of AFP (87%) and may also find applications
in the isolation of other phase specific antigens like carcino-embryonic antigen and uteroglobin.

2. Distinct ontogeny of AFP in human fetal brain, and feto-neonatal rat brain suggests that these tissues may form independent pools of AFP probably contributed by its in situ synthesis.

3. Cells and tissue explants from developing rat brain and skin have been found to synthesize and secrete estrophilic AFP in culture. AFP secreted by these tissues is immunologically, electrophoretically and with regards to molecular weight and estradiol binding similar to serum AFP. Brain cells from developing rat also synthesise albumin.

4. Age related decline in brain and skin AFP levels reflects a proportionate decrease in the rate of synthesis of the protein. Synthesis of AFP is turned off after 7, 14 and 21 d of postnatal life in brain, skin and liver of rat.

5. Dexamethasone and dbcAMP are negative regulators of AFP synthesis in different tissues with differing sensitivity.

From the foregoing it appears that expression of AFP and albumin genes is a useful tool for understanding tissue differentiation. Although final proof is still awaited, it appears that undifferentiated cells do express AFP gene. It would be of interest to quantitate mRNA levels in different tissues during development. Further, demonstration of factors that control the switch off of AFP gene during development and switch on during hepatic injury or carcinogenesis may provide some clue to mechanism of tissue differentiation as well as malignancy.
**ABBREVIATIONS & SYMBOLS**

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>A</td>
<td>Adult</td>
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<tr>
<td>Ab</td>
<td>Antibody</td>
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<td>ACTH</td>
<td>Adrenocorticotropic hormone</td>
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<td>AFP</td>
<td>Alpha-fetoprotein</td>
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<tr>
<td>AF</td>
<td>Amniotic fluid</td>
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<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
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<tr>
<td>cDNA</td>
<td>Complementary DNA</td>
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<tr>
<td>Con A</td>
<td>Concanavalin A</td>
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<tr>
<td>CP</td>
<td>Cell protein</td>
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<tr>
<td>cpm</td>
<td>Counts per minute</td>
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<tr>
<td>CM</td>
<td>Concentrated culture medium (brain)</td>
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<tr>
<td>CMS</td>
<td>Concentrated culture medium (skin)</td>
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<tr>
<td>dbcAMP</td>
<td>$N^6, O^2$, dibutyryl cyclic 3',5'-monophosphate</td>
</tr>
<tr>
<td>DEAE</td>
<td>Diethylamino ethyl</td>
</tr>
<tr>
<td>dpm</td>
<td>Disintegration per minute</td>
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<tr>
<td>3'-DAB</td>
<td>3'-methyl-diaminoazobenzene</td>
</tr>
<tr>
<td>DEN</td>
<td>Diethylnitrosamine</td>
</tr>
<tr>
<td>E</td>
<td>Day of embryonic or fetal life</td>
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<tr>
<td>$E_1$</td>
<td>Estrone</td>
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<tr>
<td>$E_2$</td>
<td>Estradiol 17-$\beta$</td>
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<tr>
<td>EDTA</td>
<td>Ethylene diamine tetraacetic acid</td>
</tr>
<tr>
<td>FE</td>
<td>Fetal extract</td>
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<td>FS</td>
<td>Fetal serum</td>
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<tr>
<td>HAFP</td>
<td>Human AFP</td>
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<td>HEPES</td>
<td>N-2-hydroxyethyl-piperazine-N'-2-ethane sulphonic acid</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>HSA</td>
<td>Human serum albumin</td>
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<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IEP</td>
<td>Immunoelectrophoresis</td>
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<tr>
<td>LCA</td>
<td><em>Lens culinaris</em> agglutinin</td>
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<tr>
<td>mRNA&lt;sub&gt;AFP&lt;/sub&gt;</td>
<td>Messenger RNA for AFP</td>
</tr>
<tr>
<td>mRNA&lt;sub&gt;albumin&lt;/sub&gt;</td>
<td>Messenger RNA for albumin</td>
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<tr>
<td>NRS</td>
<td>Normal rat serum</td>
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<tr>
<td>NHS</td>
<td>Normal human serum</td>
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<tr>
<td>P</td>
<td>Day of postnatal life</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PHC</td>
<td>Primary hepatocellular cancer</td>
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<tr>
<td>PPO</td>
<td>2,5-diphenyl oxazole</td>
</tr>
<tr>
<td>POPOP</td>
<td>1,4-bis-(5-phenyloxazolyl)benzene</td>
</tr>
<tr>
<td>PRS</td>
<td>Pregnant rat serum</td>
</tr>
<tr>
<td>RCA</td>
<td><em>Ricinus communis</em> agglutinin</td>
</tr>
<tr>
<td>RER</td>
<td>Rough endoplasmic reticulum</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
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<tr>
<td>RSA</td>
<td>Rat serum albumin</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<tr>
<td>SER</td>
<td>Smooth endoplasmic reticulum</td>
</tr>
<tr>
<td>TCA</td>
<td>Trichloroacetic acid</td>
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<tr>
<td>TP</td>
<td>Total proteins</td>
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<tr>
<td>Tris</td>
<td>(Hydroxymethyl)-2-aminomethane</td>
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INTRODUCTION
Fetus specific serum protein was first discovered by Pederson (1944) in fetal calf serum and by Borgstrand and Czar (1956) in human fetal serum. The protein migrated between albumin and α-globulin on paper electrophoresis and was called alpha-fetoprotein (AFP). Similar fetal proteins have since been demonstrated in all mammals studied so far (Gitlin and Boesman, 1967; Gitlin, 1975). However, the findings of Abelev et al (1963) brought AFP into limelight by demonstrating the presence of fetus specific alpha-globulin in the serum of adult mice with chemically induced primary and transplantable hepatocellular cancer. Shortly afterwards Tatarinov (1964) demonstrated the presence of AFP in the serum of human with primary hepatocellular cancer (PHC). This heralded exciting prospects for AFP as an immunochemical marker which could be used both in clinical diagnosis of this tumor and in epidemiological surveys. Naturally this has determined the main direction of subsequent studies. However, relatively little is known about the dynamics of the protein during the course of the disease, and even less about the reason for the resumption of its synthesis by the tumor, and finally its physiological role in the normal and abnormal conditions.
Serum albumin is the most abundant and the most studied of the proteins in circulation. One of the first protein to be recognized, its name derives from the early name for proteins, albumen, derived from Latin word albus—white in the case of white of an egg. The basic properties of albumin were recognized as early as 1837 by Ancell, who noted that albumin was needed for transport functions, for maintaining the fluidity of the vascular system and for the prevention of oedema. Serum albumin is a highly flexible, single polypeptide chain of about 65,000 molecular weight containing 583 amino acids. Albumin molecule is grouped into a series of nine disulfide-bonded loops associating into three small domains which may be specific in ligand binding (Peters, 1977; Brown, 1977; Zahringer et al, 1977). Albumin is characteristic by its extreme solubility in water, negative charge of 10 at pH 7.4, and lack of a carbohydrate moiety. Albumin (human and bovine) appears to be the longest peptide chain whose complete amino acid sequence has been determined (Brown, 1977). Known functions attributed to albumin include the binding and transport of long chain fatty acids and anions; the binding and detoxification of unconjugated bilirubin, the transport of metal ions, amino acids, metabolites, enzymes, drugs, hormones, a depot for a constant supply of amino acids, and maintenance of colloidal osmotic pressure (Mullar and Wollert, 1979; Lehane et al, 1978; Peters, 1977).
The primary sites of AFP synthesis in the developing embryo are the yolk sac and liver (Mackiewicz and Breborowicz, 1980; Breborowicz and Nishi, 1979; Gitlin, 1975; Gitlin et al, 1972). Small amount of AFP synthesis has also been demonstrated in the placenta, kidney gastro-intestinal tract (Mackiewicz and Breborowicz, 1980; Ruoslahti and Seppala, 1979; Gitlin, 1975) and immature rat uterus (Smalley and Saricione, 1978). There is no evidence for the synthesis of AFP by other fetal tissues (Gitlin, 1975). In humans, AFP is first synthesized by the yolk sac and liver and then predominantly by the liver as yolk sac becomes involuted during the first week of 2nd trimester.

Feto-neonatal estradiol binding protein has been demonstrated to be AFP (Attardi and Ruoslahti, 1976; Plapinger et al, 1973). Estradiol binding protein have also been found in immature rat skin cytosol but the identity of AFP has not been reported (Eppenburger and Hsia, 1972). The origin of $E_2$ binding protein in brain and skin is yet to be clarified.

The same pattern of AFP synthesis has been observed in other species with some variation. In the mouse, rat, chicken (Gitlin and Gitlin, 1975; Sell and Skelly, 1976) and in rabbit (Branch and Wild, 1972) synthesis by the
yolk sac continues after birth. In chicken, AFP synthesis takes place almost exclusively in the yolk sac and only small amounts are produced by liver (Gitlin, 1975; Gitlin et al, 1973). All mammalian species so far studied, including subhuman primates, carnivores, rodents, lagamorphs, ruminants, edentates, and marsupials synthesize one or more homologous of AFP during development (Gitlin, 1975). Thus, an AFP homology may exist as far back in evolution as 400 million years.

Albumin is produced in the liver, and no other site for its production has been demonstrated (Peters, 1977; Rothschild et al, 1977). Albumin may comprise 50% of the total protein synthesis in adult liver and about 14 g of albumin is synthesized daily by the human liver (Peters, 1977). Synthesis of both AFP and albumin in the human embryo can be detected as early as 29 days after conception (Rothschild et al, 1977; Gitlin et al, 1972) when liver is little more than a diverticula of hepatic buds and ducts, and there is only slight, if any, evidence of lobe formation. During subsequent development, the synthesis of other plasma proteins becomes apparent. Thus, early morphologic differentiation of the liver is accompanied by the development of hepatic plasma protein synthesis.

The actual site of AFP (Belanger et al, 1979a; Kuhlman, 1979; Gitlin, 1975) and albumin (Belanger et al,
synthesis is the hepatocytes; kupffer cells, bile duct epithelium and hepatic hematopoietic tissues or other cells which make up to 40% of the liver cell population, are not involved. Monolayer fetal hepatocytes cultures are AFP and albumin producers (Brazier et al., 1977; Tsukada and Hirai, 1975; Sell and Skelly, 1975; Leffert and Sell, 1974).

Albumin and AFP have been demonstrated to be synthesized in the same hepatocyte and that hepatocyte can also differentiate into either AFP or albumin producing ones (Guillouzo et al., 1978; Nayak and Mittal, 1977). During early embryonic life the serum concentration of AFP and albumin shows a reciprocal relationship. AFP is the major serum protein early in fetal development, whereas in the adult albumin is the major serum protein, and AFP is barely detectable (Abelev, 1971). Upto 80% of the hepatocytes in fetal liver have been found to synthesize AFP (Abelev, 1979). During postnatal development, the number of albumin producing cells increases while AFP positive cells show a continuous decline (Kuhlman, 1979; Nayak and Mittal, 1977). After two weeks of postnatal life a tremendous decrease in AFP positive cells in the liver occurs corresponding to a rapid maturation of hepatocytes and after 4 weeks AFP containing cells could no longer be detected (Kuhlman, 1979; Nayak and Mittal, 1977). It appears that as the maturation of hepatocytes occurs,
the hepatocytes switches from AFP to albumin producing one. The regulation of AFP and albumin synthesis is directly governed by the amount of mRNA. The albumin mRNA levels increase while AFP mRNA levels in the liver decline during postnatal development (Liao et al., 1980; Hoffer et al., 1979; Salatropat et al., 1979; Sell et al., 1979; Koga and Tamaoki, 1974). In the adult hepatocytes, no AFP producing cell could be observed (Kuhlman, 1979; Nayak and Mittal, 1977). The presence of small amounts of AFP in normal human sera (Ruoslahti and Seppala, 1971b) and adult sera of other species, suggests that AFP gene is not completely turned off and AFP may be produced by many cells and not by a specialised population cells (Ruoslahti and Seppala, 1979).

In normal adult rat liver only 10% hepatocytes produce albumin at any given moment and this seems to arise primarily from the cyclic activity of the hepatocytes rather than any specialised hepatocyte being albumin producer (Maurice et al., 1979; Guillouzo et al., 1976). The synthesis and secretion of AFP by fetal rat hepatocytes in vitro is related to alterations in the state of its growth while albumin synthesis is probably independent of growth cycle (Sell et al., 1975). AFP appears to be synthesized before S phase and secreted just prior to mitosis (Sell et al., 1975) and no synthesis occurs during
SI, G₁ or M phases of the cell cycle (Guillouzo et al, 1979, 1978). Albumin is synthesized from the middle period of S phase to early G₂ phase or to late G₂ phase (Tsukada and Hirai, 1975). Thus, albumin synthesis occurs at a later stage of the cell cycle and last for a much shorter period of time than AFP synthesis.

**INTRACELLULAR TRANSPORT OF ALBUMIN AND AFP**

Studies on biosynthesis of albumin have shown that it is synthesized on membrane bound polysomes (Garland and Cori, 1981; Peters and Reed, 1980; Peters, 1977; Zahringer et al, 1977; Yap et al, 1977). Following its synthesis, albumin (or its precursor) is passed successively into the channel of rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER), and golgi apparatus (GA), and finally out of the cell (Peters and Reed, 1980; Peters, 1977; Edwards et al, 1976). Intracellular albumin has been found to exist in two forms—mature serum albumin and its precursor (proalbumin). The proalbumin possesses an additional hexapeptide at the NH₂-terminus with a sequence of NH₂-Arg-Gly-Val-Phe-Arg-Arg-serum albumin (Peters and Reed, 1980; Strauss et al, 1977; Edwards et al, 1976). The proalbumin is cleaved to albumin by an intracellular membrane bound cathepsin B type enzyme and the process takes place in the golgi vesicles (Algranti and Sabatini, 1979; Judah and Quinn, 1978; Redman et al, 1978; Oda et al, 1978).
The golgi vesicles, filled with the secretory proteins, then fuse in a controlled manner forming secretory vesicles, which in turn is able to recognize and fuse with the plasma membrane to allow exocytosis of the proteins. The prealbumin and albumin do not differ in binding properties to bilirubin and palmitate (Peters and Reed, 1980). The role of the pro-form is not clear. It may serve in vivo, as the cationic propeptide to channel the nascent albumin through the liver cell in lieu of the glycosyl groups which become attached to most secreted proteins (Peters and Reed, 1980).

The product of translation of mRNAs for albumin and other secretory proteins are larger than either the intracellular form or serum form of albumin. These initial translation products are called preproteins and contain an extension (prepiece) of 15-29 amino acid residues (24 and 20 amino acid for albumin and AFP respectively) at the NH₂-terminus, popularly known as 'signal peptide' or 'signal sequence' or 'leader sequence' (Steiner et al, 1979; Strauss et al, 1977; Blobel and Dobberstein, 1975). The signal peptide contains a high percentage of hydrophobic amino acids and helps in the binding of nascent polypeptide chain to RER membrane bilayer and transports the protein across the membrane. The signal peptide is probably removed before the entire secretory protein has been
synthesized by a RER membrane bound endopeptidase cleaving at a ser-Arg peptide bond yielding proform - the proalbumin (Habener et al, 1979; Dobberstein and Blobel, 1979; Strauss et al, 1978; Shields and Blobel, 1978; Blobel and Dobberstein, 1975). The proform of albumin and the signal peptide are discharged together into the cisternae of RER, and apparently destroyed.

Essentially the same secretory pathway has also been recognized for AFP (Peters et al, 1979; Miura et al, 1979; Belanger et al, 1979a; Guillouzo et al, 1978). However, AFP is glycosylated as the peptide chain passes through the channels of ER and GA (Belanger et al, 1979a). AFP is released from membrane bound ribosomes in a nonglycosylated form. It becomes glycosylated as it moves into RER, where it receives, at least part of the core structure (N-Acetyl-galactosamine and mannose) of its oligosaccharide Unit(s). When the growing peptide enters the GA, it receives a variable number of peripheral carbohydrate residues (galactose and sialic acid) and apparently achieves a terminal lectin reactivity and microheterogeneity. The entire process is completed within 15 minutes (Belanger et al, 1979a).

PHYSIOLOGICAL PROFILES OF AFP IN BODY FLUIDS

FETAL SERUM - During human gestation, the highest AFP concentration (1-3 mg/ml) in fetal serum occurs at 12-14 weeks of gestation due to the high rate of AFP synthesis in the
liver (Seppala, 1977; Gitlin, 1975). After 14th week AFP level declines attaining very low level (13-86 µg/ml) at birth. This fall in AFP content probably reflects a large increase in volume of the blood and fetal growth rather than to a decrease in rate of AFP synthesis in liver. After 32 weeks the liver production of AFP declines sharply resulting in low level of serum AFP (Seppala, 1977; Gitlin, 1975).

In rat, the fetal serum AFP concentration at birth is in excess of 5000 µg ml⁻¹ which remains high (1000 µg ml⁻¹) for about 4 weeks when there is abrupt decline to adult level (Stillman and Sell, 1979; Sell et al, 1974). This is about the time when liver proliferation ceases, and weaning and gluconeogenesis begin (Sell et al, 1975; Leffert and Sell, 1974).

**AMNIOTIC FLUID**

In human amniotic fluid, the AFP concentration is 100-300 times smaller than in fetal serum, but the decreasing slope with advancing gestation parallels that of fetal serum AFP profile (Seppala, 1977). It is assumed that AFP enters amniotic fluid via fetal urine (Seppala and Ruoslahti, 1972b) but skin, amnion, chorion, umblical cord, gastrointestinal tract, buccal and bronchial secretions may also contribute (Seller and Berry, 1978; Sutcliffe, 1975).
MATERNAL SERUM

During human pregnancy, peak level of AFP (150-250 ng ml\(^{-1}\)) in maternal serum are obtained at 32 weeks of gestation. At birth, the maternal AFP level is 200-600 times lower than fetal serum level and correspond well with placental size (Seppala, 1975). During the postpartum period, maternal AFP concentration decreases at a rate corresponding to a half life of 4-5 days (Seppala and Ruoslahti, 1973). It has been demonstrated that AFP in maternal serum is of fetal origin and maternal tissues are not capable of synthesizing AFP (Sell and Skelly, 1976; Sell, 1974). The increase in maternal serum AFP during pregnancy is probably due to the transplacental and transamniotic passage of AFP from fetus to mother.

NEWBORN AND NORMAL SERUM

The average AFP level in human cord blood (50 μg ml\(^{-1}\)) decreases with a half life of 3-5 days and a normal adult level (4-25 ng ml\(^{-1}\)) is attained after 1-2 yrs (Masseyeff et al, 1975). In rats, there is a fall of serum AFP level owing to loss of yolk sac at birth (Gitlin, 1975; Sell et al, 1974). AFP concentration decreases gradually during postnatal life (t \(1/2 = 24\) hrs) and normal adult basal level is attained after about 10 weeks (Olsson et al, 1977; Sell et al, 1974; Masseyeff et al, 1974).
PURIFICATION AND IMMUNOASSAYS OF AFP

Early attempts to purify AFP were mainly frustrating by the similarity of the physical and chemical properties of AFP and albumin. Human AFP in amounts sufficient for biological characterization was first obtained from the AFP-anti AFP immunoprecipitate by gel filtration at low pH (Nishi, 1970; Ruoslahti and Seppala, 1971a). Since then a great variety of procedures have been described with limited success (Howard et al, 1980; Kapadia et al, 1979; Mizejewski, 1979; Gold et al, 1978; Hassoux et al, 1977; Benassayag et al, 1975; Page, 1973; Sell et al, 1972). However, the immunoaffinity or immunoadsorbent procedure has been more successful (Ruoslahti, 1976; Nishi and Hirai, 1972; Nishi, 1972), especially on immunoadsorbent employing low affinity antibodies (Ruoslahti, 1978).

All currently used methods for AFP measurement rely on its antigenicity and differ in sensitivities (Table 1). Only radioimmunoassay (RIA) and enzyme immunoassays are sensitive enough to detect AFP in adult rat or human sera. However, at high concentration of AFP in the test sample, Laurell's rocket assay has many advantages over RIA (Ruoslahti and Seppala, 1979).

PHYSICO-CHEMICAL PROPERTIES OF AFP

The main physical and chemical characteristic of AFP's from various species are listed in Table II. Extensive
### Table I: Methods for APP Measurement

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Double diffusion</td>
<td>2.5–5 μg ml⁻¹</td>
<td>Abelev, et al. 1963†</td>
</tr>
<tr>
<td>Counter electrophoresis</td>
<td>0.25–0.5 μg ml⁻¹</td>
<td>Smith, 1971</td>
</tr>
<tr>
<td>Rocket immunoassay</td>
<td>0.5–1 μg ml⁻¹</td>
<td>Norgaard-Pedersen and Gaede, 1975</td>
</tr>
<tr>
<td>Latex agglutination inhibition</td>
<td>250 ng ml⁻¹</td>
<td>Cahill et al., 1974</td>
</tr>
<tr>
<td>Immunoautoradiography</td>
<td>50 ng ml⁻¹</td>
<td>Abelev, 1971</td>
</tr>
<tr>
<td>Radioimmunoelectrophoresis</td>
<td>20 ng ml⁻¹</td>
<td>Norgaard-Pedersen, 1976</td>
</tr>
<tr>
<td>Enzyme immunoassay (EIA)</td>
<td>3 ng ml⁻¹</td>
<td>Belanger et al., 1973; Masseyeff et al., 1976</td>
</tr>
<tr>
<td>Radioimmunoassay (RIA)</td>
<td>0.5 ng ml⁻¹</td>
<td>Ruoslhti and Seppala, 1971b.</td>
</tr>
</tbody>
</table>
TABLE II: PHYSICAL AND CHEMICAL CHARACTERISTICS OF HUMAN, MOUSE, AND RAT AFP *

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Human AFP</th>
<th>Mouse AFP</th>
<th>Rat AFP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight</td>
<td>69,000 (gel filtration, SDS-PAGE)</td>
<td>70,000</td>
<td>70,000</td>
</tr>
<tr>
<td></td>
<td>64,000 (ultra-centrifugation)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mol. wt. of polypeptide chain</td>
<td>69,000 (SDS-PAGE)</td>
<td>70,000</td>
<td>70,000</td>
</tr>
<tr>
<td>Sedimentation constant S_{20, W}</td>
<td>4.5</td>
<td>4.6</td>
<td>4.43, 4.76</td>
</tr>
<tr>
<td>Partial specific volume V_{20}</td>
<td>0.726</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diffusion constant D_{20, W}</td>
<td>6.18</td>
<td>-</td>
<td>6.25</td>
</tr>
<tr>
<td>Isoelectric point (pI)</td>
<td>4.8 (major)</td>
<td>4.7</td>
<td>4.8, 5.05</td>
</tr>
<tr>
<td>E_{1 cm} (278 nm)</td>
<td>5.30</td>
<td>4.15</td>
<td>-</td>
</tr>
<tr>
<td>C-terminal amino acid</td>
<td>valine</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>4.5%</td>
<td>3.7%</td>
<td>5.3%</td>
</tr>
<tr>
<td></td>
<td>4.1-4.7%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Taken from Ruoslahti and Hirai (1978).
comparison of fetal \( \Delta \text{FP} \) with \( \Delta \text{FP} \) obtained from hepatoma serum using immunological methods, electrophoresis, molecular weight determination, amino acid analysis, carbohydrate content (Ruoslahti and Engvall, 1978; Hirai et al, 1973; Gousev et al, 1971; Ruoslahti et al, 1971a, b; Nishi, 1970) and N-terminal sequencing (Aoyagi et al, 1977; Ruoslahti et al, 1974) has not disclosed any significant differences except in electrophoretic heterogeneity (Lester et al, 1976) and a difference in estrogen binding (Benassayag et al, 1975).

The amino acid compositions of human, rat, murine, bovine, porcine, ovine, dog, rabbit and chicken \( \Delta \text{FP} \) show significant resemblance to one another and to albumin (Ruoslahti and Seppala, 1979; Ruoslahti and Engvall, 1978). However, cDNA\( \Delta \text{FP} \) cannot hydridize with mRNA\( \Delta \text{Alb} \) (Belanger et al, 1978; Tamaoki et al, 1978; Innis and Miller, 1977). It appears that relationship between \( \Delta \text{FP} \) and albumin is similar to that of fetal and adult hemoglobin (Abelev, 1971).

**MICROHETEROGENEITY OF \( \Delta \text{FP} \)**

\( \Delta \text{FP} \) was originally thought to be a relatively simple protein molecule ranging in molecular weight from 65,000-70,000 and in electrophoretic mobility from \( \alpha_1 - \beta \) region depending upon its source (Abelev, 1971). In the past 15 years, however, \( \Delta \text{FP} \) has been shown to exist as a group of closely related molecular variants and to be present in sera of humans and animals with a variety of \( \Delta \text{FP} \) inducing conditions.
An extensive and excellent review has recently appeared on the heterogeneity of AFP and its physiological and pathological correlations (Smith and Kelleher, 1980).

AFP is heterogeneous with respect to charge, size and lectin binding (Table III). Depending upon the methods of fractionation two, four or nine molecular variants of rat AFP could be obtained (Bayard and Kerckaert, 1977; Kerckaert et al, 1977; Savu et al, 1977; Nunez et al, 1976; Benassayag et al, 1975; Smith and Kelleher, 1973). Fractionation of these charge variants on Con-A and Ricinus communis lectins allows even further sub-divisions of AFP variants (Smith and Kelleher, 1980). A scheme is depicted in Fig.1 which shows the heterogeneity of AFP with respect to various procedures.

Structural studies published to date have shed little light on the basis of charge and molecular heterogeneity. No major difference have been detected in amino acid composition (Peters et al, 1978; Kerckaert et al, 1977, 1975), partial amino acid sequence (Peters et al, 1978). C-terminal amino acid residue (Kerckaert et al, 1977), circular dichorism spectra (Aubert et al, 1977; Versee and Barol, 1976) in the two charge variants of rat AFP. Only sialic acid content has been found to be different in the two variants (Kerckaert et al, 1977). However, factors other than sialic acid are thought to involved in the observed heterogeneity due to
<table>
<thead>
<tr>
<th></th>
<th>Human</th>
<th>Rat</th>
<th>Mouse</th>
<th>G.Pig</th>
<th>Bovine</th>
<th>Rabbit</th>
<th>Monkey</th>
<th>Sheep</th>
<th>Chicken</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. LECTIN BINDING VARIANTS</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. Concanavalin A</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(Mannose, glucose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>b. Ricinus communis</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(galactose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>c. Lens Culinaris</td>
<td>3</td>
<td>2</td>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(Mannose, glucose)</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>d. Wheat germ agglutinin</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(N-Acetylglucosamine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>e. Lotus tetragonolobus</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(fucose)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>f. Ulex europaeus</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>(fucose)</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td><strong>2. CHARGE VARIANTS</strong></td>
<td><strong>2</strong></td>
<td>2</td>
<td>5</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>pI</td>
<td>4.8-5.2</td>
<td>4.72</td>
<td>4.8-</td>
<td>4.9-</td>
<td>4.6</td>
<td>-</td>
<td>4.8</td>
<td>-</td>
<td>4.9</td>
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<tr>
<td></td>
<td>5.15</td>
<td>5.2</td>
<td>5.5</td>
<td>4.86</td>
<td>5.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>3. MOLECULAR SIZE VARIANTS</strong></td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Mol.wt x 10^3</td>
<td>70</td>
<td>72</td>
<td>72</td>
<td>68</td>
<td>67</td>
<td>68</td>
<td>68</td>
<td>71</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

*Not determined; **Determined by electrophoresis under non-denaturing conditions.
(For Ref. see review by Smith & Kelleher, 1980).
**Anti Ar F’^pharo5e**

**AFP B**

**PAGE**

AFP B(Fast) 40%

**RCA^-reactive**

**RCA^-non reactive**

**AFPAi**

90% (54)

**AFPA2**

10% (6)

**RCA^-non reactive**

**Con A Sepharose**

**Non reactive**

46% 27% 27% 62% 14% 24% 50% 43% 7%

(248) (146) (146) (3.7) (08) (15) (20) (172) (28)

**Weakly reactive**

FIG. 1 Isolation of Rat AFP variants From Anti-AFP Fluid And Their Relative Proportion (Values In brackets are expressed as % of the whole starting AFP)”

*Modified from Bayerd and Kerckaert (1977)*

The difference in the terminal sugars on AFP molecule gives rise to its heterogeneity on reactivity with lectins having differing carbohydrate specificities. Affinity chromatography of mouse, rat and human AFP (Bayard and Kerckaert, 1981; Smith and Kellocher, 1980; Kerckaert et al, 1979; Ruoslahti and Adamson, 1978; Ruoslahti et al, 1978; Dambuyant et al, 1978; Bayard et al, 1977; Savu et al, 1977; Smith et al, 1977) on Con-A sepharoso yielded Con-A non-reactive, weakly reactive and reactive molecular variants. Con-A nonreactive AFP variant contains more N-Acetylglucosamine, galactose and sialic acid than does Con-A reactive one and may further differ in the structure of carbohydrate chain (Fig.2) (Bayard and Kerckaert, 1981). It has been demonstrated that Con-A affinity molecular variants are glycosylated differently (Ruoslahti and Adamson, 1978; Smith and Kollocher, 1978; Wilson and Zimmerman, 1976) and have different origins. The yolk sac synthesizes Con-A nonreactive while liver exclusively synthesizes and secretes Con-A reactive AFP variant.

Heterogeneity of AFP observed on Ricinus communis agglutinin (RCA) depends on the presence of external D-galactose residue (Bayard and Kerckaert, 1981). However, difference in sialic acid content may partly account for the
**Con A-reactive AFP**

\[ \text{NeuNAc-Gal-GlcNAc-Man} \rightarrow \text{Man-GlcNAc-GlcNAc-Asn} \]

**Con A-nonreactive AFP**

\[ \text{NeuNAc-Gal-GlcNAc-Man} \rightarrow \text{Man-GlcNAc-GlcNAc-Asn} \]

\[ \text{NeuNAc-Gal'-GlcNAc-Man} \rightarrow \text{Man-GlcNAc-GlcNAc-Asn} \]

**FIG 2. Structure of oligosaccharide units of the AFP Con A affinity molecular variants (Bayard and Rückaert, 1981)**
RCA-affinity molecular variants. Not much is known about the heterogeneity of AFP due to other lectins (Smith and Kelleher, 1980; Kerckaert et al, 1979).

Relative proportion of lectin (Con-A, RCA and LCA) affinity molecular variant of rat and human AFP may differ during neonatal growth in fetal serum, amniotic fluid, and maternal serum (Smith and Kelleher, 1980; Kerckaert et al, 1979; Fournier et al, 1979; Smith and Kelleher, 1978; Smith et al, 1977; Bayard et al, 1977) and in sera of hepatoma bearing rats, mice and human (Kelleher et al, 1979; 1978; McMahon et al, 1979; Smith et al, 1979, 1977). Con-A nonreactive AFP variant has also been found to decrease in certain abnormal pregnancies (Brock, 1979; Kelleher et al, 1979; Smith et al, 1979). It appears that lectin affinity molecular variants may be useful in the differential diagnosis of various diseases or abnormal pregnancies where AFP levels are known to be affected (Smith and Kelleher, 1980).

PHYSIOLOGICAL ROLE OF AFP: CORRELATION WITH MOLECULAR HETEROGENEITY

The physiological function(s) of AFP have not been established with certainty. Several investigators have shown evidence for (Innis and Miller, 1980; Ruoslahti and Engvall, 1978; Versee and Barol, 1978b; Grigurova et al, 1977; Ruoslahti and Terry, 1976) or against (Sell et al,
homology with serum albumin. It is thought that AFP may be a substitute for the latter, and that albumin replaces AFP in much the same way as adult hemoglobin replaces fetal hemoglobin (Abelev, 1971). AFP may also share some of the binding and transporting functions of albumin (Berde et al., 1979).

**BINDING OF ESTROGENS AND OTHER SUBSTANCE**

The estrogen binding property of rodent AFP's are firmly established (Uriel, 1979; Nunez et al., 1979; Versee and Barel, 1978; Attardi and Ruoslahti, 1976). AFP may also bind diethylstilboesterol (Savu et al., 1979; Aussel et al., 1973) and other norandrogens which do not possess estratriene skeleton (Versee and Barel, 1978). The association constant of AFP for estradiol is of the order of $10^8$ M$^{-1}$ (Aussel and Masseyeff, 1977; Bonassayag et al., 1975; Raynaud et al., 1971). Stoichiometry of rat AFP-estrogen interaction is controversial. One group finds two types of binding sites with different affinities and binding sites less than one (Vallett et al., 1977; Bonassayag et al., 1975) while others (Versee and Barel, 1978; Aussel and Masseyeff, 1977; Soloff et al., 1976) have found no heterogeneity and one binding site per mole of AFP. Similar discrepancies exist in case of the estrogen binding with various charge and lectin affinity molecular variants of AFP. One group has demonstrated that
AFP molecular variants differ in the estrogen binding and possess fractional binding sites (Vallett et al, 1979, 1977; Nunez et al, 1976; Benassayag et al, 1975) and propose that oligosaccharide unit has a role in estrogen binding. While several other investigators found no difference in estrogen binding by various AFP molecular variants and reported one binding site and propose that oligosaccharide moiety is not important for estrogen binding (Aussel et al, 1979, 1978, 1977; Soloff et al, 1976). The source of these discrepancies have not yet been clarified.

According to most studies human, guinea pig, hamster, bovine, rabbit and chicken AFP's are devoid of demonstrable estrogen binding activity (Ruoslahti and Seppala, 1979; Kiang et al, 1978; Nunez et al, 1976, 1974; Swartz and Soloff, 1975; Savu et al, 1974). It is proposed that lack of estrophilicity of human AFP may be due to very low proportion of Con-A nonreactive AFP variant (Vallett et al, 1977) which is high affinity estradiol binding component of rat AFP.

The physiological significance of estrogen binding is not known. Although speculation on a possible role of AFP in protecting the rat or mouse fetal brain against excessive maternal estrogens have been popular, only a few aspects have been experimentally investigated. The first one deal with the participation of AFP in the action of estrogen on
uterus (Uriel et al, 1976; Raynaud et al, 1971). Secondly, rat AFP has been shown to compete with the estrogen metabolizing tissue enzymes for the estrogens (Aussel et al, 1976). The overall result of this effect is likely to be inhibition of estrogen metabolism and to decrease tissue uptake of estrogens (German et al, 1978; Pool et al, 1978). Finally, the presence of AFP in the serum may influence the concentration of hormones with estrogen dependent expression in the circulation of neonatal rat (Aussel et al, 1980; Nunez et al, 1976).

**AFP AND IMMUNOREGULATION**

The idea that AFP would be immunosuppressive originates with the report of Parmely and Hsu (1973) and found confirmation in case of mouse, rat and human by several authors (Lester and Yachnin, 1979; Auer et al, 1979; Gupta and Good, 1977; Keller and Tomasi, 1976). Mouse AFP strongly suppresses the plaque forming cell response to sheep RBC in spleen cell cultures. Primary IgM, secondary IgM, IgA and IgG response were all inhibited (Murgita and Tomasi, 1975a,b). AFP suppresses certain T lymphocyte functions in vitro but does not directly inhibit B cell function and acts probably through induction of T cell with capacity to inhibit helper T cell (Alpert et al, 1978; Murgita et al, 1977). The significance of immunosuppressive activity of AFP is not clear. Although, immunosuppression of the mother by fetal
AFP would offer a convenient explanation for the survival of the fetus as an allograft, there is no direct evidence that AFP would be immunosuppressive in vivo. Evidence to the contrary has been presented (Sheppard et al, 1977; Sell et al, 1977; Belanger et al, 1976). Further, immunosuppression by human, rat and mouse AFP is also controversial. Sheppard et al, (1977) and Sell et al (1977) in extensive studies on the effect of mouse and rat AFP on immune response, could not confirm the findings reported by Murgita, Tomasi and co-workers. Several others (Littman et al, 1977; Charpentier et al, 1977; Goeken and Thompson, 1977) found no consistent immunosuppression by human AFP and propose that immunosuppressive activity is likely to be of no biological significance.

AFP has been demonstrated to inhibit the binding of myasthenia gravis antibody to acetylcholine receptor and thus provide clinical remission from the symptoms of the disease during pregnancy (Brenner et al, 1980). It is also proposed to perform similar role in other autoimmune diseases.

Human and rat AFP can also bind a variety of biologically active small molecular weight ligands (Table IV). It is proposed that AFP play a role in the transport of these biologically active substances (Ruoslahti and Seppala, 1979).
<table>
<thead>
<tr>
<th>LIGANDS</th>
<th>SPECIES</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>Rat, Mouse only</td>
<td>Kiang et al, 1978; Swartz and Soloff, 1975.</td>
</tr>
<tr>
<td>Bilirubin</td>
<td>Rat, Human, Bovine</td>
<td>Berde et al, 1979; Versee and Barel, 1979.</td>
</tr>
<tr>
<td>Retinoic acid</td>
<td>Rat</td>
<td>Ruoslahti, 1979.</td>
</tr>
<tr>
<td>Dyes</td>
<td>Human</td>
<td>Endo et al, 1974.</td>
</tr>
<tr>
<td>Copper and nickel</td>
<td>Human</td>
<td>Aoyagi et al, 1978.</td>
</tr>
</tbody>
</table>
MOLECULAR BASIS OF REGULATION OF AFP AND ALBUMIN SYNTHESIS

The striking variation of AFP and albumin production accompanying cell differentiation and carcinogenesis indicate that effective mechanisms of turning the AFP synthesis on and off must exist. However, very little is known about the exact molecular nature of the events that are behind the expression of AFP production.

Various hormones markedly suppress serum AFP levels in newborn rats and in hepatocyte culture in vitro (Belanger et al., 1978a, 1975). Glucocorticoids (prednisolone, dexamethasone, hydrocortisone) induces a dramatic decrease in serum AFP level while insulin, glucagon, deoxycorticosterone, testosterone, progesterone and estradiol revealed no consistent effects. Suppression of AFP synthesis by glucocorticoids is mediated by a direct decrease in the amount of AFP mRNA (Commer et al., 1979). Conversely, in animals submitted to AFP inducing condition (CCL₄ intoxication, 3'-DAB carcinogenesis) corticoids increased the AFP levels (Belanger et al., 1978a). ACTH, dbcAMP, thyroxine and epinephrine may also induce an accelerated decrease of serum AFP in newborn rat, while iron deficiency has an opposite effect. The accelerated decrease of AFP from serum by a factor present in adult mouse organ (Tumyan et al., 1975) might have contained more of hormones known to suppress serum AFP levels. It has been observed that experimental induction
of AFP is accompanied by an increase in methionine levels with a depletion of ATP pool (Belanger et al, 1978b, 1976) and ATP administration may prevent induction of AFP (Taketa et al, 1976). This hypothesis implies that methionine activation and AFP expression are functionally related and altered transmethylation event might determine a modification of gene expression towards incomplete differentiation which includes re-expression of AFP.

Genetic experiments on the regulation of AFP gene have revealed that AFP is controlled by a single Mendelian gene, the Raf gene (Rat AFP gene) (Olsson et al, 1979; Lindahl et al, 1978). How direct the regulation of AFP by this gene is, remains to be elucidated.

Several laboratories have, during the past couple of years, been tooling up to study the regulation of the AFP gene using methods of molecular biology. The mRNA for AFP has been isolated and cDNA probe prepared (Law et al, 1980; Innis and Miller, 1980, 1979; Simon et al, 1980; Gorin and Tilghman, 1980; Miura et al, 1979). Using this AFP cDNA probe it has been demonstrated that the control of AFP synthesis takes place at the transcription level. The change in AFP levels during normal fetal development (Liao et al, 1980; Nishi et al, 1979; Miura et al, 1978; Iio et al, 1976; Tamaoki et al, 1976; Koga and Tamaoki, 1974) and in carcinogenesis or liver regeneration (Atryzek et al, 1980; Salatrep et al, 1979; Chiu et al, 1979; Miura et al, 1979;
Sell et al, 1979; Innis and Miller, 1979) is directly related to the amount of AFP mRNA and does not arise from any change in the translation activity.

**REGULATION OF ALBUMIN SYNTHESIS**

Albumin concentration in serum is the net result of its synthesis, degradation and distribution. Several factors are responsible for modulation of albumin metabolism (Rothschild et al, 1977). The most important factor is nutrition. Deficiency of dietary protein results in the decrease of albumin synthesis (Pain et al, 1978; Morgan and Peters, 1971) with a corresponding decrease in albumin synthesizing cells (Chandrashekharan, 1967) and a decrease in albumin mRNA (Pain et al, 1978). Fasting also decreases albumin synthesis (Yap et al, 1978). Thyroid hormone and cortisone increase albumin synthesis by a direct increase in albumin mRNA level (Keller and Taylor, 1979). Similarly, hypophysectomy results in 50% decrease in albumin synthesis with a corresponding decline in its mRNA (Keller and Taylor, 1979; Feldhoff et al, 1977). Growth hormone treatment may restore albumin mRNA level and normal rate of albumin synthesis in hypophysectomized rats.

Several conditions like CCl₄ intoxication, ethanol administration partial hepatectomy or alloxan induced diabetes decrease albumin synthesis due to disaggregation of polysomes from RER and a decrease in albumin mRNA level
Certain hereditary disorders of albumin metabolism like analbuminemia (complete absence of albumin in serum) has been shown to be due to the complete absence of functional albumin mRNA in liver (Sugiyama et al., 1980). Thus there appears to be a close resemblance in the amount of albumin mRNA, albumin synthesizing polysomes, and albumin synthesis in liver.

The exact mechanism or site(s) for the catabolism of albumin are not known. Since, no single organ has yet been identified as the major site of albumin catabolism, the possibility of diffused catabolism throughout the body involving reticuloendothelial system or the vascular endothelium may be the major site of albumin catabolism (Baynes and Thorpe, 1981; Waldman et al., 1977).

**AFP IN LIVER DISEASES AND GERM CELL TUMORS**

**LIVER CELL PROLIFERATION**

The proliferation of hepatocytes in adult liver is generally associated with AFP production (Abolev, 1974). This has been demonstrated in animal experiment following CCl₄ intoxication, partial hepatectomy and chemically induced liver necrosis (Chiu et al., 1981; Ruoslahti and Seppala, 1979; Stillman and Sell, 1979; Engelhardt, 1976; Sell et al., 1974). AFP production during regeneration takes place in mature hepatocytes due to derepression of AFP gene resulting in increased level of AFP mRNA (Chiu et al., 1981;
Abelev, 1979). In humans, elevated serum AFP levels are found in hepatitis, liver necrosis or cirrhosis and tend to occur in regeneration phase (Lehman and Wegner, 1979; Ruoslahti et al, 1974; Silver et al, 1974). Elevation of AFP levels in serum after ligation of the common bile duct is proposed to be due to the ductular cell proliferation (Wu et al, 1981).

**HEPATOCARCINOGENESIS**

Feeding of various hepatocarcinogens (3'-methyl-DAB, AAF, ethionine, DEN etc) results in the elevation of AFP level (Stillman and Sell, 1979). An early rise occurs within a few weeks or even hrs after the initiation of the treatment or with a subcarcinogenic, nontoxic dose of carcinogen without any apparent liver injury (Stillman and Sell, 1979; Kroes et al, 1975; Becker and Sell, 1974). The final elevation of AFP is usually followed with the appearance of malignant cells with increasing size of the tumor and with metastatic spread. There seems to be a general agreement that 'Oval cells' or transitional basophilic hepatocyte like cells are responsible for early rise of AFP and may give rise to cancer cells (Atryzek et al, 1980; Guillouzo et al, 1978; Kuhlman, 1978a). Whether all tumor cells go through a stage of AFP production some time during their life span, or whether AFP production is a function of differentiated subpopulation of tumor cells, is not clear (Abelev, 1979).
HEPATOCELLULAR CANCER

Highly elevated levels of AFP in serum have been found to be specific for PHC (Ruoslaiti and Seppala, 1979; Ruoslaiti et al, 1974; Abelev, 1974; McIntire, 1972). The occurrence of AFP in serum of patients with PHC shows a striking geographical variation. The highest positive rates are found in those areas where tumor occurs most commonly e.g. Indonesia 87%, South Africa 78%, Taiwan 73%, compared to 29% in Britain and 28-50% in USA (Ruoslaiti and Seppala, 1979). The reason for this variation is not known but it may reflect the difference in the etiology of the tumor in endemic and nonendemic parts of the world. A survey of 500,000 individuals in China revealed several new cases of PHC (Nature editorial, 1977, coordinating group for Res. on liver cancer, 1974). Malignant cells or hepatocytes grown in culture synthesize AFP (Suganuma et al, 1980; Kuhlman, 1978a,b; Masseyeff et al, 1973). AFP producing cells (malignant) have been found to be situated near vessels surrounding the central veins or scattered throughout the tumor (Nishioka et al, 1972; Gousse et al, 1971). As might be expected, serum AFP level falls rapidly after resection of the tumor (Stillman and Sell, 1979; Sugahara et al, 1973; Alpert et al, 1971).

It is apparent that AFP assays can be used for the detection and localization of PHC and to assess completeness
of surgical resection of the tumor and for the subsequent surveillance of the patients with regard to recurrence or growth of metastasis.

**GERM CELL AND ENDODERMAL CELL TUMORS**

The association of elevated serum AFP level with teratocarcinomas of the ovary and testis is now well documented (Eyben et al, 1980; Kurman et al, 1977; Grigor et al, 1977; Kohn et al, 1976; Tsuchida et al, 1975; Wahren et al, 1974). Yolk sac tumors produced in mice by murine sarcoma virus also produce AFP (Hooghe and Zeicher, 1974). Immunohistochemical studies have shown that AFP is localized in the yolk sac tumor (Kurman et al, 1977; Teilum et al, 1974). In patients with yolk sac tumor, the serum AFP concentration reflects the activity of the disease and proved to be useful in monitoring of the treatment (Norgaard-Pederson, 1978; Lange et al, 1977). AFP has also been reported to occur in a wide range of endodermal tumors without hepatic metastasis. Gastrointestinal tract tumor seems to be common site for AFP production (Akai and Kato, 1973; Montplaisis et al, 1973).

Germ cell tumors, developing primarily in the skull, comprise about 5% of all brain tumors. Several patients with primary intracranial germ cell tumors have been found to have elevated serum AFP level (Arita et al, 1980; Norgaard-Pedersen, 1978). Several other kind of tumors like dysgerminomas, retinoblastomas and various carcinomas of
breast, lung, basal cell carcinoma of nose (Lie-Injo et al, 1976; Michelson et al, 1976; Tsuchida et al, 1975) and in the epidermal cell tumor (Bowen's disease) (Soltani et al, 1978) may show high levels of AFP.

**AFP IN OTHER LIVER DISEASES**

AFP is elevated in about 10% of the patients with liver diseases other than PHC. These include acute viral hepatitis, toxic hepatitis, chronic active hepatitis, cirrhosis, liver trauma, congenital biliary artresia and neonatal hepatitis (Ruoslanti and Seppala, 1979; Lehman and Wegner, 1979) with an elevation of AFP level from slight to moderate. A strong association has been observed between PHC and evidence for a contact with hepatitis B virus (Tabor et al, 1977). Vaccination against the virus may prove to be an effective measure to prevent liver cancer (Ziegler et al, 1978). Tyrosinemia, which is also associated with elevated AFP levels, often leads to the development of PHC and repeated testing of such individuals may reveal patients at a curable stage (Belanger et al, 1976).

**AFP AS A POSSIBLE TARGET MOLECULE FOR TUMOR IMMUNOPREVENTION AND IMMUNOTHERAPY**

Several reports indicate that heterologous antibodies to AFP inhibit the growth of hepatoma cells *in vitro*, inhibit the growth of hepatoma transplanted in rat and human testicular tumor transplanted into nude mice, and
inhibition of tumor development in hepatocarcinogenesis (Hirai, 1979). It has been proposed that anti-AFP may coat tumor cells and in this way increases their immunogenecity to the host or in some way facilitate recognition of their foreignness to the host. Small amount of AFP may be bound to the cell surface and binding of antibody induce a cytostructural alteration of the cell membrane which would eventually lead to the death of the tumor cell (Wepsic et al, 1980).

Effect of anti-AFP on pregnancy is controversial. Degenerative changes have been reported to occur in the fetal liver of rabbit with autologous antibodies. However, production of homologous antibodies did not elicit any abnormality (Goussev et al, 1974). Injection of heterologous antiserum into pregnant animal have been aruged to have an abortogenic effect (Mizejewski and Grimley, 1976; Slade, 1973), but this has not been confirmed by several investigators (Hirai, 1979; Ruoslahti and Seppala, 1979; Leung et al, 1977).

The use of radiolabelled antibodies against AFP has proved to be useful in tumor localization (Goldenberg, 1980). The possibility of a clinical application of the antiserum treatment, either heterologous or homologous, to the patients with hepatoma, yolk sac tumor or cirrhosis may prove very useful and needs further clinical trials.
The AFP assays have recently become important in the prenatal diagnosis of certain fetal defects and a number of other situations in which it is necessary to monitor the fetal condition. Some excellent reviews have recently appeared on these aspects (Ruoslahti and Seppala, 1979; Brock, 1979, 1977; Seppala, 1977; Kjessler and Johansson, 1977). Table V summarizes the possible diagnostic value of AFP in different trimesters of pregnancy (Lau and Linkins, 1976).

Normal levels of AFP in amniotic fluid, maternal serum and fetal serum at various stages of pregnancy has already been established (Seppala, 1977). Abnormal values of AFP in these compartments may arise due to abnormal development of pregnancy and presence of various fetal morphogenetic defects, such as open neural tube defect (NTD), hereditary congenital nephrosis (Finnish type), omphalocele, pitonidal sinus, esophageal artresia and others (Ruoslahti and Seppala, 1979).

The classic observation of Brock and Sutcliffe (1972) that great majority of pregnancies with fetuses affected by open NTD (anencephaly, myelocoele, cervical meningocoele hydrocephalus) are associated with elevated levels of AFP in amniotic fluid, is now extremely valuable in obstetric
<table>
<thead>
<tr>
<th>TABLE V: AFP IN THE DIAGNOSIS OF FETAL PATHOLOGY</th>
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<tbody>
<tr>
<td><strong>FIRST TRIMESTER</strong></td>
</tr>
<tr>
<td>Predict outcome of threatened abortion</td>
</tr>
<tr>
<td>Select cases of cervical incompetence</td>
</tr>
<tr>
<td>Screen for early birth defects</td>
</tr>
<tr>
<td>Differentiation of male and female pregnancy</td>
</tr>
<tr>
<td>Detection of missed abortion and incomplete abortion.</td>
</tr>
<tr>
<td><strong>SECOND TRIMESTER</strong></td>
</tr>
<tr>
<td>Assessment of severity of diabetes, Rh-immunization and toxaemia</td>
</tr>
<tr>
<td>Detection of late congenital defects</td>
</tr>
<tr>
<td>Screening for twins</td>
</tr>
<tr>
<td><strong>THIRD TRIMESTER</strong></td>
</tr>
<tr>
<td>All the above conditions</td>
</tr>
<tr>
<td>Determination of gestational age</td>
</tr>
<tr>
<td>Detection of fetal distress and impending fetal death</td>
</tr>
<tr>
<td>Assessment of uterine bleeding</td>
</tr>
<tr>
<td>Detection of cancer in pregnancy</td>
</tr>
</tbody>
</table>
practice. The frequency of open NTD in various countries is as follows:

<table>
<thead>
<tr>
<th>Country</th>
<th>Rate (per 1000 pregnancies)</th>
</tr>
</thead>
<tbody>
<tr>
<td>North Ireland</td>
<td>7.2</td>
</tr>
<tr>
<td>Wales</td>
<td>5.8</td>
</tr>
<tr>
<td>Scotland</td>
<td>5.6</td>
</tr>
<tr>
<td>England</td>
<td>4.0</td>
</tr>
<tr>
<td>USA</td>
<td>4.5</td>
</tr>
<tr>
<td>India</td>
<td>3.7</td>
</tr>
<tr>
<td>India (North)</td>
<td>6.7</td>
</tr>
</tbody>
</table>

The presence or absence of certain cells (neural, epithelial, peritoneal, and placental cells) in amniotic fluid originating from fetal brain and spinal cord may further prove to be helpful in more precise detection of various fetal abnormalities.

Determination of AFP in maternal serum in symptom free pregnancies, not known to be at increased risk of having affected offspring, may provide valuable information. Maternal serum AFP levels may generally be increased in twin pregnancies and NTD (Brock, 1977, 1975; Collaborative report of UK, 1977) hereditary congenital nephrosis, Meckle's syndrome and intrauterine fetal death (Kjessler and Johansson, 1977, 1975; Seppala, 1977).

Screening of all pregnancies by maternal serum AFP is now contemplated in several countries. Such screening
would yield about 3000 cases with elevated values per 100,000 pregnancies. In a repeat serum sample, the number would be reduced to 500 requiring amniocentesis and likely to reveal at least 50 cases of NTD (report of the UK collaborative study, 1977) along with several cases of congenital nephrosis, twin pregnancies, low birth weight infants and intrauterine fetal death.
MATERIALS & METHODS
DEAE-cellulose (DE-52), bovine serum albumin, human serum albumin (Cohn fraction IV), ribonuclease, cytochrome C, polyethylene glycol-6000, collagenase (type IV), trypsin, insulin, cycloheximide, N⁶, O², dibutyryl 3',5'-cyclic adenosine monophosphoric acid (dbcAMP), dexamethasone, estrone (E₁), estradiol-17 β (E₂), diethyl-stilboesterol (DES) sodium dodecyl sulphate (SDS) and β-mercaptoethanol were procured from Sigma Chemical Company, St. Louis, MO, USA. Agarose, commassie brilliant blue, 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethane sulphonylic acid (HEPES), sepharose-4B and 6B, sucrose, 2,5-diphenyloxazole (PPO), 1,4-bis-(5-phenyloxazolyl) benzene (POPOP), tris (hydroxymethyl)-aminomethane (Tris), glycine, amino acids were purchased from SISCO Research Laboratories, Bombay, India. Acrylamide, bisacrylamide (bis), ammonium persulphate, ethylene diamine tetraacetic acid (EDTA), N,N,N',N'-tetramethylene diamine (TEMED) were products of Eastman, Kodak, Rochester, New York, USA. Leibowitz-15 (L-15) medium, leucine free Eagle's MEM-199, penicillin, streptomycin, fungizone, were obtained from GIBCO, USA. Concanavalin-A was a product of PL Biochemicals, USA. Lysozyme, pepsin, and amido black -1OB were procured from E.Merck, Darmstadt, Germany. Complete Freund's adjuvant (CFA) was supplied by Difco, England. Cibacron blue -F3GA
and epichlorohydrin were a generous gift from Mr. Mohd. Akhlaq, Department of Biophysics, C.D.R.I., Lucknow. 6,7-[^3]H-estradiol (specific activity 44.0 Ci/m mol) was procured from New England Nuclear, Boston, USA, while DL-1-[^14]C-leucine (specific activity 28.9 mCi/m mol) was supplied by Bhabha Atomic Research Centre (BARC), Bombay, India. All other chemicals and reagents were of AR grade.

**ANIMALS**

Inbred albino rats (Charles Foster strain) were drawn from Central Drug Research Institute animal colony. The animals were housed in air-conditioned quarters and fed standard balanced pellet diet (Hind Lever, Bombay, India) and water *ad libitum* throughout the experiments. Adult albino rabbits and guinea pigs were also drawn from our institute animal colony.

Fetal and neonatal rats of known ages were obtained from timed pregnancies. The female rats were mated in the pre-estrus phase of estrus cycle as determined by vaginal smears. The day after mating was designated as day zero of pregnancy. The fetuses were removed from the pregnant females on day 16, 18 and 20 of gestation. Postnatal rats after 1, 3, 7, 14, 21, 28 days were also used taking day of birth as day 1 of postnatal life.
NORMAL RAT SERUM (NRS) AND PREGNANT RAT SERUM (PRS)

Blood was withdrawn through hepatic portal vein from several adult rats and from pregnant rats (20 d gestation). Sera obtained were pooled separately and labelled as NRS and PRS and stored frozen.

AMNIOTIC FLUID (AF) AND FETAL SERUM (FS)

Intact fetuses at 16, 18 and 20 d gestation were removed from the uterus and washed in normal saline. Amniotic fluid was collected by puncturing the amniotic membrane. The fluid obtained was centrifuged at 4000 rpm for 15 min. at 4°C and stored frozen. Fetal blood was collected from the decapitated fetuses. Sera obtained from several fetuses of each age group were pooled separately and stored frozen.

FETAL EXTRACT (FE)

The fetuses removed from pregnant rats at 16 d of gestation were washed in phosphate buffered saline, pH 7.4 and homogenized. The homogenate (50% v/v) was cleared at 40,000 rpm at 4°C. The supernatant was termed as fetal extract and stored frozen in small aliquots.

PURIFICATION OF RAT AND HUMAN SERUM ALBUMIN

PREPARATION OF BLUE-SEPHAROSE

Epichlorohydrin cross-linked sepharose-6B was prepared according to the procedure of Porath et al (1971).
20 g of sepharose 6B was treated with 18 ml of 1M NaOH and 2 ml of epichlorohydrin at room temperature in the presence of 40 mg of sodium borohydride. The suspension was heated to 60°C with stirring and the reaction was stopped after 2 hr. The gel was washed free of alkali with distilled water.

Blue-sepharose-6B was synthesized by coupling cibacron Blue-F3 GA to epichlorohydrin cross-linked sepharose-6B essentially according to the procedure of Bohme et al (1972). A solution of 200 mg of cibacron Blue-F3 GA in 50 ml of water was added dropwise with vigorous stirring to a 10 g suspension of epichlorohydrin cross-linked sepharose-6B in 350 ml of water at a temperature of 60°C. After stirring for 30 min 45 g of NaCl was added and the stirring continued for 1 hr. 4 g Na₂CO₃ was added to the mixture heated to 80°C. It was then stirred at this temperature for 2 hrs. After cooling to room temperature the gel was filtered by suction on a Büchner funnel and washed repeatedly with 0.02 M acetate buffer, pH 4.5, and 0.02 M bicarbonate buffer, pH 9.0 containing 2 M NaCl. After washing at pH 9.0, the gel was washed thoroughly with water. The extent of dye binding was determined by hydrolyzing the gel in 6M HCl at 40°C for 30 min, reading the absorbance at 650 nm and comparing the values to a standard curve prepared with the pure dye. The dye concentration was 0.69 μ mol/ml of packed gel volume.
Purification of Rat Serum Albumin from NRS by PEG Precipitation and Affinity Chromatography on Blue Sepharose

Rat albumin was partially purified from NRS by polyethylene glycol precipitation procedure of Jimenez et al. (1974). NRS was treated with 25% polyethylene glycol (mol. wt. 6000) at 4°C with constant stirring for 30 min. The precipitate was discarded and to the supernatant, ethanol was added to a final concentration of 40% at -5°C. After keeping it for 4 hrs at 4°C it was centrifuged and supernatant discarded. The precipitate was dissolved in water and ethanol added to a final concentration of 40% at pH 5.0 and -5°C. This process was repeated thrice and the precipitate was finally dissolved in 0.05 Tris-HCl, pH 7.4 containing 0.1 M KCl. This preparation showed a minor band along with a major band of albumin on PAGE and was further purified by affinity chromatography on blue sepharose (Smith and Kelleher, 1979).

Partially purified rat serum albumin (20 mg) was applied on blue-sepharose column (1.0 x 15.0 cm) equilibrated with 0.05 Tris, pH 7.0, containing 0.1 M KCl and washed thoroughly with this buffer. Albumin was eluted with the starting buffer containing 1.5 M KCl. Elution of the protein was followed by measuring absorbance at 280 nm. Protein rich fractions were pooled and dialysed against water for 24 hrs and lyophilized. The purity of albumin
was checked on polyacrylamide gel electrophoresis, immunoelectrophoresis, and double diffusion.

**PURIFICATION OF HUMAN SERUM ALBUMIN (HSA)**

Human serum albumin was purified from its crude preparation (Cohn fraction IV) by affinity chromatography on blue sepharose as described above.

**ANTISERA**

**RABBIT ANTI-NORMAL ADULT RAT SERUM (ANTI-NRS)**

Anti-NRS was obtained by immunizing rabbits four times with 1.0 ml of normal adult rat serum mixed with an equal volume of complete Freund's adjuvant (CFA) administered subcutaneously at one week interval. The animals were administered two booster doses of 1.0 ml NRS intramuscularly at 10 days intervals and were bled after 10th day of last injection when antibody titre was maximum. Antibody titre was evaluated by double diffusion.

**RABBIT ANTI-NORMAL ADULT HUMAN SERUM (ANTI-NHS)**

Anti-NHS was obtained by immunizing rabbits with NHS using the same protocol as described for anti-NRS.

**GUINEA PIG ANTI-NORMAL RABBIT SERUM (ANTI-NRbS)**

Immunization schedule followed to raise antibody against normal adult rabbit serum (NRbS) in guinea pigs was similar to that used for anti-NRS except that 0.5 ml NRbS was used for initial immunization.
RABBIT ANTI-NORMAL ADULT RAT SERUM ALBUMIN (ANTI-RSA)

Purified RSA obtained by affinity chromatography on blue sepharose was used for immunizing rabbits. Four subcutaneous injections of 2, 4, 4 and 6 mg each of purified RSA in 1.0 ml normal saline mixed with an equal volume of CFA were administered weekly. Then three booster doses, 6 mg each of RSA, were administered intramuscularly at 10 day intervals. The animals were bledd when antibody titre reached peak value as analyzed by double diffusion.

RABBIT ANTI-NORMAL ADULT HUMAN SERUM ALBUMIN (ANTI-HSA)

Anti-HSA was obtained by immunizing rabbits with HSA purified by affinity chromatography on blue sepharose. The immunization schedule was similar to that used for anti-RSA.

RABBIT ANTI-RAT AMNIOTIC FLUID SERUM (ANTI-AF)

Antibody against pooled rat amniotic fluid was raised in rabbits by following the immunization protocol similar to that used for anti-NRS.

PREPARATION OF ANTI-AFP*

Anti-AF serum was made specific for AFP by repeated absorption with lyophilized NRS (0.3 mg/ml anti-AF) till no visual immunoprecipitation occurred. The anti-serum thus prepared did not reacted with any adult rat serum
proteins and was specific for AFP. It was termed as anti-AFP*.

**RABBIT ANTI-HUMAN AMNIOTIC FLUID SERUM (ANTI-HAF)**

The rabbits were immunized with human amniotic fluid (14th week of gestation) as described for anti-NRS.

Anti-HAF was also adsorbed with lyophilized adult human serum and made specific for AFP and termed as anti-HAFP*.

**ISOLATION OF IMMUNOGLOBULINS (IgG) FROM ANTI-NRS AND ANTI-NHS BY DEAE-CELLULOSE CHROMATOGRAPHY**

Antiserum (anti-NRS or anti-NHS) was precipitated at 4°C by addition of ammonium sulphate at a final concentration of 40%. The precipitate was washed twice with 40% ammonium sulphate solution and dissolved in 0.01 M phosphate buffer, pH 8.0 (one third volume of initial antiserum) and dialyzed against the same buffer for 24 hrs at 4°C with several changes of the dialyzing buffer.

The crude immunoglobulin sample (300 mg protein) was applied to DEAE-cellulose column (2.5 x 30 cm) pre-equilibrated with 0.01 phosphate buffer, pH 8.0 (buffer I). Unadsorbed proteins were washed through the column with buffer I at a flow rate of 50 ml/hr. Protein content in the effluent was followed by measuring the absorbance at 280 nm. IgG which does not bind to the adsorbant column, under
these conditions, was collected in the unadsorbed peak. Immunological activity of the protein rich fraction (IgG) was checked on double diffusion against NRS. IEP of the isolated immunoglobulins and subsequent diffusion with guinea pig anti-normal rabbit serum did not revealed any precipitin arc in α or β region. This indicates that the immunoglobulins preparation contain proteins with γ-mobility only (IgG). The IgG was concentrated by lyophilization and stored frozen in small aliquots.

**ANALYTICAL TECHNIQUES**

**OUCHTERLONY’S DOUBLE DIFFUSION.**

Double immuno diffusion method was basically according to Ouchterlony (1958). Hot agarose solution (1% in PBS containing 0.02% sodium azide) was poured on microscopic slides or glass plates placed on levelling table and kept at room temperature for 15 minutes to allow the formation of gel. Wells of appropriate sizes were punched out. Antigen and antibody were then filled in appropriate wells and allowed to diffuse at room temperature for 24 hrs in a humid chamber. After formation of precipitin lines, the gel plates were washed in normal saline for several days at 4°C and stained with amido black (0.25% in 7% glacial acetic acid and 5% methanol) to visualize the precipitin lines.
IMMUNOELECTROPHORESIS (IEP)

The procedure developed by Grabar and Williams (1955) was used to perform immunoelectrophoresis on 1% agarose plates buffered at pH 8.3 with 0.05 M barbital-HCl buffer. Antigens were filled in the wells and electrophoresed at constant current of 2 mA/slide for 2 hrs at room temperature. Bromophenol blue was used as indicator dye. After electrophoresis, appropriate antisera were filled in the troughs and allowed to diffuse at room temperature for 24 hrs in a humid atmosphere. The plates were washed and stained as described above.

Immunological identity of the antigen samples was revealed by diffusing the antigens (after electrophoresis) between the two parallel troughs; one containing the known antigen (with which the immunological identity is being investigated) and the other corresponding antiserum (Osserman, 1970).

POLYACRYLAMIDE GEL ELECTROPHORESIS (PAGE)

Analysis of the proteins on polyacrylamide gel electrophoresis was performed on 10% gels as described by Benassayag et al (1975) with the following gel composition: 8 g acrylamide, 0.16 g bis, 56 mg ammonium persulphate, and 0.023 ml TEMED in 80 ml of Tris (0.3 M)/HCl (0.06 M) buffer, pH 8.9. The migration of the proteins was
performed at room temperature, unless otherwise stated, in a Tris (0.025 M)/glycine (0.2 M) buffer, pH 8.3, for 3 hrs at a constant current of 1.5 mA per tube. After electrophoresis, the gels were stained with coomassie brilliant blue (0.25% coomassie blue in 7% glacial acetic acid and 5% methanol) for 30 min. Destaining of the gels was performed in 7% glacial acetic acid and 5% methanol solution.

**DETERMINATION OF MOLECULAR WEIGHT BY SDS-PAGE**

Molecular weight of the pure protein was determined by the procedure of Weber and Osborn (1969). Cyt C (11,700), RNase (13,700), lysozyme (14,300), pepsin (35,000), and BSA (66,000) were used as known molecular weight protein markers.

In some experiments AFP and albumin were immunoprecipitated from culture medium using specific antiserum. The immunoprecipitates were processed for the determination of molecular weight of dissociated antigens (AFP and albumin) according to Schapiro et al (1967).

**ESTRADIOL BINDING ASSAY OF RAT AFP**

Interaction of $[^3H]$-estradiol with AFP was studied as described by Plapinger et al (1973). Aliquots of AFP (5 μg) in 0.1 M phosphate buffer, pH 7.4, in triplicates were incubated with $[^3H]$-E$_2$ (2-100 nM) with or without 1000 fold excess of cold E$_2$ for 3 hrs at 4°C. After incubation, 50 μl dextran coated charcoal (DCC)[1% dextran (T-70) and 10%
charcoal (Norit A) was added to each tube and mixed vigorously followed by centrifugation at 800 x g for 10 min. Radioactivity was measured in the supernatants in a Tricarb liquid scintillation counter with an efficiency of 50%.

4 g PPO and 100 mg POPOP dissolved in one litre 1:1 mixture of toluene and 2-methoxy ethanol was used as a scintillation cocktail. Association constant and number of binding sites for E sub 2 per mol of AFP were calculated from Scatchard plot (1949).

**QUANTITATION OF AFP AND ALBUMIN BY ROCKET IMMUNOASSAY**

AFP and albumin were quantitated in various samples by Laurell's rocket immunoassay (1965). Agarose (2% in 0.05 M barbital-HCl buffer, pH 8.3, containing 0.02% sodium azide) was melted in a water bath and maintained at 45° C. Suitably diluted specific antiserum (anti-rat AFP or anti-rat albumin, or anti-human AFP) was mixed with equal volume of 2% agarose solution and mixed thoroughly. Antibody-agarose solution was then poured on pre-warmed glass plates kept on a levelling table. Amount of agarose plated was adjusted to give a uniform gel thickness of 1.5 mm. After formation of the gel, series of wells were punched out and kept in humid atmosphere at 4° C.

Serial dilutions of appropriate samples (10 μl) were filled in the wells and electrophoresis was carried out at room temperature for 4 hrs using 0.05 M barbital-HCl buffer,
pH 8.3, at a constant voltage of 5 V/cm of agarose gel. After electrophoresis immunoprecipitin rocket height was measured under a magnifying lens. Amount of AFP or albumin in unknown sample was determined from the standard curve for that particular antigen. Purified rat serum albumin and AFP served as the standards. Standard human AFP was a 72/225 preparation of cord serum (WHO, Lyon, France, 1975). This preparation has 50,000 international units (IU) of AFP per ml of standard cord serum. One I.U. corresponded to 1.18 ng human AFP. Sensitivity of this assay, in our hands, is 2-3 μg/ml for rat AFP, 3-5 μg/ml for rat albumin and 1-2 μg/ml for human AFP.

A typical standard curve for rat AFP is shown in Fig.3A. Insert in Fig.3A shows the rocket pattern (in duplicate) of three serial dilution of rat AFP. Fig.3B shows a typical rocket assay plate used for quantitation of rat AFP in samples containing variable amounts of the protein. Large rockets (open peaks) are due to the high concentration of the protein.

**ESTIMATION OF PROTEIN**

Protein estimation was carried out according to Lowry et al (1951) using bovine serum albumin as standard.
FIG. 3 QUANTITATION OF AFP BY ROCKET IMMUNOASSAY

3A. Standard curve for rat AFP. Insert in Fig. 3A shows a typical pattern of rockets for a series of three standard concentration (in duplicate) of rat AFP.

3B. A typical rocket assay plate for the quantitation of rat AFP. Sample wells contain varying concentration of AFP (open rockets are due to the high concentration of AFP).
PREPARATION OF VARIOUS AFFINITY MATRICES

PREPARATION OF CYANOGEOB BROMIDE (CNBr)

CNBr was synthesized in this laboratory according to the procedure described in organic synthesis Vol. 2. A white crystalline product with melting point of 49-51°C and boiling point of 60-61°C was obtained. Crystalline CNBr was dissolved in acetonitrile (2 g CNBr/ml CH₃CN) and stored frozen in small aliquots.

CNBr ACTIVATION OF SEPHAROSE-4B

Sepharose-4B was activated using CNBr (200 mg CNBr/ml of gel) essentially according to March et al (1974). Amount of CN⁺ ions linked to the gel was quantitated by a colorimetric procedure of Kohn and Wilchek (1978). Usually 6-8 μ moles of cyanide ions were bound per ml of the packed gel volume.

PREPARATION OF CONCANAVALIN A-SEPHAROSE (CON A-SEPHAROSE)

Con-A was coupled to CNBr activated sepharose-4B according to Porath et al (1967). Usually about 5 mg Con-A was coupled per ml of packed sepharose-4B volume.

PREPARATION OF ANTI-RAT AFP SEPHAROSE

Rabbit anti-rat AFP IgG was isolated from 20 ml of rabbit anti-rat AFP serum as described earlier. It was subsequently coupled to the CNBr activated sepharose-4B as described above.
Anti-AFP sepharose immunoadsorbent was used to isolate rat AFP from amniotic fluid or fetal serum as described by Ruoslahti (1976). AFP purified by this procedure was homogeneous by various criteria used for AFP (see characterization of rat AFP). However, the coupled ligand (IgG) showed a tendency to leak out (dissociate) slowly from the column.

**PREPARATION OF FETO-NEONATAL RAT TISSUE EXTRACTS**

The fetuses (18 and 20 E) and postnatal rats (1, 3, 7, 14, 21, 28 d old) were obtained from timed pregnancies. Neonatal rats were killed by putting them on cracked ice or by ether anaesthesia. Various feto-neonatal organs (brain, thymus, heart, lung, liver, stomach, intestine, spleen, pancreas, kidney and adrenals) were rapidly excised from each rat and washed with cold phosphate buffered saline (PBS) (8.0 g NaCl, 0.2 g KCl, 2.0 g Na$_2$HP0$_4$, 0.4 g KH$_2$PO$_4$ per litre). Full thickness dorsal skin was dissected off and washed several times with PBS. The skin was placed on a petridish, stratum corneum side down, and freed off the subcutaneous tissues by scraping to produce thin pieces of skin. Homogenates of skin (after freezing and thawing the tissue three times) and of various other feto-neonatal tissues were prepared in PBS, pH 7.4 and cleared at 22000 x g. Clear supernatants (tissue extracts) from various tissues were collected and used for various analyses.
PREPARATION OF HUMAN FETAL BRAIN EXTRACTS

Human fetal brain samples were obtained from legally aborted fetuses and still births 2-4 hrs after abortion, with due consent from women undergoing treatment at Queen Mary Hospital, King George Medical College (KGMC), Lucknow. Fetal age was ascertained from menstrual age and from crown to lump length of the fetuses. Amniotic fluid and cord serum samples were also collected at KGMC, Lucknow. The brain samples were stored frozen at -20°C and used within a week after excision. Brain tissue homogenates were prepared in PBS in a Potter Elvehjem homogenizer using a motor driven teflon coated pestle. The homogenates were centrifuged at 22,000 x g for 1 hr at 4°C and the supernatants obtained were treated as brain extracts and used for various analyses.

DETERMINATION OF FETAL BLOOD CONTAMINATION IN VARIOUS RAT AND HUMAN TISSUE EXTRACTS

In order to determine whether serum like antigenic component (AFP and albumin) detected in rat and human brain as well as other tissue extracts reflected artifactual contamination, heme concentrations in the fetal blood and various tissue extracts was determined. For this experiment, various tissue extracts 100 mg wet tissue weight 0.1 ml were prepared in 10 mM Tris, 1.5 mM EDTA, pH 7.4 (TE buffer). The buffer used is hypotonic (low ionic strength) and was
chosen to ensure hemolysis of red blood cells that might have been present in the tissue extracts. Blood was diluted and homolyzed 1:100, 1:200, 1:400, 1:800 with TE buffer. Relative heme concentrations in blood and tissue extracts were estimated from spectrophotometric absorbance at 414 nm. The minimal blood concentration required for detection of antigen (AFP and albumin) was estimated by correlating O.D. at 414 nm with the development of precipitin lines by double diffusion method. When fetal blood was diluted to yield concentration of heme in various tissue extracts, it failed to form a detectable precipitin line against anti-AFP or anti-albumin on double diffusion. This observation indicates that AFP and albumin in various tissue extracts are not entirely due to the fetal blood contamination. The magnitude of blood contamination in various tissue extracts was calculated by comparing heme concentration with the standard fetal blood samples.

PREPARATION OF BRAIN CELLS BY MECHANICAL DISSOCIATION PROCEDURE

Mechanical dissociation procedure (Benda et al, 1975) for the preparation of brain cells was used with some modifications. The whole brains (18-20 fetal and 1-7 postnatal rats) were excised from the cranium at the level of medulla oblongata and washed in isotonic buffer (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na$_2$HPO$_4$, 0.22 mM KH$_2$PO$_4$, 5.5 mM
glucose, 59.0 mM sucrose, pH 7.2). Each brain was carefully cleaned of pia matter and blood vessels, washed twice with isotonic buffer and minced to about 1-2 cubic mm pieces with fine scissors. The minced tissue (1-2 g) was suspended in L-15 medium (supplemented with 25 mM HEPES buffer and 6 mg/ml glucose) and mechanically dissociated by passing the tissue several times through a fine tipped Pasteur pipette. The solution was allowed to stand for 5 min and the procedure repeated thrice on decanted pellet. The pooled supernatant containing the cell suspension was centrifuged at 50 x g for 3 min. The cells were recovered and washed twice with L-15 medium. For demonstration of protein synthesis (AFP and albumin), brain cells were also washed once with leucine-free Eagle's MEM medium containing 25 mM HEPES, 6 mg/ml glucose, 1 mM glutamine, 100 \( \mu \)g ml\(^{-1} \) penicillin, 100 \( \mu \)g ml\(^{-1} \) streptomycin, and 0.25 \( \mu \)g ml\(^{-1} \) fungizone. The procedure routinely yielded suspension of 90-95% viable cells (0.2% trypan blue exclusion) with very few clusters of 5-10 cells.

**Preparation of Brain Cells by Enzymatic Procedure**

Brain cells were also prepared from 7-14 d old postnatal rats by trypsin digestion procedure as described by Raizada et al (1980) with a few modifications. Briefly, the minced brain tissue (1-2 g) was suspended in 50 ml of isotonic buffer containing 0.25% w/v trypsin at 37°C for
15 min with a constant and gentle stirring. After incubation, tissue suspension was allowed to stand for 5 min. The undissociated tissue was triturated several times with a Pasteur pipette followed by trypsin treatment and subjected to the above procedure. The cell suspension were pooled and the cells collected by centrifugation at 50 x g for 3 min. About 90% of the dissociated brain cells seems to be viable (0.2%, trypan blue exclusion).

RAT SKIN EXPLANT CULTURES

Cultures of skin explants from newborn rat were set up as described by Halprin et al (1979). Full thickness dorsal skin was dissected off, washed first with 70% alcohol (2 min) and then thoroughly with PBS. The skin was placed on a petridish, stratum cornium side down, and freed of the subcutaneous tissues by scraping to produce thin pieces of skin and then chopped into small pieces (2 mm²). Eight skin explants were placed in each culture flask, dermal side down, and allowed to stand for 5 min and maintained in leucine-free MEM medium with supplement mentioned earlier. Skin explants cultures of 7, 14, and 21 d old rat were also set up by the procedure of Halprin et al (1979) and Fusenig et al (1979).

PREPARATION OF HEPATOCYTES

Hepatocytes were prepared from rat liver by slight modification of the collagenase perfusion method of Berry
and Friends (1969) and Seglen (1976). The initial wash out perfusion, through the hepatic portal vein, was performed in the anaesthetized animal (21 d old, 1 month old, and adult rats) at room temperature with Ca$$^{++}$$ and Mg$$^{++}$$ free PBS, pH 7.4 supplemented with 25 mM HEPES buffer, 0.1% BSA and 0.1% glucose (buffer A). The liver were then perfused with buffer containing Ca$$^{++}$$ (10 mM) and 0.04 mg/o collagenase for 10-15 min until the liver began to disintegrate visibly. The perfusion was repeated with buffer A for about 5 min. The liver was then excised and dispersed with blunt forceps in buffer A. The cell suspension was passed through muslin cloth and the cells recovered by centrifugation at 50 x g for 2 min. The cell pellet was washed by resuspending in buffer A and recovering the cells by centrifugation. Finally, hepatocytes were washed with L-15 medium supplemented with 25 mM HEPES, 1 mM glutamine, 7 mM glucose and antibiotics. The viability of cells was checked by trypan blue (0.2%) exclusion. Usually 90-95% viable preparation of hepatocytes could be obtained. The hepatocytes were counted with the help of a hemocytometer and suitable quantity of cells (1-3 x 10$$^6$$ cells) were used for the determination of the synthesis and secretion of AFP and albumin in short term culture of hepatocytes.

Hepatocytes from fetal (18 and 20 E) and neonatal (1, 7, 14 d old) rat liver were prepared by dissociation of liver slices by collagenase digestion.
DETERMINATION OF INCORPORATION OF $[^{14}C]$-LEUCINE INTO AFP AND ALBUMIN BY RAT BRAIN CELLS, HEPATOCYTES, AND SKIN EXPLANT CULTURES

Incorporation of $[^{14}C]$-leucine into AFP and albumin by brain cells, hepatocytes, and skin explant cultures maintained in leucine free medium for 6 hrs was determined by following immunoprecipitation of labelled AFP and albumin in cell lysates (synthesis) and medium (secretion). Aliquots of the cell lysates or the culture medium were mixed with 100 µl of newborn rat serum (diluted ten times) to provide sufficient carrier AFP and albumin for immunoprecipitation of labelled AFP and albumin. AFP was then precipitated by the addition of 0.5 ml anti-AFP and the mixture was kept at 37°C for 1 hr and overnight at 4°C. The following day, the immunoprecipitates were obtained by centrifugation at 4000 rpm for 15 min at 4°C. The supernatants obtained were then used for immunoprecipitation of albumin by addition of 0.7 ml of anti-RSA. The amount of anti-rat AFP and anti-RSA used here was determined by preliminary titrations to give maximum precipitation of labelled AFP and albumin. It was confirmed by the observations that (a) a second addition of anti-rat AFP or anti-RSA to the supernatants (after immunoprecipitation of AFP and albumin) did not result in visible immune precipitation, and (b) a second addition of carrier fetal serum, followed by further addition of anti-rat AFP or anti-RSA to these supernatants did not result in
the appearance of additional radioactivity in either AFP or albumin immunoprecipitates. The order of immunoprecipitation of AFP and albumin was also reversed in order to see whether it affected the amount of radioactive AFP or albumin precipitation. No significant differences were noted. The immunoprecipitates were washed several times with PBS containing 0.1% leucine (PBS-leucine) to remove nonspecific adsorption of [3H]-leucine. Finally, the immunoprecipitates were dissolved in 0.3 ml 0.1 M NaOH and [3H]-radioactivity was determined in a liquid scintillation counter (Packard model 3330).

**DETERMINATION OF TOTAL PROTEIN SYNTHESIS AND SECRETION**

Total protein synthesis (in cell lysates) and secretion (in medium) was determined by following the [14C]-leucine incorporation into TCA insoluble fractions. Aliquots (0.5 ml) of cell lysates and the medium were mixed with 10 μl NRS (carrier protein) and the proteins were precipitated by the addition of 0.5 ml 10% TCA. The tubes were mixed and kept for 3 hrs at 4°C. The samples were then centrifuged at 4000 rpm for 10 min at 4°C. The precipitates were washed several times with cold 5% TCA containing 0.1% leucine. Finally, the precipitates were dissolved in 0.5 ml 0.1 M NaOH and radioactivity was determined in an aliquot of 0.25 ml in a Tricarb liquid scintillation counter.
RESULTS
SPECIFICITY OF ANTI-AFP

Rabbit anti-AF serum was absorbed successively with lyophilized NRS (0.3 mg/ml), till no visual immunoprecipitation occurred by further addition of NRS, and thus made specific for AFP. The specificity was confirmed by obtaining single precipitin arc on IEP with amniotic fluid, fetal serum (FS), fetal extract (FE) and pregnant rat serum (PRS) (Fig. 4A and 4B) and no reaction with NRS. Double diffusion analysis of anti-AFP* also yielded single line showing complete line of identity with AFP from rat AF, FS and FE (Fig. 5A). Anti-AFP* was used for initial detection of AFP during the purification of rat AFP by the procedure described below.

AN IMPROVED PROCEDURE FOR ISOLATION OF RAT AFP

DIRECT NEGATIVE IMMUNOABSORPTION OF AMNIOTIC FLUID (AF) WITH ANTI-NRS IgG

Adult rat serum proteins were removed from amniotic fluid (18 d of gestation fetuses) by successive addition of rabbit anti-NRS IgG at optimal precipitation proportion. The mixture (AF and IgG) was incubated for 2 hrs at 37°C and overnight at 4°C; followed by removal of the immunoprecipitate. The absorption of AF with anti-NRS IgG was continued till no visual immunoprecipitation occurred. The
Fig. 4 Immunoelectrophoretic (IEP) analysis of rat AFP during its isolation from amniotic fluid.

FB, fetal extract; PRS, pregnant rat serum; FS, fetal serum; AF, amniotic fluid; AFP, y glob., AFP-IgG mixture; AFP, alpha-fetoprotein; Anti-AFP*, antiserum made specific for AFP; Anti-AF, anti-amniotic fluid.
FIG. 5 CHARACTERIZATION OF RAT AFP

5A.a: Double immunodiffusion analysis of purified rat AFP.
Peripheral well 1, purified rat AFP; 2, fetal serum; 3, purified AFP; 4, fetal extract; 5, amniotic fluid. Central well contain anti-AFP.

5A.b: Double immunodiffusion analysis of AFP.
Peripheral well 1, amniotic fluid; 2, AFP γ-globulin mixture; 3 and 4, purified AFP; 5, fetal serum. Central well contain anti-amniotic fluid (anti-AF).

5A.c: Double immunodiffusion analysis of AFP.
Peripheral well 1, anti-RSA; 2, anti-AF; 3, anti-AFP; 4, anti-NRS; 5, anti-NRBS. Central well contain purified AFP.

5B: PAGE (10%) of isolated 'slow' and 'fast' variant of rat AFP.
Gel a and c, 'slow' and 'fast' variant of AFP (20 μg each); b, total purified AFP (50 μg).

5C: Double diffusion analysis of isolated 'slow' and 'fast' rat AFP variants.

5C.a: Peripheral well 1, 'slow' AFP variant; 2, total AFP; 3, 'fast' AFP variant; 4, amniotic fluid. Central well contain anti-rat AFP.

5C.b: Peripheral well 1-4 similar to 5C.a. Central well contain anti-rat AFP 'slow' variant.

5D: Crossed-immunoelectrophoresis of purified rat AFP.
6, polyacrylamide gel (after electrophoresis of AFP).

5E: Double immunodiffusion analysis of Con A-affinity molecular variants of rat AFP.
Peripheral well 1, purified AFP (total); 2, Con-A reactive; 3, Con-A nonreactive variant; 4, amniotic fluid; 5, fetal serum. Central well contain anti-AFP.

5F: Molecular weight determination of purified rat AFP by SDS-PAGE.

5G: An electrophoretogram (10% PAGE) of amniotic fluid proteins.
immunoprecipitate thus formed were removed by centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was then analysed for ΔFP and its contaminants. Double diffusion analysis of the supernatant revealed no albumin or other adult rat serum proteins (Fig.5A.b) except ΔFP and IgG of rabbit origin (Fig.5Ab). Presence of excess of Igs in the supernatant was revealed by formation of several precipitin arcs around the wells on IEP due to cross reaction between anti-NRS Ig and anti-ΔFP* which contained excess of NRS (Fig.4C).

**SEPARATION OF ΔFP FROM ΔFP AND IMMUNOGLOBULINS MIXTURE**

The supernatant (350 mg protein) containing ΔFP and rabbit immunoglobulins (IgG) was fractionated on DEAE-cellulose column (2.5 x 10 cm) pre-equilibrated with 10 mM phosphate buffer, pH 8.0 (buffer I). Unadsorbed proteins were collected with two bed volumes of buffer I. The adsorbed proteins were eluted stepwise with buffer I containing 0.05 M NaCl and 0.2 M NaCl (Fig.6). The elution profile was monitored by following absorbance at 280 nm. Three protein peaks thus obtained were analyzed for ΔFP, albumin and rabbit IgG by double diffusion and IEP using appropriate antisera (Table VI). ΔFP was found to be present in the third peak only with no apparent contamination of the proteins of either rat or rabbit origin. Protein rich fractions from the third peak were pooled, dialyzed against water and lyophilized.
FIG 6. Fractionation Of AFP-IgG Mixture By DEAE-Cellulose Chromatography.

Arrows indicate the tube where buffer composition is changed.
### TABLE VI: DOUBLE DIFFUSION ANALYSIS OF THE THREE PEAKS OBTAINED FROM DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

<table>
<thead>
<tr>
<th>Eluant</th>
<th>Peak No.</th>
<th>NRS</th>
<th>Anti-AF</th>
<th>Anti-AFP*</th>
<th>RSA</th>
<th>Anti-NRbS</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M phosphate buffer, pH 8.0</td>
<td>I</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>0.01 M phosphate buffer, pH 8.0 containing 0.05 M NaCl</td>
<td>III</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>0.01 M phosphate buffer, pH 8.0 containing 0.2 M NaCl</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHARACTERIZATION OF RAT AFP

Purified AFP yielded single precipitin line with complete immunological identity with other AFP's on double diffusion against anti-AFP or anti-AFP* (Fig.5A.a and 5A.b). It also formed a single line on IEP (Fig.4D and 4E). No albumin or other serum proteins of either rat or rabbit origin could be detected by double diffusion (Fig.5A.c). Unlike AFP IgG mixture (Fig.4C), no precipitin arcs around the wells were obtained with purified AFP when the troughs were developed with anti-AFP*, thereby, demonstrating complete elimination of immunoglobulins of rabbit origin (Fig.4F).

PAGE OF RAT AFP: ISOLATION OF 'SLOW' AND 'FAST' MOVING VARIANTS BY PREPARATIVE POLYACRYLAMIDE SLAB GEL ELECTROPHORESIS

Rat AFP revealed heterogeneity on PAGE (10%), yielding 'slow' and 'fast' moving variants (Fig.5B.b). The two variants of AFP were isolated by preparative polyacrylamide slab (3 mm) gel (10%) electrophoresis of AFP for 6 hrs at 4°C at a constant voltage of 10 V/cm of the gel. After completion of electrophoresis, a portion of the slab gel was stained with coomassie brilliant blue. Portions of the gel corresponding to the two stained bands of 'slow' and 'fast' AFP variant were cut separately and minced by passing through a syringe. Subsequently, the protein ('slow' and 'fast' AFP variant) were separately extracted with 0.02 M
phosphate buffer, pH 7.3, dialyzed in the same buffer and
lyophilized.

Each of the two variants isolated by preparative
PAGE was found to be homogeneous on re-electrophoresis on
PAGE under similar conditions (Fig.5B.a and 5B.c). The two
variants of AFP (slow and fast) also migrated with their
original electrophoretic mobility on PAGE.

PRODUCTION OF ANTI-AFP AND ANTI-AFP 'SLOW' VARIANTS:
IMMUNOLOGICAL IDENTITY OF 'SLOW' AND 'FAST' RAT AFP
VARIANTS

Purified AFP was injected in rabbits to study
whether it induced AFP specific antiserum. Initially 200
µg AFP, containing both slow and fast variants, was
emulsified in CFA and administered through foot pads.
After one week, 200 µg AFP in CFA was injected intradermally
at multiple sites at 10 day intervals (3 injections). After
the last injection, the animals received two subcutaneous
booster doses (without CFA) of 500 µg AFP. The animals
were bled after last injection, analyzed for antibody
titre, and bled when the titre was maximum. Specificity
of the antiserum was analyzed by double diffusion and IEP.

Rabbit anti-AFP showed single precipitin line on
double diffusion with rat amniotic fluid and purified AFP
with a line of fusion(Fig.5C.a). It did not reveal any
reactivity with normal adult rat serum, indicating the
specificity of the antiserum for AFP.
Anti-rat AFP 'slow' variant (anti-AFP 'S') was also raised in rabbits following the immunization schedule described for total AFP. Both anti-AFP and anti-AFP 'S' showed similar specificity. The two AFP variants individually formed single precipitin line against either anti-AFP or anti-AFP 'S' (Fig.5C.a and 5C.b). A complete immunological identity was also displayed among AFP (total), AFP 'slow', AFP 'fast' and AFP present in amniotic fluid. These observations clearly indicate that two bands of AFP observed on 10% PAGE were its charge variants which have common antigenic determinant site.

Heterogeneity of rat AFP was also displayed on crossed immunoelectrophoresis carried out according to Clark and Freeman (1967). Rat AFP was resolved on 10% PAGE and after electrophoresis, the gel was cut longitudinally into two halves. One half was placed on 1% agarose plate containing uniformly distributed anti-AFP and electrophoresed in 2nd dimension at right angle to the first (PAGE). AFP yielded two precipitin peaks fusing with each other (Fig.5D) indicating again that AFP is heterogenous on PAGE yielding two variants which are immunologically identical. Heterogeneity of rat AFP on PAGE is well documented and probably arises due to a difference in the sialic acid content of the two charge variants (Smith and Kelleher, 1980; Aussel and Masseyoff, 1977; Versee and Barel, 1976; Watanabe et al. 1975; Benassayag et al. 1975).
Purified rat AFP was chromatographed on Con A-sepharose column to evaluate Con A-reactive and non-reactive AFP content according to Smith et al (1977). AFP was applied on Con A-sepharose column (0.8 x 10 cm) and unadsorbed fraction was eluted with the starting buffer (0.05 M Tris-HCl, pH 7.6 containing 1 M NaCl, 1 mM MgCl₂, 1 mM MnCl₂ and 1 mM CaCl₂). Adsorbed fraction (Con-A reactive AFP) was then eluted with the starting buffer containing 0.1 M mannose. Elution profile was monitored by measuring optical density at 280 nM. AFP content in the unadsorbed and adsorbed fractions was analyzed by rocket assay. Con A-nonreactive and reactive variants were 52 ± 2% and 47 ± 4% of the total AFP. Con A-nonreactive AFP variant when rechromatographed on the same Con A-sepharose column, it did not reveal any binding indicating that it was not contaminated with over loaded Con A-reactive variant. Our values corresponds well with the reported value for Con A-reactive and non-reactive AFP variant (Smith and Kelleher, 1980).

Con A-reactive and non-reactive rat AFP variants were also analyzed for their immunological relatedness by double diffusion. The two variants individually formed a single precipitin line with a complete fusion among each other and with AFP (total) and amniotic fluid (Fig.5E). This observation indicate that the two variants obtained
from Con A-sepharose affinity chromatography are immuno-
logically identical. Heterogeneity of rat AFP on Con-A
sepharose have now been shown to be due to a difference in
the amount of N-acetylglucosamine, galactose and sialic
acid in the two variants (Bayard and Kerckaert, 1981). It
has also been demonstrated that Con A-affinity molecular
variants of AFP have different origins and are glycosylated
differently (Smith and Kelleher, 1980; Ruoslahti and Adamson,
1978). The yolk sac synthesizes Con A-nonreactive while
fetal liver exclusively produces Con A-reactive AFP variant.

**ESTRADIOL BINDING ASSAY OF RAT AFP**

Interaction of estradiol with rat AFP was studied by
dextran coated charcoal procedure. Scatchard analysis of
$[^3H]$-estradiol binding assay of AFP (Fig. 7) yielded an
association constant $K_a = 1.4 \times 10^8 \text{ M}^{-1}$ with about one
binding site per mol of AFP. This stoichiometry of estradiol
interaction with rat AFP is in agreement with observations of several authors (Aussel et al, 1979, 1978; Versee
and Barel, 1978a; Aussel and Massyeff, 1977; Soloff et al,
1976). Scatchard plot of $E_2$ was curvi-linear with uniform
slope. This observation also supports the view that there
is no heterogeneity in AFP with regard to $E_2$ binding
property. However, several others (Vallett et al, 1977;
Nunez et al, 1976; Benassayag et al, 1975) have consistantly
observed the existence of two classes of binding sites with
different affinities for estradiol and with a fractional
number of $E_2$ binding sites per mol of AFP. The source of these discrepancies is not clear. It may be due to the technical differences and biological source of AFP.

Estradiol binding of rat AFP variants was also analyzed on PAGE. AFP was incubated with $[^3H]$-E$_2$ in presence or absence of excess estrone (E 1) and treated with DCC; the supernatants, obtained by centrifugation were electrophoresed on 10% PAGE. After electrophoresis, gels were sliced at 1 mm intervals and each slice was dissolved in 0.3 ml $H_2O_2$ at 55°C for 3 hrs and $[^3H]$-radioactivity determined. The results are depicted in Fig.8. The two charge variants of AFP showed $E_2$ binding which could be displaced by incubation with excess estrone prior to electrophoresis. These results, although inconclusive, indicate that the two charge variants bind estradiol with similar capacity and high specificity. This observation supports the results of several authors who also find that the charge variants of AFP do not differ in their estradiol binding properties (Aussel et al, 1979; Vorsee and Barel, 1978a).

**Molecular Weight Determination of Purified Rat AFP**

Molecular weight analysis of purified rat AFP by PAGE in the presence of SDS and $\beta$-mercaptoethanol (under denaturing conditions) shows that it is composed of a single polypeptide chain with a molecular weight of 70,000 daltons (Fig.5F). This value is in good agreement with the molecular weight of rat AFP purified by various procedures (Ruoslahti
FIG 7. Scatchard Plot For The Interaction Of Estradiol With Purified Rat AFP

FIG 8. Estradiol Binding Of Rat AFP Variants On PAGE. $E_2$ Binding in presence $\circ \circ$, and absence $\bullet \bullet$, of excess estrone ($E_1$).
RECOVERY OF AFP FROM AMNIOTIC FLUID BY DIRECT NEGATIVE IMMUNOABSORPTION PROCEDURE

A flow sheet of the present procedure for isolation of AFP from amniotic fluid is presented in Fig. 9. The loss of AFP at each step was checked by rocket assay. It appears that there is no significant loss of AFP in the first step (Table VII). Total recovery of AFP from amniotic fluid is about 97%.

PURIFICATION OF HUMAN AFP

Amniotic fluid (14th week of gestation) served as the source for isolation of human AFP. Negative immunoabsorption of amniotic fluid with rabbit anti-normal human serum IgG was used essentially as described for rat AFP isolation.

CHARACTERIZATION OF HUMAN AFP

Purified human AFP yielded single precipitin line with anti-human AFP on double diffusion showing complete line of identity with AFP's present in cord serum and amniotic fluid (Fig. 10 A). It also did not reacted with anti-HSA and anti-NHS. Antiserum raised against human AFP did not cross react with any adults human serum proteins. It also formed a single precipitin arc at the corresponding position of AFP present in its native source (AF) (Fig. 10 B). Purified human AFP was homogeneous on 10% (PAGE) (Fig. 10 C).
Amniotic Fluid
1. AFP = 50%
2. ALB ≈ 40-45%
3. Trans = 5-10%
4. γglob.

Step 1. Immunoprecipitation of AF proteins
- IgG
  - at 37°C for 2 hrs.
  - and 4°C for 24 hrs., centrifuge

Immunoprecipitate
- ALB, Trans, γglob, etc.

Supernatant
- (AFP and excess IgG)

Step 2.
- DEAE-Cellulose Chromatography
- 0.02M buffer pH 8

Adsorbed
- (AFP and traces of γglob.)
  - Elute with buffer containing 0.02M NaCl

Eluted traces IgG-II

Unadsorbed
- (IgG-I)

AFP
<table>
<thead>
<tr>
<th>Amniotic fluid</th>
<th>4500</th>
<th>-</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFP. γ-globulin mixture</td>
<td>4366</td>
<td>97</td>
</tr>
<tr>
<td>Purified preparation of AFP</td>
<td>3917</td>
<td>87</td>
</tr>
</tbody>
</table>

*Amniotic fluid was arbitrarily assigned to contain 1000 units of AFP per ml as quantitated by rocket immunosassay.
FIG. 10 CHARACTERIZATION OF HUMAN AFP

10.A: Double immunodiffusion analysis of purified human AFP and fetal brain AFP.
Peripheral well 1, purified human AFP; 2, human cord serum; 3, human amniotic fluid; 4, human fetal brain extract (20th week); 5, fetal brain extract after immunoprecipitation of AFP.

10.B: IEP analysis of purified human AFP. HAFP, purified human AFP; AF, human amniotic fluid; AbHAFP, anti-human AFP.

10.C: IEP analysis of human fetal brain AFP. BE, human fetal brain extract (20th week); HAFP, purified human AFP; Ab HAFP, anti-human AFP.

10.D: PAGE (10%) of purified human AFP.
Estradiol binding activity of the cord serum and purified human AFP was analyzed by DCC procedure. Both cord serum and human AFP were devoid of $E_2$ binding. Our observations also support the numerous reports indicating the non-estrophilic nature of human (Smith and Kelleher, 1980; Ruoslahti and Seppala, 1979).

**CHARACTERIZATION OF RSA AND HSA**

RSA purified by affinity chromatography on blue-sepharose was homogeneous on PAGE (10%) (Fig.11A). Antiserum raised against purified RSA formed single precipitin arc on IEP with RSA and NRS (Fig.11C). Purified HSA was also homogeneous on PAGE (Fig.11B). Antiserum raised in rabbits against HSA also formed single precipitin arc with NHS and HSA (Fig.11D).

**DISTRIBUTION AND ONTOGENY OF AFP IN FETONEONATAL TISSUES OF RAT**

AFP was found to be present in the tissue extracts, arranged in order of decreasing concentration of AFP at 18E as liver skin brain kidney stomach lung heart intestine adrenal. Thymus, spleen and pancreas were devoid of AFP when analyzed by double diffusion (Fig.12A). High levels of AFP were found in all tissue during fetal life (18 and 20 E) and its concentration registered a continuous decline with development. Ontogeny of AFP in various tissues of rat, as presented in Fig.13 indicates that AFP disappears after: 7P in brain and adrenals; 14P in lung, heart, stomach,
FIG. 11  CHARACTERIZATION OF RAT AND HUMAN SERUM ALBUMIN

11.A: PAGE (7%) of purified rat serum albumin

11.B: PAGE (7%) of purified human serum albumin

RSA, rat serum albumin; NRS, normal rat serum; AbRSA, anti-RSA

HSA, human serum albumin; NHS, normal human serum; AbHSA, anti-human serum albumin.
FIG. 12  CHARACTERIZATION OF RAT TISSUES AFP

12.A: Double immunodiffusion analysis of various newborn rat tissues AFP.

1. fetal serum  7. lung
2. amniotic fluid  8. brain
3. spleen  9. heart
4. pancreas  10. kidney
5. thymus  11. intestine
6. liver  12. stomach

Wells in the central row contain anti-AFP.
Various tissue extracts, fetal serum and amniotic fluid were adjusted to contain 50 µg AFP/ml as determined by rocket assay.

12.B: IEP analysis of various tissues AFP

1. kidney  2. fetal serum
3. skin  4. brain

Trough contain anti-AFP.

12.C: Diagrammatic representation of the analysis of various tissues AFP by PAGE coupled with immunodiffusion.

Polyacrylamide gel 1, fetal serum; 2, skin; 3, brain; 4, kidney.

Trough contain anti-AFP.
FIG 13. Ontogeny Of AFP In Various Tissues Of Rat

A, 18E; B, 20E; C, 1P; D, 3P; E, 7P; F, 14P; G, 21P; *, Not determined.
intestinal and kidney: 21P in liver and skin. The fetal blood contamination in various tissue extract preparations could not account for more than 5-10% of the observed levels in various tissues.

**IMMUNOLOGICAL AND ELECTROPHORETICAL PROPERTIES OF TISSUES AFP**

AFP present in various tissues was immunologically identical and displayed a complete line of identity (Fig. 12A). IEF of AFP from serum, brain, skin and kidney also showed similar electrophoretic mobility (Fig. 12B). Aliquots of brain, skin and kidney cytosol (100 μg total protein) and fetal serum were electrophoresed on 10% (PAGE) and after electrophoresis gels were cut longitudinally into two halves and one half of each gel was placed on 1% agarose. Anti-AFP was then filled in troughs made parallel to each gel and allowed to diffuse for 24 hrs. Each gel yielded single precipitin arc for AFP at the same position (Fig. 12C). This observation further indicates that AFP derived from these tissues is also similar with respect to charge.

**ESTROGEN BINDING CHARACTERISTICS OF BRAIN AND SKIN AFP**

Estradiol binding of brain and skin cytosol was studied by DCC procedure as described for rat AFP. Scatchard plots of E₂ interaction with brain and skin AFP yielded an association constants of 1.3 x 10⁸ M⁻¹ and 1.0 x 10⁸ M⁻¹ respectively. These values are similar to AFP purified from rat amniotic fluid. Our results of brain AFP also corresponded well with the association constant
reported earlier (Attardi and Ruoslahti, 1976; Plapinger et al, 1973). It would thus appear that AFP present in brain and skin also did not differ in their estrogen binding characteristics.

**ANALYSIS OF CON A AFFINITY MOLECULAR VARIANT IN BRAIN AND SKIN AFP**

Brain and skin cytosol prepared from newborn rat were chromatographed on Con A-sepharose column as described earlier. Amount of Con A-reactive and non-reactive AFP variant were determined by rocket assay. Amount of Con A-nonreactive AFP variant were 37 ± 3% and 46 ± 4% in skin and brain cytosol respectively. The amount of Con A-reactive variant in newborn rat serum was 45 ± 2.5%. This is in close agreement with the reported value at this stage of development (Smith and Kelleher, 1980). The difference in the relative content of Con A-nonreactive AFP variant in serum, brain and skin may reflect a difference in the amount of carbohydrate moiety (sialic acid, mannose, glucose etc.) attached to AFP variant in these tissues. Other tissues like yolk sac and liver are known to differ in the relative proportion of Con A-affinity molecular variants (Smith and Kelleher, 1980; Ruoslahti and Adamson, 1978).

**ONTOGENY AND DISTRIBUTION OF AFP IN FETO-NEONATAL RAT BRAIN**

Whole brains were removed from fetal (18 and 20 E), neonatal rats (1, 3, 7, 14 and 21 d old) and pregnant rat (at 20 E), and washed several times with 0.01 M Tris, 1.5 mM
EDTA, pH 7.4 (TB buffer). The brains from several rats of each age group, after stripping the meninges, were macroscopically dissected into olfactory, cerebrum, cerebellum and the remaining portions. The homogenates prepared from whole brain or pooled discrete regions of the brains in TE buffer were cleared by centrifugation at 22,000 for 30 min at 4°C. The clear supernatants were used for AFP determination by rocket assay to study the distribution of AFP in developing rat brain. Extent of blood contamination in various samples of brain extract was determined by measuring heme concentration as described earlier.

Distribution of AFP in whole brain and its discrete regions during development is shown in Fig. 14. AFP seems to be distributed uniformly throughout the brain. Different regions of the brain contained AFP in the following order: olfactory cerebrum cerebellum remaining portions.

Ontogeny of AFP either in whole brain or in its discrete regions is similar. High levels of AFP were observed in each region during fetal development which show almost similar decline with development. AFP disappeared simultaneously from olfactory cerebrum cerebellar as well as rest portions of the brain after one week of postnatal development. It is possible that AFP may still be present (after 7P) in brain but is not detectable by the present assay system. Attardi and Ruoslahti (1976) reported that feto-neonatal estradiol binding protein (AFP) is present in mouse brain in very low level, detected by
Figure 1: Ontogeny and Distribution of APP in Rat Brain

1. Whole Brain
2. Olfactory
3. Cerebrum
4. Cerebellum
5. Rest portion
radioimmunoassay, up to 3 weeks of postnatal development.

Fetal blood contamination in cytosol of different regions of the brain was not more than 3-5% as determined by measuring heme concentration. Several authors (Palpinger et al, 1977, 1973) have also reached to the same conclusion and suggest that contamination of AFP from fetal blood could not account for the high levels of AFP in immature rat or mouse brain. This is also supported by the fact that pregnant rat brain was devoid of AFP, although serum concentration in pregnant rat (20 E) is significantly high (Stillman and Sell, 1979).

AFP is present in various discrete regions of rat brain could bind estradiol with high affinity. It is very likely that binding characteristics of AFP in various regions are similar to that observed in whole brain cytosol (Fig. 7).

**ONTOGENY OF AFP IN HUMAN FETAL BRAIN**

AFP was also found to be present in human fetal brain during early embryonic life. Ouchterlony's double diffusion analysis of brain extracts yielded single precipitin line and complete line of identity with AFP's present in cord serum, amniotic fluid and purified AFP, indicating the antigenic similarity of brain with other tissues AFP (Fig. 10A). Aliquots (0.2 ml) of fetal brain extract (20th week) and cord serum samples were incubated with anti-AFP human (0.5 ml) at room temp. for 1 hr and at 4°C overnight. The immunoprecipitates were removed by centrifugation and supernatants
analyzed by double diffusion to determine whether immuno-recipitation of AFP from human fetal brain extracts (20th week) removed protein species reactive with anti-human AFP. The supernatant (after immunoprecipitation) failed to give any precipitin line with anti-human AFP in both fetal brain extract (Fig.10A) and cord serum indicating that AFP is the reactive protein species. IEP of brain extract also yielded a single precipitin arc with electrophoretic mobility similar to that of purified human AFP (Fig.10C). These observations indicate that human fetal brain AFP is immunologically and electrophoretically identical to serum AFP.

AFP could be detected in fetal brain after 12 weeks of gestational age. It registered a peak level in fetal brain at 20th week and declined rapidly to low levels after 24th weeks of gestation and was absent, as measured by rocket assay, in the fetal brain of subsequent ages.

Developmental profile of AFP in human fetal brain, as summarized in Fig.15, is different from that observed in amniotic fluid and fetal serum where peak level of AFP are found around 14th week of gestation and from maternal serum attaining peak level at about 28-32 weeks of gestation (Ruoslahti and Seppala, 1979; Gitlin, 1975; Seppala, 1975). Interestingly, when AFP level in amniotic fluid and fetal serum register a decline, AFP in fetal brain starts showing steady rise. However, the peak level of AFP in brain coincides with the period at which the AFP level are also
FIG 15. DEVELOPMENTAL PROFILE OF AFP IN HUMAN FETAL BRAIN

- Number of samples

<table>
<thead>
<tr>
<th>Gestation Period in Weeks</th>
<th>Samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>12</td>
<td>2</td>
</tr>
<tr>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
</tr>
<tr>
<td>24</td>
<td>3</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
</tr>
<tr>
<td>32</td>
<td>2</td>
</tr>
<tr>
<td>36</td>
<td>3</td>
</tr>
</tbody>
</table>

Not detectable
higher in cerebrospinal fluid (CSF) (Adinolfi and Adinolfi, 1976).

The high concentration of AFP found in human fetal brain could not be accounted for by fetal blood contamination which could contribute only 7-10% of the observed levels of AFP as determined by heme concentration in various fetal brain extracts. Further, no AFP was detected in fetal brain after 24th week of gestational life when AFP level continues to remain high in both fetal serum and amniotic fluid. The origin of AFP in brain is not clear. Intracellular localization of AFP in developing rat (Toran-Alterand, 1980; Trojan and Uriel, 1979; Benno and Williams, 1978) and human (Mollgard et al, 1979) brain is suggestive of its synthesis by brain cells. Uptake from CSF or fetal blood may, in part, account for the observed AFP levels in human brain.

E₂ binding of AFP was measured in human fetal brain extract and cord serum samples by DCC procedure. Aliquots (0.3 ml) of brain extract (5 mg protein/ml) in triplicate or cord serum (diluted 10 times with saline) were incubated with 5 µl of [³H]-E₂ in various concentrations (0.25-100 mM) with or without 1000 fold concentration of cold E₂ for 3 hrs at 0°C. After incubation 50 µl dextran coated charcoal (10% charcoal and 1% dextran T 70 in 0.1 M phosphate buffer, pH 7.3) was added to each tube and mixed thoroughly, followed by centrifugation at 800 x g for 10 min. Radioactivity was measured in the clear supernatants. Further,
aliquots of brain extracts and cord serum were mixed with a little excess of anti-human AFP, incubated for 24 hrs at 4°C and centrifuged at 20,000 x g for 30 min. The supernatants thus obtained were assayed for AFP by double diffusion and E₂ binding by DCC procedure.

E₂ binding assays in whole fetal brain extract, cord serum and their supernatants obtained after immunoprecipitation of AFP did not show any specific binding of [³H]-E₂ (Table VIII). This observation further supports that human AFP, irrespective of its source, is devoid of E₂ binding ability (Smith and Kelleher, 1980; Mendelson et al, 1980; Ruoslahti and Seppala, 1979; Swartz et al, 1974; Nunez et al, 1974; Savu et al, 1974).

SYNTHESIS AND SECRETION OF AFP AND ALBUMIN BY NEWBORN RAT BRAIN CELLS IN CULTURE

Synthesis and secretion of AFP and albumin was studied by incubating 1-2 x 10⁶ brain cells in each culture flask containing 3.0 ml leucine free medium supplemented with 0.6% glucose, 1 mM glutamine, 25 mM HEPES, and antibiotics (Penicillin 100 U/ml, streptomycin 100 µg/ml) at 37°C in a metabolic shaker. Radioactive pulse experiments were initiated by adding 3 µCi DL [l-¹⁴C]-leucine. Cycloheximide was added into some flasks 15 min prior to addition of labelled leucine at a final concentration of 10 µg/ml. Incubation was terminated at specific intervals by removing the flasks and chilling the solution in ice bath. The cells
**TABLE VIII:** ESTRADIOL BINDING ANALYSIS OF HUMAN FETAL BRAIN EXTRACTS AND CORD SERUM

<table>
<thead>
<tr>
<th>Samples</th>
<th>$E_2$ binding *</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cord serum</td>
<td>127 cpm</td>
</tr>
<tr>
<td>Cord serum (after immunoprecipitation of AFP)</td>
<td>146 cpm</td>
</tr>
<tr>
<td>Brain extract (20th week)</td>
<td>166 cpm</td>
</tr>
<tr>
<td>Brain extract (after immunoprecipitation of AFP)</td>
<td>152 cpm</td>
</tr>
</tbody>
</table>

*For details see text.*
were separated by centrifugation and incubation medium recovered. The cells were washed several times with phosphate buffered saline containing 0.1% DL-Leucine (PBS-leucine). The cell lysate was prepared by sonication of brain cells for 3 min at 0-4°C.

Intracellular synthesis (in cell lysate) and secretion (in medium) of AFP and albumin by brain cells was determined by following the incorporation of $^{14}$C-leucine into immunoprecipitable AFP and albumin, and total proteins (TCA insoluble) as described earlier (Material and Methods).

Incubation of dissociated newborn brain cells with medium containing $^{14}$C-leucine resulted in linear incorporation of radioactivity into immunoprecipitable AFP, albumin and TCA insoluble total proteins synthesized in the brain cells (Fig. 16A). The radioactivity into the secreted labelled AFP, albumin and total proteins also showed a linear increase during 6 hrs in culture (Fig. 16B). The addition of cycloheximide into the cultures inhibited the incorporation of $^{14}$C-leucine into AFP and albumin (Fig. 16A) and their secretion into the medium (Fig. 16B). Labelled-AFP accounted for 11-13% and albumin 5% of the total proteins synthesized in the brain cells and their secretion into the medium accounted for 27-30% and 11-13% of the total proteins secreted by the brain cells. It appears that bulk of the proteins synthesized in the brain cell are not secreted. AFP and albumin, the two secretory proteins, together constitute
Fig. 30. \^{[1]}\textsuperscript{3}H-proline incorporation in Rat Brain Cells in Culture. **A**.)AFP, Albumin, and Total Protein Synthesis and Secretion in the Presence of \textsuperscript{14}C-lysine. **B.**AFP and Albumin Synthesis and Secretion in the Presence of \textsuperscript{3}H-proline.
about 40% of the total proteins present in the culture medium of newborn rat brain cells.

Intracellular content of AFP and albumin in cell lysates and their secretion into the medium (10 times concentrated) by brain cells during 6 hrs incubation period were determined by rocket immunoassay. Intracellular content of both AFP and albumin did not change significantly during 6 hrs incubation period (Fig.17 A). However, quantitable amounts of AFP and albumin linearly accumulated (secreted) into the culture medium, as a function of incubation period (Fig.17 B), indicating a continuous production of these proteins by brain cells. Further, total amounts of AFP and albumin secreted by these cells during 6 hrs culture period is far in excess of intracellular content of the two proteins in brain cells at the initiation of cultures. These observations clearly indicates that AFP and albumin are synthesized in situ in newborn rat brain cells.

CHARACTERIZATION OF AFP AND ALBUMIN SECRETED BY NEWBORN RAT BRAIN CELLS IN CULTURE

The medium collected after incubation of brain cells for 6 hrs in culture medium was dialyzed extensively against PBS-leucine and concentrated (about 10 times) by lyophilization and will be referred to as concentrated medium (CM). CM was analyzed by various immunological and E2 binding assay to characterize the AFP and albumin secreted by newborn brain cells.
FIG 17. Quantitation Of AFP And Albumin In Newborn Rat Brain Cell Cultures. The values are represented as mean ± SD of eight cultures.
IMMUNOLOGICAL IDENTIFICATION AND CHARACTERIZATION OF AFP AND ALBUMIN SECRETED BY NEWBORN RAT BRAIN CELLS

IEP of the CM revealed the presence of both AFP and albumin (Fig. 18 A) with complete line of identity and electrophoretic mobility similar to corresponding serum AFP (Fig. 18 B) and albumin (Fig. 18 C). Aliquots of CM were separately treated with anti-RSA and anti-AFP to remove albumin and AFP from CM. The immunoprecipitates were removed by centrifugation and supernatants collected. Aliquots of the supernatants (albumin and AFP free) thus obtained and CM (without removal of AFP and albumin) were electrophoresed on 10% PAGE to observe the profile of secreted (labelled) proteins. After electrophoresis the gels were sliced at 1 mm intervals, each slice dissolved in 0.3 ml H2O2 at 55°C for 3 hrs, and [14C]-radioactivity measured. A separate gel [after 10% PAGE of the CM] was cut longitudinally and one half stained with commassie blue and another half placed on 1% agarose and diffused against anti-AFP and anti-RSA to locate the AFP and albumin. Further, another gel (after 10% PAGE of CM) was sliced at 2-3 mm intervals (transversely) and slices were kept serially on a glass plate on which agarose gel (1% agarose) was poured later on. After diffusing the slices for about 2 hrs at room temperature, they were allowed to diffuse overnight against a series of wells containing anti-AFP and anti-RSA to further identify the position of AFP and albumin on the polyacrylamide gel.
FIG. 18  CHARACTERIZATION OF AFP AND ALBUMIN SECRETED
BY NEWBORN RAT BRAIN CELLS IN CULTURE

CM, concentrated medium obtained after 6 hrs culture of newborn rat brain cells; 
\( \text{Ab}_1 \), anti-RSA; \( \text{Ab}_2 \), anti-AFP.

18.B: Immunological identity of AFP secreted by brain cells with serum AFP.
FS, fetal serum.

18.C: Immunological identity of albumin secreted by brain cells with rat serum albumin. 
RSA, rat serum albumin.

18.D: Identification of AFP and albumin present in CM on PAGE (10%) coupled with immunodiffusion.
G, polyacrylamide gel (10%) after electrophoresis of CM. G was cut longitudinally and diffused against specific antiserum (\( \text{Ab}_1 \) and \( \text{Ab}_2 \)).

18.E: Identification of AFP and albumin present in CM by PAGE (10%) coupled with immunodiffusion. G, polyacrylamide gel sections (2-3 mm). The gel was cut transversely after electrophoresis of CM and each section diffused against specific antiserum (\( \text{Ab}_1 \) and \( \text{Ab}_2 \)). Arrow number 1 shows the formation of precipitin line for albumin. Arrow number 2 shows the lines for AFP.

18.F: An electrophoretogram of CM (10% PAGE) showing the proteins secreted by newborn rat brain cells in culture.
PAGE pattern of the total proteins secreted by newborn rat brain cell is depicted in Fig. 19A (upper curve). Specific immunoprecipitation of AFP (Fig. 19B) and albumin (Fig. 19C) from CM removed the radioactivity from the corresponding regions. That AFP and albumin are the two faster moving component (as is evident from Fig. 19A) was further confirmed by diffusing the gel (after PAGE of CM) against respective antisera and subsequent formation of single precipitin arc (Fig. 18D). When the same gel was cut transversely and diffused against anti-AFP and anti-RSA, the precipitin lines were only formed in the slices corresponding to the position AFP and albumin (Fig. 18E) as observed in Fig. 19A (upper curve) and Fig. 18D. An electrophoretogram of the stained gel after PAGE of CM is shown in Fig. 18F. It is evident from Fig. 18F and Fig. 18A that brain cells of newborn rat secretes a number of proteins along with two major proteins identified as AFP and albumin. These results are in accordance with the high amount of radioactivity found in immunoprecipitable AFP and albumin secreted by brain cells (Fig. 16).

IDENTIFICATION AND CHARACTERIZATION OF E2 BINDING PROTEIN SECRETED BY NEWBORN RAT BRAIN CELLS IN CULTURE

E2 binding activity of the proteins secreted by brain cells and identification of the active protein species was investigated as follows. Aliquots of CM (total protein 100 μg) were incubated with [3H]-E2 with or without 100 μM
FIG 19. PAGE Pattern Of The Proteins Secreted By Newborn Rat Brain Cells In Culture
A-Total proteins (CM); B-CM after removal of AFP; C-CM after removal of albumin

Estradiol Binding Analysis Of CM
Fig.19A: CM with and without excess of cold estradiol
nonradiolabelled E₂ for 3 hrs at 0°C. After incubation 50 µl DCC was added to each tube, mixed and centrifuged at 800 x g for 5 min at 0°C. Resulting supernatants were electrophoresed on 10% PAGE. After electrophoresis, the gels were sliced, dissolved, and [³H]-radioactivity counted. In another set of experiment CM was incubated with [³H]-E₂, treated with DCC, and AFP immunoprecipitated by anti AFP. The immunoprecipitate and the supernatant were recovered and [³H]-radioactivity counted to determine whether specific immunoprecipitation of AFP removed the E₂ binding component from the CM.

[³H]-radioactivity profile, as presented in Fig.19A (middle curve) demonstrates that AFP and the protein binding [³H]-E₂ co-migrate on PAGE. Further, [³H]-E₂ binding could be almost completely displaced if CM was incubated with excess of cold E₂ before electrophoresis (Fig.19A, lower curve). Immunoprecipitation of AFP also removed the E₂ binding ability of CM (4770 cpm/100 µg total protein) and almost all [³H]-radioactivity was recovered (4527 cpm) in the immunoprecipitate. These observations clearly indicate that AFP is the only active protein species secreted by brain cells of newborn rat that binds E₂ with high specificity.
Molecular weights of $[^{14}\text{C}]$-labelled AFP and albumin synthesized and secreted de novo were determined by specific immunoprecipitation of AFP and albumin from CM and subsequent analysis of the immunoprecipitate on SDS-PAGE (Schapiro et al, 1967). $[^{14}\text{C}]$-labelled AFP and albumin were immunoprecipitated using anti-AFP and anti-albumin as described earlier. The immunoprecipitates were washed with PBS and dissolved in 0.2 ml 3% sodium dodecyl sulphate (SDS)-tris glycine buffer, pH 8.6 by incubation for 18 hrs at $37^\circ\text{C}$. Aliquots of clear supernatants containing 75-100 $\mu$g protein were analyzed on 10% PAGE containing 0.1% SDS. After electrophoresis the gels were sliced into 1 mm section, each slice dissolved and counted for $[^{14}\text{C}]$-radioactivity. Mobility of the radioactive proteins was compared to known molecular weight protein markers electrophoresed simultaneously. Newly synthesized and secreted AFP and albumin gave a molecular weights of 70,000 and 65,000 daltons respectively (Fig.20). These values are in close agreement with molecular weights reported for these proteins (Ruoslahti and Seppala, 1979; Attardi and Ruoslahti, 1976).
FIG 20. Molecular Weight Determination Of AFP And Albumin Secreted By Newborn Rat Brain Cells In Cultures.
DEVELOPMENTAL CHANGES IN THE RATE OF SYNTHESIS OF AFP AND ALBUMIN IN BRAIN: CORRELATION WITH THEIR BRAIN CONTENT DURING DEVELOPMENT

The changes in the synthesis of AFP and albumin accompanying the maturation of the rat brain were studied by measuring the rate of synthesis of the two proteins by the brain cells, prepared from rat brain at various stages of fetal-neonatal development. The results are expressed as dpm $[^{14}\text{C}]$-leucine incorporated into immunoprecipitable AFP and albumin in the brain cell lysates (synthesis) per hr per mg cellular proteins.

The results presented in Fig. 21 summarizes the developmental changes in the rate of synthesis of AFP and albumin in brain along with their tissue levels. It is apparent that AFP and albumin are synthesized at a higher rate during fetal life and postnatal development of the brain is accompanied by a rapid decrease in the synthesis of the two protein. Cells prepared from rat brain after 1 week of postnatal life revealed no incorporation of $[^{14}\text{C}]$-leucine into AFP or albumin. This indicates that synthesis of AFP and albumin in the brain is turned off after 1 week of postnatal development. AFP and albumin levels in brain during development also registered a rapid decline and the two protein disappears, as determined by rocket assay, after 1 week of postnatal development.
FIG. 21 Developmental Changes in AFP and Albumin Level and Their Rate of Synthesis in Brain.
A correlation between AFP and albumin level in brain and their rate of synthesis by brain cells in culture, during development is presented in Fig. 22 A and Fig. 22 B, respectively. Results are expressed as percent decrease in AFP and albumin level in brain and rate of their synthesis by brain cells during development over the values observed in 18 E fetal brain (control). A linear relationship was displayed. It is clear from Fig. 22 that high levels of AFP and albumin in brain could be accounted for due to their high rates of synthesis in the brain cells. The decline in the brain AFP and albumin level with age is due to a proportionate decrease in the rate of synthesis of these proteins. It may be concluded that levels of AFP and albumin in brain directly reflects their rate of synthesis in the brain.

**EFFECT OF HORMONES ON THE RATE OF AFP SYNTHESIS AND SECRETION BY NEWBORN RAT BRAIN CELLS IN CULTURE**

Newborn rat brain cells were prepared and maintained in culture for 24 hrs. Various hormones (Insulin, 0.05 IU ml\(^{-1}\); dbcAMP, 100 µg ml\(^{-1}\); dexamethasone, 2 µg ml\(^{-1}\); estradiol, 3 µg ml\(^{-1}\)) were added at the initiation of cultures. AFP synthesis and secretion were determined after 24 hrs culture of brain cells as described earlier. Amount of AFP and albumin secreted into the medium were determined by rocket assay. The alterations in the rate of synthesis and secretion of AFP by brain cells due to
FIG 22. Correlation Of Rates Of AFP And Albumin Synthesis With Their Contents In The Developing Rat Brain.

Results are expressed as % change over control (18 E rat brain). a, 20 E; b, 1 P; c, 7 P.
incubation with hormones is expressed as percent change over the control values (the cells incubated with the diluent only).

Results summarized in Fig. 23 indicate that dexamethasone and dbcAMP were effective in reducing the rate of AFP synthesis and secretion. However, reduction in AFP synthesis by dexamethasone was most effective (40% decrease) than dbcAMP (28% decrease). Similar effects were also exhibited in the amount of AFP accumulated (secreted) in the culture medium over a 24 hrs culture period. It would thus appear that synthesis and secretion of AFP and albumin is a coupled process. A change in the rate of synthesis of the protein is related to a proportionate change in the amount of the protein secreted into the medium. Insulin and estradiol did not reveal any significant change in either the synthesis or secretion of AFP by newborn rat brain cells. None of the hormones affected the rate of albumin synthesis and secretion.

**DEMONSTRATION OF AFP SYNTHESIS BY IMMATURE RAT SKIN**

New born rat skin explants were maintained in leucine free medium containing 1.0 μCi/ml [¹⁴C]-leucine as radioactive tracer. Control flasks included cycloheximide (100 μg/ml medium) added 30 min prior to the addition of labelled leucine. Culture flasks were incubated at 37°C in a metabolic shaker. Incubation was terminated after 2, 4 and 6 hrs and culture medium recovered by decantation.
FIG 23. Effect of Hormones on the Rate of AFP Synthesis in Newborn Rat Brain Cells in Cultures

Figure inside the bars is the mean of three experiments and represent the percent change over control (C). Insulin (I), estradiol (E), dbcAMP (A), Dexamethasone (D).

<table>
<thead>
<tr>
<th>Condition</th>
<th>% Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>C (Control)</td>
<td>(96.53%)</td>
</tr>
<tr>
<td>I (Insulin)</td>
<td>(102.46%)</td>
</tr>
<tr>
<td>E (Estradiol)</td>
<td>(85.10%)</td>
</tr>
<tr>
<td>A (dbcAMP)</td>
<td>(60.25%)</td>
</tr>
<tr>
<td>D (Dexamethasone)</td>
<td>(63.43%)</td>
</tr>
</tbody>
</table>
Skin explants were washed several times with PBS-leucine and homogenized in PBS. The homogenates were centrifuged at 22,000 x g for 30 min at 4°C. The clear supernatants were collected and treated as skin lysate. Incorporation of $^{14}$C-leucine into immunoprecipitable AFP and albumin was determined, as described earlier, in aliquots of skin lysates (synthesis) and culture medium (secretion). Total protein synthesis and secretion was determined by following $^{14}$C-leucine incorporation into TCA insoluble proteins. Results are expressed as dpm of $^{14}$C-leucine incorporated into AFP or total proteins per mg wet weight of skin explants cultured.

Time course of $^{14}$C-leucine incorporation into immunoprecipitable AFP in skin lysates (synthesis) and in medium (secretion) during 6 hrs culture period is presented in Fig.24. Incorporation of $^{14}$C-leucine into AFP and TCA insoluble fraction (total proteins) both in skin lysates (synthesis) Fig.24A and in medium (secretion) Fig.24B was linear during 6 hrs culture period. Addition of cycloheximide inhibited the incorporation of $^{14}$C-leucine into AFP (synthesis)(Fig.24A) indicating that AFP is indeed synthesized by newborn rat skin explants in culture. Accumulation of labelled AFP into the medium (secretion) was also inhibited by cycloheximide. Newly synthesized AFP was 10-12% of the total protein synthesis in these explants. $^{14}$C-labelled AFP secreted into the medium accounted for about 22-30% of the total labelled proteins secreted by these cultures.
FIG 24. Time Course Of \(^{14}C\) -Leucine Incorporation Into Total Proteins & AFP By Newborn Rat Skin Explants. Total Proteins, AFP, and AFP in presence of cycloheximide.
No significant incorporation of $^{14}$C-leucine into immunoprecipitable albumin was found in these cultures. It appears that albumin is not synthesized in the skin.

**Nature of AFP Secreted by Newborn Rat Skin Explants in Culture**

The medium obtained after 6 hrs culture of newborn rat skin explant was dialyzed extensively against first with PBS-leucine and then with PBS only. It was concentrated (10 times) by lyophilization and will be referred as CMS (concentrated medium from newborn rat skin explant cultures). CMS was subjected to various analyses to characterize AFP secreted by skin.

IEP analysis of CMS yielded single precipitin arc for AFP in $\alpha_1$-region with an electrophoretic mobility similar to serum AFP (Fig.25). The immunological identity of skin AFP to that of serum AFP is also apparent by a complete line of fusion between the two AFP's (Fig.25).

Active $E_2$ binding protein species among the proteins secreted by newborn rat skin explants was identified in CMS by the procedure similar to that used for brain AFP characterization. $[^3H]E_2$ binding observed in the CMS [7730 cpm/100 $\mu$g protein] could almost completely be displaced by excess of cold $E_2$ (587 cpm). Further, immunoprecipitation of AFP by anti-AFP from the CMS precipitated almost all the $[^3H]$-radioactivity (7190 cpm) and the supernatants did not show any significant $E_2$ binding.
FIG. 25 CHARACTERIZATION OF AFP SECRETED BY NEWBORN SKIN EXPLANTS IN CULTURE

25.A: Immunological identification of AFP secreted by skin explants in culture with serum AFP.
FS, fetal serum; CMS, culture medium (concentrated) obtained after 6 hrs culture of newborn rat skin explant; AbAFP, anti-AFP.

FIG. 26 25.B: Molecular weight determination of AFP secreted by newborn rat skin explants in culture by SDS-PAGE.

CMS (culture medium of newborn rat skin explant)

CMS (culture medium of 3 weeks old rat skin explant).
Similar results were also obtained in newborn rat skin cytosol. These observations clearly indicate that AFP is the only active protein secreted by newborn rat skin that binds $E_2$ with high specificity.

Molecular weight of $^{14}$C-labelled AFP secreted by newborn rat skin explants was determined following immunoprecipitation of labelled-AFP from CMS by anti-AFP and subsequent analysis of the immunoprecipitate on SDS-PAGE (see brain AFP mol. wt. determination). The medium obtained from skin explants culture of 3 weeks old rat (non producer of AFP; see below) was also processed in a similar manner and included as control. AFP yielded single band on SDS-PAGE corresponding to molecular weight of 68,000 daltons (Fig. 26) which is similar to that observed for purified rat AFP and brain AFP. Control gel did not reveal any radioactivity corresponding to AFP position, as observed in CMS, indicating that anti-AFP used for immunoprecipitation of AFP from CMS is monospecific and that 3 weeks old rat skin explant do not produce AFP.

ONTOGENY OF AFP IN SKIN

Developmental profile of AFP in rat skin was studied by measuring AFP level in skin cytosols, at various stages of development, by the rocket assay. The results are presented in Fig. 27A. High levels of skin AFP, observed during fetal life (18 and 20E) decreased progressively to very low levels at 21P and the protein disappeared thereafter.
Developmental Changes in AFP Levels and Its Rate of Synthesis in Developing Rat Skin
Measurement of heme concentrations in various skin cytosol samples revealed that fetal blood contamination could contribute about 5-10% of the total AFP levels observed in skin. It became apparent that an independent pool of AFP contributed by in situ synthesis may probably exist in skin which could account for high AFP levels in developing rat skin.

**AGE RELATED CHANGES IN THE RATE OF AFP SYNTHESIS IN RAT SKIN**

Rat skin explant cultures were established from fetuses of 18 d gestational age and 1, 7, 14, 21 and 28 d old postnatal rats. Synthesis of AFP was determined as described earlier. Results are expressed as rate of \(^{14}\text{C}\)-leucine incorporated into immunoprecipitable AFP per hr per mg of skin explants cultured. The change in the rate of AFP synthesis with age is depicted in Fig. 27B. It appears that maturation of skin with age results in continuous decrease in the rate of AFP synthesis. The AFP synthetic rates in 14 d old rat skin were greatly reduced (12.05% of the values at 18E). No incorporation of \(^{14}\text{C}\)-leucine into immunoprecipitable AFP was observed in the skin explant cultures established from 3 and 4 weeks old rat. It appears that synthesis of AFP in skin is turned off after two weeks of postnatal life.
CORRELATION OF AFP CONTENT WITH ITS RATE OF SYNTHESIS IN SKIN

AFP level as well as its rate of synthesis in skin explant cultures showed a continuous decline during development. A correlation between the rate of AFP synthesis and its content in skin during development is presented in Fig. 28. Results are expressed as percent change in these parameters over the values observed in 18 E fetal rat skin (control). A linear relationship between the AFP level and its rate of synthesis by skin explants in culture demonstrate that high level of AFP in skin is due to its high rate of synthesis in skin. Age related decline in AFP content in skin reflects a proportionate decrease in the rate of its synthesis.

EFFECT OF HORMONES ON THE RATE OF AFP SYNTHESIS BY NEWBORN RAT SKIN

Effect of incubating the skin explant cultures with hormones (insulin, dbcAMP dexamethasone, estradiol) on the rate of AFP synthesis over a 24 hrs culture period of newborn rat skin explant is depicted in Fig. 29. Results are expressed as percent change in the rate of AFP synthesis over the control (18 E fetal skin explant culture). Dexamethasone and insulin were effective in decreasing the rate of synthesis upto 26% and 11% respectively. Estradiol increased the rate of AFP synthesis by 10% over control. dbcAMP did not show any significant change.
FIG 28. Correlation Of AFP Level And Its Rate Of Synthesis In Skin During Development Of Rat
Results are expressed as % change over control values (18E rat skin) A, 1P; B, 7P; C, 14P.

FIG 29. Effect Of Hormones On The Rate Of AFP Synthesis In Newborn Rat Skin Explant Cultures
Results are expressed as % change over control. (C) Insulin; A, AM; D, dexamethasone; E, estradiol.
SYNTHESIS AND SECRETION OF AFP AND ALBUMIN BY NEWBORN RAT HEPATOCYTES IN CULTURE

Hepatocytes were prepared from newborn rat liver and maintained in leucine free medium for 6 hrs. Incorporation of $^{14}$C-leucine into immunoprecipitable AFP and albumin in cell lysates (synthesis) and in the medium (secretion) was determined to study the specific protein synthesis. Fig. 30 summarizes the kinetics of the incorporation of $^{14}$C-leucine into AFP, albumin and total proteins. Newborn rat hepatocytes synthesize AFP at a very high rate, accounting for about 80% of the total protein synthesis. Albumin synthesis in these cells was about 9% of the total protein synthesis. AFP and albumin together constitute about 90% of the total protein synthesis in the liver. Similar results have been reported by other authors (Sell et al, 1979). Secretion of AFP by the hepatocytes into the medium was about 76% and for albumin about 10% of the total secreted proteins. Cycloheximide can inhibit the synthesis and secretion of AFP and albumin (Fig. 30A and 30B).

SERUM AFP AND ALBUMIN PROFILES DURING DEVELOPMENT
RELATIONSHIP BETWEEN AFP AND ALBUMIN CONTENT IN LIVER AND SERUM LEVELS OF THESE PROTEINS WITH THEIR RATE OF SYNTHESIS IN THE DEVELOPING HEPATOCYTES

Blood was collected from fetal (18 and 20 E), post-natal (1, 3, 7, 14, 21, 28 and 35 d old) and adult rats. Sera were separated and AFP and albumin levels determined by
FIG. 30 Incorporation of $^{14}$-leucine into Total Proteins AFP and Albumin By Newborn Rat Hepatocytes.

Total Proteins (TP) $\Delta$,AFP $\bullet$ – Albumin $\circ$–AFP and Albumin In presence of cycloheximide $\rightarrow$, CP, Cell protein.
rocket assay. Amount of AFP and albumin was also determined in the cytosol samples prepared (see Material and Methods) from rat liver at various stages of development. The hepatocytes were prepared from rat liver at the specified ages and maintained in leucine free medium. Rates of AFP and albumin synthesis by these hepatocyte cultures were determined as described earlier.

Developmental changes in serum level of AFP, its content in liver, and rate of synthesis are depicted in Fig.31. The same parameters were also studied for albumin and the results are summarized in Fig.32. Serum levels of AFP and albumin showed an inverse relationship during development (Fig.31A and Fig.32A). Serum AFP concentration decline from 11.5 mg ml\(^{-1}\) at 18 E fetuses to 130 \(\mu\)g ml\(^{-1}\) in 28 d old neonatal rats. During this period AFP concentration in serum declined to about 1/100th to that observed in 18 E fetuses, while albumin level increased about 35 times during the same period. Liver contents of AFP and albumin also displayed similar relationship (Fig.31B and Fig.32B). AFP content in liver declined by about 92% in 28 d old liver (results are compared to 18 E fetal liver). Albumin content of the liver showed a constant increase during development. These observations indicates that age related changes in the serum levels of AFP and albumin and their content are closely related and might be a reflection of their rate of synthesis in liver.
FIG 31. Developmental Changes in Serum AFP Level, Liver Content and Rate of AFP Synthesis in The Hepatocytes

CP, Cell protein; TP, Total protein.
FIG 32. Developmental Changes in Serum Albumin Level, Liver Content, and Rate of Albumin Synthesis in the Hepatocytes. CP, Cell protein; TP, Total proteins
A comparative study of the synthesis of the two protein in the hepatocytes revealed that maturation of the hepatocyte is marked by a specific pattern of development of AFP and albumin synthesis. Immature rat hepatocytes (18 and 20 E fetuses) showed a very high rate of AFP synthesis in comparison to albumin. Rate of AFP synthesis in the hepatocyte cultures established from rat liver at various stages of development showed a continuous decline with age (Fig.31 C). Hepatocyte cultures from 4 weeks old rat liver did not incorporated $[^{14}C]$-leucine into AFP. It appears that synthesis of AFP is turned off at this stage of development. Rate of AFP synthesis during development (maturation) of hepatocytes were 80.7%, 70.9%, 43.2%, 32.4% and 0.45% in 20 E, 1 P, 7 P, 14 P and 21 P rat respectively (Results are expressed as % decline over the rate of AFP synthesis observed in 18 E fetal liver).

On the other hand, age related development of hepatocyte is associated with an increase in the rate of albumin synthesis (Fig.32 C), liver content (Fig.32 B) and serum level (Fig.32 A). Rate of albumin synthesis in the hepatocytes during foeto-neonatal development were 14.07%, 22.05%, 27.94%, 61.76%, 70.58% and 92.64% in 18 E, 1 P, 7 P, 14 P, 21 P and 28 P rat respectively (Results are compared with the rate of albumin synthesis obtained in the hepatocytes prepared from 35 d old rat liver).
It is apparent that maturation of hepatocyte is accompanied by a decline in the rate of AFP synthesis and an increase in the rate of albumin synthesis. Ratios of the rate of albumin to AFP synthesis in the hepatocytes were 0.05, 0.10, 0.28, 0.35 and 48 in 18 E and 1, 7, 14 and 21 d old rats.

**CORRELATION OF AFP AND ALBUMIN CONTENT IN RAT LIVER WITH THEIR RATE OF SYNTHESIS IN THE HEPATOCYTES DURING DEVELOPMENT**

The liver content of AFP and albumin and rates of their synthesis were directly correlated (Fig.33). Results are presented as percent change in the rate of AFP synthesis in the hepatocytes and its liver content over the values observed in 18 E fetal liver (control) (Fig.33A). Rate of albumin synthesis and its liver content are presented as percent change in these parameters over the control (35 d old rat). It would appear from Fig.33A and 33B that liver content of AFP and albumin depends upon their rates of synthesis in the developing hepatocyte. Age related decrease in AFP and increase in albumin content in liver directly reflects magnitude of their synthesis in liver.

**CORRELATION OF SERUM AFP AND ALBUMIN LEVEL WITH RATES OF THEIR SYNTHESIS IN THE DEVELOPING HEPATOCYTES**

A correlation of serum albumin level and its rates of synthesis presented in Fig.34A. Results are expressed as percent change in these two parameters over the control.
FIG 33. Correlation Of Liver Contents Of AFP And Albumin With Their Rates Of Synthesis In The Hepatocytes During Development. Results are expressed as % change over their respective controls. The values observed in 18E rat and 35P rat are taken as control in figures A and B respectively. A, 18E; B, 20E; C, 1P; D, 7P; E, 14P; F, 21P; G, 28P.
FIG 3. Correlation Of Serum Albumin And AFP With Their Rates Of Synthesis In The Hepatocytes During Development
Results are expressed as % change over their controls. The values observed in 35P rat and 18E rat are treated as control in figure A and B respectively: A,20E; B, 1P; C,7P; D,14P; E,21P; F,28P.
values (35 d old rat). An increase in serum albumin level may be accounted due to proportionate increase in the rate of AFP synthesis and secretion by the developing hepatocyte. Relationship between AFP level in serum and its rate of synthesis by the developing hepatocyte is presented in Fig. 34 B. Serum AFP level during first two weeks of postnatal development decrease more rapidly than its rate of synthesis in the hepatocyte. During this period rate of AFP synthesis decreased by 32.4% while serum AFP level declined by 7.7% over the values observed in 18 E fetal rat. In other words serum AFP level were lower than its rate of synthesis and secretion by the hepatocytes. It may be due to a large increase in the body weight (total blood volume) resulting in greater dilution of AFP secreted by liver during first two weeks of postnatal life, and due to a decreased rate of AFP synthesis and secretion by the developing hepatocyte. The loss of yolk sac at term is also responsible for a more rapid fall in serum AFP level. In rat, yolk sac is active in the synthesis and secretion of AFP throughout the gestation period and at term it may account about half of the total serum AFP level (Gitlin, 1975). The curve represented by dotted lines in Fig.33 B would have been true if AFP synthesis and its serum level were declining at the same rate.
HORMONAL CONTROL OF AFP AND ALBUMIN SYNTHESIS IN HEPATOCYTE OF NEWBORN RAT

Hepatocytes from newborn rat were cultured in leucine free medium as described earlier. Effect of various hormones (insulin, dbcAMP, estradiol and dexamethasone) on the rate of AFP and albumin synthesis in these cells was determined over a 24 hrs culture period. Effect of these hormones on the rate of amount of AFP and albumin secreted into the medium during the same culture period in presence of various hormones was also studied.

Dexamethasone and dbcAMP reduced the rate of AFP synthesis by 45% and 7% over the corresponding control values (Fig.35 B). Insulin increased the rate of AFP synthesis by 21%. Similar effects of these hormones were also exhibited in the rate of secretion of AFP when expressed as μg/hr/mg cellular protein (Fig.35 A). Estradiol did not affect either the rate of synthesis or secretion of AFP.

Effects of insulin, dexamethasone, dbcAMP seems to be specific for AFP since albumin synthesis or secretion by newborn hepatocyte was not significantly altered under these conditions.

EFFECTS OF HORMONES ON SERUM AFP LEVEL IN NEWBORN RATS IN VIVO

Various hormones were administered to newborn rats to study their effects on serum AFP level. Dexamethasone (2 μg g⁻¹ d⁻¹), estradiol (3 μg g⁻¹ twice a week), insulin
Flg 35. Effect of Hormones On The Rate Of AFP Synthesis And Secretion In Newborn Rat Hepatocytes In Culture

The values inside the bars represents the mean of three experiments expressed as percent change over control (C). Insulin (I), dbcAMP (A), dexamethasone (D), estradiol (E).
(0.03 IU g\(^{-1}\) d\(^{-1}\)) and dbcAMP (10 \(\mu g\) g\(^{-1}\) d\(^{-1}\)) were injected intraperitoneally into newborn rats, while control group received equal volume of saline. Blood samples were collected from hormone treated and control rats after specified intervals of time. Serum AFP and albumin levels were determined by rocket assay. Results are depicted in Fig. 36.

Control group showed the normal disappearance of AFP from serum after 4 weeks of postnatal life. Dexamethasone induced a premature fall in serum AFP and it disappeared 8 days earlier than control group. Dibutyryl cAMP also suppressed AFP level and resulted in its disappearance from serum about 4 days earlier. Estradiol and insulin did not affect AFP level significantly.
FIG 36. Effect Of Hormones On Serum AFP Level In Newborn Rats. Each point represent the mean of five observations.
DISCUSSION
AFP is one of the best characterized oncofetal antigens known to be expressed primarily in yolk sac visceral endometrium and fetal liver parenchyma (Gitlin, 1978, 1975). Both cell types have common origin during ontogenesis. AFP is re-expressed in tumors of these cell types and thereby it has attained a great significance as a diagnostic aid for hepatocellular carcinoma and germ cell teratocarcinomas (Ruoslahti and Seppala, 1979). Slowly it is being appreciated that other developing tissues also express AFP gene.

Albumin and AFP are useful differentiation markers. They are invaluable in investigating differentiation related activation or quiscence of specific gene expression. This dissertation is aimed at developing relevant methodology and gaining an insight into regulation of AFP and albumin genes by looking at ontogenesis of synthesis and levels of these proteins in different developing tissues.

AN IMPROVED PROCEDURE FOR ISOLATION OF AFP

Isolation of homogeneous AFP by a direct negative immunoabsorption followed by DEAE-cellulose column chromatography is a definite and significant improvement over the procedures reported for purification of AFP (Ruoslahti and Seppala, 1979). AFP and albumin are major proteins (~80%) of the amniotic fluid (Tomasi et al., 1977) and they share many physicochemical properties (Ruoslahti
and Seppala, 1979; Ruoslahti and Engvall, 1978; Smith and Kelleher, 1973). This has rendered their separation, by conventional means, difficult.

Partial success in purification of AFP has been achieved by an array of physicochemical procedures including gel filtration, isoelectric focussing, ion exchange chromatography, solid phase lectin/estradiol affinity chromatography (Ruoslahti and Seppala, 1979). Such procedures have not only been multistep and time consuming but have also resulted in poor recoveries of the protein. This leaves immunochemical procedure as the method of choice for isolation of the protein. Affinity procedure involving adsorption of the protein to anti-AFP sepharose column has proved useful (Ruoslahti and Seppala, 1979; Sell et al., 1972). However, this procedure has obligatory requirement for a highly specific antiserum against AFP, for the preparation of immunoadsorbent, which may not be available initially. Moreover, elution of adsorbed AFP from anti-AFP sepharose column needs drastic conditions (low pH, 8 M urea etc.) which may affect the biological potency of AFP (estradiol binding, immunosuppression, binding of various ligands etc.). However, no comparison has yet been reported regarding the biological activity of AFP purified by different procedures. This may indicate the reported discrepancies regarding the stoichiometry of E₂-AFP interaction, and magnitude of immunosuppression by different preparations of AFP (Ruoslahti and Seppala, 1979).
Classical antibody affinity chromatography frequently leads to the constant leakage of covalently coupled ligand (IgG) from the agarose column matrices during elution of AFP (Ruoslahti, 1976; Pihko et al, 1973), thereby, necessitating another immunoadsorbent column or gel filtration step to get rid of the contaminating IgG.

Absorption of antibody with antigen(s) is commonly used to make the heterogenous antiserum specific for a 'phase specific antigen' (see preparation of anti-AFP*). Similarly undesired antigen(s) can also be removed by absorption with monospecific or polyvalent antisera to precipitate out the undesired antigen(s). Present procedure based on this principle could yield homogeneous AFP with properties identical to literature reports for AFP purified by other procedures. Moreover, specific antiserum raised in rabbits against AFP purified by the procedure was also used for the preparation of anti-AFP sepharose. Rat AFP isolated from amniotic fluid by these two procedures did not reveal any difference with respect to molecular weight, estradiol binding, and Con A-affinity molecular variants.

Negative immunoabsorption procedure resulted in high yield (87%) of AFP. Further, the procedure employed very gentle isolating conditions. The inherent disadvantage associated with the present procedure lies in the use of relatively larger amount of purified anti-NRS IgG. While immunoadsorbent having anti-AFP (IgG) coupled to sepharose
can be reused for many times. The present procedure for isolation of AFP may also be useful for isolation of other phase specific antigens like carcinoembryonic protein and uteroglobin.

**ONTGENY AND DISTRIBUTION OF AFP IN FETO-NEONATAL TISSUES OF RAT AND HUMAN FETAL BRAIN**

The expression of a common protein in a tissue during feto-neonatal life and in carcinogenesis have favoured the concept of common gene expression during development and oncogenesis. AFP is known to be mainly synthesized by the yolk sac, fetal liver and to a small extent by the gastrointestinal tract, kidney and placenta of all mammals so far studied (Mackiewicz and Breborowicz, 1980; Breborowicz and Nishi, 1979; Mackiewicz et al, 1978; Gitlin, 1975). Recently, immature rat uterus has also been demonstrated to synthesize AFP (Smalley and Sarcione, 1980). However, ontogeny of AFP in these tissues has not been studied. Most of the studies of AFP were concentrated on neoplastic tissues and the possibility that the protein may also be of significance in the development of tissues other than liver and yolk sac has not attracted much attention. Studies on AFP conducted during the last decade have made it clear that tumors arising in the adult tissues which are known to synthesize AFP during early development (Liver, yolk, sac, gastrointestinal tract) often lead to elevated levels of AFP in serum (Ruoslahti and Seppala,
1979). In addition, tumors arising in other tissues (non-procedures of AFP) such as testis, ovary lung, skin and brain may also produce elevated levels of serum AFP (Ruoslahti and Seppala, 1979; Arita et al, 1980; Soltani et al, 1978). In view of these observations various feto-neonatal tissues were investigated for the presence of AFP and its ontogeny.

AFP was found to be present in most of the fetal tissues except pancreas, spleen, and thymus. AFP present in serum, brain, skin, and kidney was immunologically, electrophoretically, and with regard to estradiol binding identical. Immunological identity of AFP produced by human fetal organs such as yolk sac, liver, intestine and kidney has been already demonstrated (Mackiewicz and Breborowicz, 1980). This may indicate the expression of AFP gene in these physiologically and functionally different tissues. However, the origin of AFP in various tissues is not clear at present. It may be speculated that fetal blood contamination, having high concentration of AFP, may give rise to detectable levels of AFP in various tissues. The level of AFP in various tissues at a specific stage of development was found to be different. In addition, AFP disappeared from various tissues at a different yet specific period after postnatal development. These observations strongly suggest that AFP in various tissues does not arise due to the contamination from fetal blood. Otherwise, ontogeny of AFP in various tissues should have
been alike. Determination of heme concentrations in various tissue extracts showed that fetal blood contamination could, at the most, contribute up to 10% of the observed AFP levels in various fetal-neonatal tissues of rat. However, this test would not account for AFP in extra vascular fluids (i.e. AFP may distribute in both intravascular and extra vascular compartments whereas heme in blood cells is present only in the intravascular compartment). It may also be argued at this stage that various tissues differ in their capacity to absorb or concentrate AFP from fetal circulation, thereby, differing in tissue content of AFP. No direct evidence is available to prove or disprove any of the above alternatives.

Another possibility which can account for the observed tissue levels of AFP is: in situ synthesis of AFP by different tissues. Synthesis of AFP has already been demonstrated in the yolk sac, fetal liver, stomach, intestine, kidney and placenta (Mackiewicz and Breborowicz, 1980; Breborowicz and Nishi, 1979; Gitlin, 1975). While evidence for synthesis of AFP by developing rat brain and skin is presented in this dissertation. Further, support for this hypothesis is derived from the reports that AFP produced by human yolk sac, fetal, liver, intestine and kidney may differ from serum AFP with respect to its relative proportion binding to Con-A and lentil agglutinin (Mackiewicz and Breborowicz, 1980). Our results also
indicate that brain and skin cytosol differ from serum in their relative amounts of Con A-nonreactive AFP variant. These observations also support that contamination of AFP in these tissues from fetal blood may partly account for the observed AFP levels in these tissues.

**SYNTHESIS OF AFP BY DEVELOPING RAT BRAIN, SKIN, AND LIVER**

Feto-neonatal estradiol binding protein in rodent brain has earlier been reported as AFP (Attardi and Ruoslahti, 1976; Plapinger and McEwen, 1975; Plapinger et al, 1973). Intracellular localization of both AFP and albumin has also been confirmed in the central and peripheral nervous systems of developing rodents (Toran-Allerand, 1980; Trojan and Uriel, 1979; Benno and Williams, 1978) and human fetuses (Mollgard et al, 1979) by immunohistochemical procedures. Studies by Eppenburger and Hsia (1972) have indicated the presence of an $E_2$ binding protein in the skin cytosol of immature rats. However, no information is available regarding its origin in these tissues. The presence of high levels of AFP and its distinct ontogeny in brain and skin led us to propose that AFP might be synthesized in these tissues and may account for all the above observations.

An extensive biochemical and morphological characterization of dissociated mammalian brain cells in primary culture (Raizada et al, 1981, 1980; Hansson et al, 1980; Labourdette et al, 1979; Benda et al, 1975) and skin
explant cultures (Vorma and Boutwell, 1980; Hawley-Nelson et al., 1980; Halprin et al., 1979; Epstein, 1979) has provided a good in vitro model systems to study the biochemical events accompanying the growth and differentiation of these tissues. Dissociated newborn rat brain cells and skin explant cultures were used to answer the following questions: Is \( E_2 \) binding protein in newborn rat brain and skin AFP? If so, where does this protein originate from and what is its ontogenetic profile in different tissues?

Synthesis of AFP and albumin by immature rat hepatocytes is well established (Erazier et al., 1977; Tsukada and Hirai, 1975; Sell and Skelly, 1975; Leffert and Sell, 1974). This system was used in the present study to compare with AFP synthesis in brain cell culture and skin explant.

Our observations, derived from \(^{14}\text{C}\)-leucine incorporation into immunoprecipitable AFP and albumin by dissociated brain cells and skin explant maintained in short term cultures provide evidence for the synthesis of AFP and albumin in brain, and AFP in skin of newborn rat. Cycloheximide mediated inhibition of incorporation of \(^{14}\text{C}\)-leucine into: AFP and albumin in brain cells and into AFP in skin explant cultures strongly suggests that neonatal rat brain and skin are sites for synthesis of these proteins. This observation also eliminated the possibility of the labelled AFP having arise from an artifact of culture such as AFP being released from dead cells or carried over from fetal serum and released subsequently later in
cultures or due to nonspecific adsorption of free $^{14}$C-leucine.

Further, intracellular content of AFP and albumin in brain cell did not change significantly during 6 hrs culture period. To the contrary, these cells demonstrated a linear secretion of AFP and albumin, as a function of incubation time. Total amount of AFP as well as albumin secreted into the culture medium was also far in excess of the intracellular content of these proteins at the initiation of brain cell cultures. In view of these observations, it may be concluded that AFP and albumin observed in brain cell cultures arise entirely as a result of in situ synthesis of the two proteins in the brain. AFP and albumin secreted by the brain cells and newborn rat of skin explants were immunologically, electrophoretically and with regards to molecular weight and $E_2$ binding properties similar to serum AFP. Demonstration of the synthesis of estrophillic AFP by developing brain and skin also provide an explanation for the presence of $E_2$ binding protein in the cytosol of rat brain (Attardi and Ruoslahti, 1976; Flapinger et al, 1973) and skin (Eppenburger and Hsia, 1972). Intracellular pool of AFP and albumin found in the diverse region of the developing rodent (Toran-Allerand, 1980; Trojan and Uriel, 1979; Bonno and Williams, 1978) and human brain (Mollgard et al, 1979) and the presence of AFP in human fetal brain cultures (Mackiowicz et al, 1978) may also arise from their
in situ synthesis in the brain.

One of the important questions which remain unexplained is the fate of AFP and albumin secreted by developing rat brain and skin. Whether AFP and albumin synthesized in the brain are restricted to brain only or find their way to the common pool i.e. fetal blood. The blood-brain barrier is not fully developed till 8 d of postnatal life (Davson, 1976). It may be speculated that there may be an exchange of proteins between the two compartments. Equilibration of AFP and albumin from the brain to the blood may not be possible because levels of these proteins are extremely lower in brain in comparison to serum. Despite this large concentration gradient, equilibration of these proteins between the two pools (including fetal blood to brain) does not occur as is evident from the level of these proteins in serum and brain. Developmental profile of AFP in human fetal brain as observed in the present study was similar to that reported in cerebrospinal fluid (Adinolfi and Adinolfi, 1976). It is possible that fetal brain might be contributing to CSF levels of AFP and albumin. It appears that AFP and albumin synthesized and secreted by the developing rat brain are probably restricted to this compartment only. Clarification of these speculations may have a direct bearing on the physiological role of these proteins in mammalian brain.
ONTODY AND DISTRIBUTION OF AFP AND ALBUMIN IN RAT BRAIN: CORRELATION WITH THEIR RATE OF SYNTHESIS

Regional distribution of AFP in olfactory, cerebrum, cerebellum and the rest portion of the brain did not reveal any difference indicating a uniform distribution of this $E_2$ binding protein. Feto-neonatal development of the rat brain was accompanied by a striking change in AFP levels in either whole brain or its discrete regions. During first week of postnatal life, AFP and albumin levels in the brain were reduced drastically and the protein could not be detected after 7 d of postnatal development. The rates of AFP and albumin synthesis in brain also declined rapidly and no synthesis of these two proteins could be demonstrated after one week of postnatal development. Interestingly, intraneural localization of both AFP and albumin has been observed only before 8 d of postnatal life (Toran-Allerand, 1980). The proposition that intracellular AFP and albumin in brain may arise from the uptake of these proteins by brain cells from fetal blood (Mollgard et al, 1979) may not thus explain intracellular pool of AFP and albumin in brain. If this were so, localization of these proteins in brain should have been demonstrated even after 8 d of postnatal life because the serum AFP and albumin levels remain high upto 3 weeks of postnatal life.

Postnatal development of the rat brain is known to be accompanied by a rapid decrease in protein synthesis in
either whole brain (Johnson, 1976; Gilbert and Johnson, 1972; Andrews and Tata, 1971) or various regions of the CNS (Dunlop et al, 1978; Dunlop et al, 1977). Rate of protein synthesis is quite high in fetal rat brain and its maturation is accompanied by a rapid decline in the same during first week of postnatal life and by 12 d protein synthesis in rat brain is only 10% of that observed in newborn rat (Johnson, 1976; Gilbert and Johnson, 1972). The deceleration of protein synthesis during early development is presumably due to a change in intracellular mechanism (Johnson, 1976). It is difficult to conclude at this stage whether inhibition of the synthesis of AFP and albumin during brain development is a reflection of overall inhibition of protein synthesis observed during this process. AFP and albumin synthesis takes place on membrane bound polysomes (Peters and Reed, 1980; Belanger et al, 1979). It has been observed that decline in the rate of protein synthesis with age is more intense for the proteins synthesized on membrane bound polysomes than for those synthesized on free polysomes (Andrews and Tata, 1971). In view of this observation the rates of synthesis of AFP and albumin are expected to show faster decline with age. This has indeed been observed during the present investigation. However, the significance of a decrease in specific protein synthesis (AFP and albumin) in brain remains to be elucidated.
Co-existence of AFP and albumin has been demonstrated within the same brain cell (Toran-Allerand, 1980). It is possible that the synthesis and secretion of AFP and albumin takes place in the same cell. In the present study we have demonstrated the synthesis of AFP and albumin by brain cells (derived from whole brain) of developing rat which represent a heterogeneous population of various cell types. It is not clear, at the moment, whether all cell types of the brain synthesize AFP and albumin or whether it is restricted to any special cell type amongst astrocytes, neurones, and oligodendrocytes etc. Maturation of the brain is known to accompany a change in the relative proportion of neuronal and glial cells (Johnson and Sellinger, 1971) which may further differ in the rate of protein and RNA synthesis (Yanagihara, 1979). In view of these observations, it appears that the synthesis of AFP and albumin by isolated neuronal and glial cells in culture or brain cells enriched in particular cell type(s) would prove helpful in determining the precise role of AFP and albumin in the differentiation and growth of mammalian brain.

**Ontogenic Profiles of Serum AFP and Albumin: Correlation with Rate of Their Synthesis in the Hepatocytes**

Synthesis and secretion of AFP and albumin takes place exclusively in the hepatocytes and other cell types of the liver are not known to synthesize these proteins.
(Abelev, 1979; Gitlin, 1975). Human fetal liver starts synthesizing AFP and albumin and secreting them into the serum as early as 29 days after conception (Rothschild et al, 1977; Gitlin, 1975). Considerable information is available regarding the change in serum profiles of AFP and albumin accompanying the development of rat and human (Ruoslhhti and Seppala, 1979; Stillman and Sell, 1979; Gitlin, 1978, 1975; Nayak and Mittal, 1977; Abelev, 1971) but a direct correlation with their rate of synthesis throughout the development of hepatocyte is not documented.

Feto-neonatal development of rat liver is accompanied by a striking change in the serum levels of AFP and albumin. AFP is the major serum protein during early fetal life and its level shows a continuous decline with postnatal development. To the contrary, serum albumin levels registered a continuous increase. The changing serum profile of these protein and their contents in liver correlated well with their rate of synthesis in the developing hepatocytes. AFP is the major protein species synthesized and secreted by the fetal hepatocytes and its synthesis may account up to 85% of the total protein synthesis. However, as the hepatocytes undergo maturation, the rate of AFP synthesis shows a continuous decline which is also reflected in the proportionate decrease in serum AFP content. AFP synthesis in the hepatocytes seems to be turned off after 3 weeks of postnatal development. At 3 weeks postpartum rate of AFP
synthesis in the hepatocyte was only 3% of that observed in 18 d gestation hepatocytes. During the same period, serum AFP level also declined by more than 95%. Concomitantly serum albumin concentration and its rate of synthesis in the hepatocyte shows an increase with age. It is now apparent that differentiation and maturation of hepatocytes is accompanied by a sequential switch over from AFP to albumin as the major protein species synthesized and secreted by the hepatocytes. Similar conclusion has been drawn from the immunocytochemical localization of AFP and albumin in the developing hepatocyte (Kuhlman, 1979; Nayak and Mittal, 1977). In fetal liver as much as 80% hepatocytes may contain AFP (Abolev, 1979). During postnatal development, the number of albumin producing cells are known to increase while AFP positive cells exhibits a continuous decline and after 4 weeks, AFP containing hepatocytes could no longer be detected (Kuhlman, 1979; Nayak and Mittal, 1977). AFP and albumin have been localized in the same hepatocyte (Kuhlman, 1979; Nayak and Mittal, 1977) indicating that these two proteins may be synthesized and secreted by the same cell. It is not clear whether age related decrease in the rate of synthesis of AFP reflects a simultaneous and slow decline in AFP gene expression in all the AFP synthesizing hepatocytes or AFP gene expression is sequentially turned off in different hepatocytes. A decrease in the number of AFP positive
hepatocytes with increasing age seems to favour the later proposal. However, such a process would involve a highly specific regulatory mechanism capable of selecting a specific population of hepatocytes.

Recently, some of the aspects of the regulation of AFP and albumin synthesis during development have been clarified. It was demonstrated that AFP and albumin synthesis is directly regulated, at the level of transcription, by controlling the amount of mRNA of these protein in the liver (Liao et al, 1980; Salatrepat et al, 1979; Sell et al, 1979; Belanger et al, 1979; Hofer et al, 1979; Nishi et al, 1979; Miura et al, 1978; Iio et al, 1976; Tamaoki et al, 1976; Koga and Tamaoki, 1974). The albumin mRNA levels increase while AFP mRNA levels in the liver decline during postnatal development and lack of AFP synthesis in the adult hepatocytes is due to the absence of its mRNA. Several conditions (CCl₄ intoxication, partial hepatectomy, carcinogenesis) which induce AFP synthesis in the adult liver have also been attributed to a direct increase in the amount of mRNA AFP (Atryzek et al, 1980; Liao et al, 1980; Commer et al, 1979; Salatrepat et al, 1979; Chiu et al, 1979; Miura et al, 1979; Sell et al, 1979; Belanger et al, 1979; Innis and Miller, 1979) rather than any change in translation activity. However, the molecular mechanism responsible for regulating the AFP gene activity during normal development and carcinogenesis remains to be elucidated.
AFP IN SKIN: AGE RELATED CHANGES IN THE SKIN AFP PROFILE AND ITS RATE OF SYNTHESIS

The present study provides evidence that estradiol binding protein in skin cytosol from developing rat is AFP. Eppenberger and Hsia (1972) have also found an E₂ binding protein in skin cytosol with a molecular weight of 58,000 daltons determined by gel filtration. We have demonstrated that such E₂ binding protein (AFP) arises in skin as a result of its independent synthesis in the tissue. Analysis of the total proteins secreted into the medium by newborn skin explants maintained in culture revealed that AFP is the only E₂ binding protein. This conclusion is arrived at from the observation that immunoprecipitation of AFP by anti-AFP precipitated the E₂ binding protein from both the skin cytosol (newborn rat) as well as the medium obtained from newborn rat skin explant cultures. It would appear that E₂ binding protein observed by Eppenberger and Hsia (1972) is indeed AFP. They had not characterized E₂ binding protein beyond molecular weight determination. The discrepancies between our results and Eppenberger and Hsia (1972) regarding the molecular weight may be due to a difference in techniques used for the evaluation of the molecular weight of E₂ binding protein (AFP). No other reports are available regarding the characterization of skin E₂ binding protein (AFP).
Like other tissues of rat, AFP levels in skin also showed an age dependent decline with a proportionate fall in the rate of AFP synthesis in the tissue. During first two weeks of postnatal development, rate of AFP synthesis and its level in skin declined by 90%. It is likely that AFP synthesis in skin during development is also regulated by controlling the amount of its mRNA as in other tissues.

**EXPRESSION OF AFP AND ALBUMIN GENES IN THE DEVELOPING RAT BRAIN, LIVER, AND SKIN: A COMPARATIVE STUDY**

Although we have demonstrated that genes for AFP and albumin are expressed in developing rat brain and skin as well but a marked difference exists in the rate of their synthesis in brain, skin, and liver.

A comparative study of the pattern of AFP synthesis during development revealed that it is turned off in brain, skin and liver after 7, 14, and 21 day of postnatal development, respectively. Magnitude of AFP synthesis in newborn rat skin, brain and liver showed a ratio of 19:2:1. Similarly, albumin synthesis in brain is turned off after 1 week of postnatal life while liver shows a continuous increase throughout postnatal development and attaining an adult basal rate of albumin synthesis by about 8-9 weeks after birth. On the other hand, albumin is not synthesized in the skin. Magnitude of albumin synthesis in developing brain and liver showed a striking change during development. Ratios of the rate of albumin synthesis in liver:brain at
18E, 1 P and 7 P were 1, 2 and 16. It appears that the genes for AFP and albumin are expressed in developing liver, brain and skin of rat and probably regulated independently. An effective way of doing this would be through the control of the amount of functional (translatable) mRNA$_{AFP}$ and mRNA$_{albumin}$ available in different tissues. This concept is supported by the findings of Liao et al (1980) who demonstrated that amount of mRNA$_{AFP}$ are different in rat yolk sac and liver at a specified period of postnatal development. Such a situation may also be valid for synthesis of AFP and albumin in developing brain, skin and liver of rat depending upon the functional requirement of these proteins in a given tissue.

**HORMONAL CONTROL OF AFP AND ALBUMIN SYNTHESIS IN NEWBORN RAT LIVER, BRAIN, AND SKIN**

Ontogeny related decline in the rate of AFP synthesis in various tissues suggests that an effective mechanism for regulating AFP synthesis exists. Amongst different hormones studied, dexamethasone was the most potent inhibitor of AFP synthesis in hepatocytes, brain cells and skin explants. Administration of the hormone also lead to a significant fall in serum AFP level in the newborn rat. However, response in different tissues varied significantly. Rate of AFP synthesis in skin explant cultures decreased by 25% in comparison to 45% and 40% decline observed in hepatocyte and brain cell cultures respectively.
Dibutyryl cAMP mediated decrease in rate of AFP synthesis also showed variable response in various tissues. It was more intense in brain cells (-15%) followed by hepatocyte (-7%) while skin explants were almost refractory. Acceleration of the rate of decline of AFP after dbcAMP administration supports that the above in vitro observation also holds true under in vivo conditions for intact newborn rat.

Insulin exhibited a dual effect on the rate of AFP synthesis in various tissues. It increased AFP synthesis in the hepatocyte (+21%) and showed an opposite effect in brain cells (-15%) and skin (-4%). However, insulin did not reveal any significant effect on serum AFP levels in vivo. Discrepancies in in vivo and in vitro effects of insulin and a differential response to various tissues is not clear at present.

Because AFP in rats binds estradiol with high affinity and specificity, its administration is expected to increase AFP synthesis in various tissues in vitro and to delay the normal disappearance of serum AFP in vivo. However, estradiol did not significantly affect the rate of AFP synthesis in hepatocyte and brain cells in culture. Similar conclusion may be drawn from the observation that induction of AFP synthesis in rats does not affect the serum estradiol (Aussel et al, 1980). However, estradiol caused a decrease of about 10% in the rate of AFP synthesis.
in skin explants in culture which seems to be unusual on the basis of expected estradiol and AFP relationship.

Dexamethasone, dbcAMP, insulin and estradiol did not show any significant and consistent effect on the rate of albumin synthesis in the hepatocyte or brain cells in culture. Serum levels of albumin in newborn rats were also not affected by these hormones. It appears that effects of dexamethasone, dbcAMP and insulin are specific for AFP synthesis.

A few reports are available to compare the effects of various hormones on AFP synthesis in vitro and in vivo and that too in liver only (Belanger et al, 1979, 1975). These authors also reported that exogeneously administered glucocorticoids, ACTH, thyroxin, adrenalin and dbcAMP lead to premature fall in serum AFP without affecting albumin and total protein levels. Recently a few workers have tried to investigate the mechanism of hormone mediated control of AFP gene expression. Commer et al (1979) have demonstrated that suppression of AFP synthesis by glucocorticoids is mediated by a direct decrease in the amount of mRNA_{AFP}. However, dexamethasone also showed an opposite effect during induction of AFP (CCL4 intoxication, 3'-DAB carcinogenesis) and may increase serum AFP levels (Belanger et al, 1978). Neonatal adrenalectomy (at day 6), radiothyroidectomy (day 0), ovariectomy (day 3) do not significantly alter the neonatal curve of serum AFP level
(Belanger et al, 1978). Additionally, neonatal adrenalectomy does not modify the effect of prednisolone but suppresses the response to ACTH and reduces the effect of thyroxine. It appears that glucocorticoids may have a permissive effect on thyroxine mediated regulation of AFP synthesis.

However, not all in vitro effects reproduce in neonatal animals in vivo. Insulin shows a stimulating effect on AFP production by hepatocytes but has no apparent effect in vivo. Similar results have also been observed by Belanger et al (1979) for insulin and glucagon. Further, some hormones may also exhibit variable in vitro effect on AFP synthesis, depending on dosage and/or culture conditions (Belanger et al, 1979).

It may be stressed that the postnatal repression of AFP in rats normally takes place during a distinct developmental stage of biochemical differentiation of the liver, the third postnatal week, which coincides with a complete expression of several hormone function (Greengard, 1973). It is also notable that the AFP suppressive agents (glucocorticoids, thyroxin, dbcAMP) are known to exert a number of differentiation events in various fetal and neonatal tissues. Glucocorticoids in particular induce multiple biochemical maturation processes in the liver (Belanger et al, 1979). How this relates to the quiescence of the AFP gene function remains to be clarified. The dual
effects of some hormone on different tissues also needs much more attention.

**PHYSIOLOGICAL ROLE OF AFP IN DEVELOPMENT**

Although a bulk of information is available on the course of AFP appearance in normal and abnormal development, exact role of AFP is far from clear (Ruoslahti and Seppala, 1979; Smith and Kelleher, 1980; Stillman and Soll, 1979). It is now well established that rodent AFP binds estrogens with high affinity and specificity (Ruoslahti and Seppala, 1979; Nunez et al, 1979; Uriel, 1979). AFP has also been identified as estrogen binding protein in immature rat uterus (Attardi and Ruoslahti, 1977; Radanyi et al, 1977; Uriel et al, 1976), brain (Attardi and Ruoslahti, 1976; Uriel et al, 1976; McEwen et al, 1975) and bone (Chen and Feldman, 1978) cytosol. Our studies have also recognized AFP as $E_2$ binding protein in skin. It has been proposed that AFP modulates the transport of estrogens in estrogen target tissues and may prevent the uterotrophic action of natural estrogen by decreasing their uterine uptake (Uriel, 1979; Nunez et al, 1979; Raynaud et al, 1971). Alternatively, AFP may also compete with the estrogen metabolizing tissue enzymes (Aussel et al, 1976). However, the physiological implications of these observations are yet to be recognized.

Estrophilic AFP is found to be distributed throughout fetoneonatal rat brain (Toran-Allerand, 1980; Trojan and
Uriel, 1979; Benno and Williams, 1978; Plapinger and McEwen, 1975; Plapinger et al, 1973). It is known that sexual differentiation of rodent, which occurs during the early neonatal period, is influenced by steroid hormones (Fox et al, 1978; McEwen et al, 1974). Steroid administration to females before the 10th day postnatal life has resulted in failure to stimulate ovulation, decreased behavioral responsiveness to exogenous ovarian hormones, and masculinization of external morphological features (Clemens et al, 1978; Hart, 1977). Since rodent AFP avidly binds estrogens, it is suggested that AFP acts as estrogen scavenger and protects the developing female rodent brain against the effects of high concentration of circulating maternal estrogens (Trojan and Uriel, 1979; Attardi and Ruoslahti, 1976; Plapinger and McEwen, 1975; Plapinger et al, 1973). This concept is further supported by the observation that 'masculinization or neonatal androgenization' can also be brought about by the neutralization of neonatal brain AFP by its homologous antibody (Miczewski et al, 1980). However, such a function may not operate in human fetal brain where AFP is nonestrophilic in nature as observed in the present study. Induction of AFP in adult rats does not affect serum E₂ concentration indicating that AFP is not directly related with circulatory E₂ level (Aussel et al, 1980). These observations are compelling to consider some more general role for AFP. Recently, it was demonstrated that AFP inhibits the
growth of $E_2$ sensitive cells (Soto and Sonnenschein, 1980). It is possible that AFP plays a role in determining the relative growth rate of brain cell types - a phenomenon crucial to brain differentiation.

The present observations reveal that skin AFP level are high during first two weeks of postnatal life (anagen phase of hair growth). Skin is not only sensitive to steroid hormones but also capable of transforming them to an active form which in turn influences cellular activities (Rampini et al, 1971). Administration of exogenous estradiol inhibits hair growth during this period by modulating growth rate and increasing duration of telophase (resting phase) (Davis, 1963). It is possible that AFP acts as $E_2$ trap for excess of circulating estrogens and probably protects developing rat skin from the premature growth inhibitory action of estrogens.

Overwhelming evidence supports that most of the mammalian species (human, guinea pig, hamster, bovine, rabbit and chicken), unlike rodents (rat and mouse), are devoid of demonstrable estrogen binding (Ruoslahti and Soppala, 1979; Nunez et al, 1979; Uriel, 1979; Kiang et al, 1978; Swartz and Soloff, 1975; Savu et al, 1974). It would thus appear that estradiol mediated role proposed for AFP may not be applicable to species having nonestrophilic AFP.
Developmental profile of AFP in rat and human brain is different. Human fetal brain AFP disappears during fetal life while it persists in rat, at least, until 7 d of postnatal life. The critical hypothalamic differentiation is reported to occur both in rat (Toran-Allerand, 1976) and human (Dorner, 1978) when fetal brain AFP level is high. It is, therefore, tempting to speculate that species specific appearance of AFP has some, as yet unidentified, role in the development of mammalian brain.

Human and rat AFP can bind a variety of small molecular weight ligands (fatty acids, bilirubin, deoxycholate, Cu^{++} and Ni^{++} etc.) (Ruoslahti and Seppala, 1979). It may, therefore, mediate or modulate the intracellular transport of these biologically active substances in various developing tissues. This property of AFP may play an important role in differentiation and detoxification during development.
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SUMMARY & CONCLUSION
Ontogeny of tissue specific protein markers provides useful experimental model for investigating regulation of gene expression during differentiation. Alpha fetoprotein and albumin are important specific differentiation markers and they bear a reciprocal relationship during ontogeny. The following lines summarize the results obtained regarding ontogeny of AFP and albumin in different tissues of rat as well as developmental profile of AFP in human brain.

AN IMPROVED PROCEDURE FOR ISOLATION OF AFP

A direct negative immunoabsorption procedure was developed to isolate homogeneous preparation of rat and human AFP from amniotic fluid. It requires no more than two steps: (a) removal of adult serum proteins present in amniotic fluid by a direct negative immunoabsorption (immunoprecipitation) with rabbit anti-normal adult rat or human serum IgG, and (b) removal of excess of immunoglobulins using DEAE-cellulose chromatography. Purified AFP was characterized by double immunodiffusion, immunoelectrophoresis, crossed immunoelectrophoresis, PAGE, SDS-PAGE, Con-A affinity chromatography, and estradiol binding. Rat AFP has $K_a = 1.4 \times 10^8 \text{ M}^{-1}$ for estradiol-17β with one binding site for estradiol per mole of AFP.

Isolation of homogeneous AFP by a direct negative immunoabsorption is a significant improvement over the time consuming, multistep procedures reported for AFP. In
addition, the present procedure did not require specific antiserum against AFP and employed very gentle isolating conditions. The procedure yielded 87% recovery of AFP.

**ONTOGENY AND DISTRIBUTION OF AFP IN FETO-NEONATAL TISSUES OF RAT AND HUMAN FETAL BRAIN**

Most of the feto-neonatal tissues, except thymus, spleen and pancreas, of rat were found to contain elevated levels of AFP. High levels of AFP observed during fetal life in various tissues registered a decline with development. Ontogeny of AFP in these tissues was different from that observed in serum. AFP disappears after 7 d of postnatal life (P) in brain and adrenals; 14 P in lung, heart, stomach, intestinal and kidney; 21 P in liver and skin. Determination of heme concentration in these tissue extract revealed that contamination of AFP from fetal blood could not account for more than 5-10% of the observed levels of AFP in various feto-neonatal tissues of rat.

AFP present in various feto-neonatal tissues of rat was immunologically identical to serum AFP. AFP from serum, brain, skin and kidney also displayed a similar electrophoretic mobility on 10% PAGE and IEP. Estradiol binding assay of AFP in brain and skin gave an association constant, $K_a = 1.3 \times 10^8 \text{ M}^{-1}$ and $1.0 \times 10^8 \text{ M}^{-1}$. However, brain and skin cytosols from newborn rat were found to
differ in the relative amounts of Con A-non reactive AFP variant and contained 37% and 42% of this variant respectively. In view of these observations it appears that AFP in various feto-neonatal tissues of rat does not entirely represent fetal blood contamination and probably arises as a result of its independent synthesis in each tissue.

Studies on the distribution of AFP in feto-neonatal rat brain have revealed that it is distributed uniformly throughout the brain. Ontogeny of AFP in olfactory region, cerebrum, cerebellum and rest portions of the rat brain was similar to that in whole brain and it disappeared in these discrete regions after 7 d of postnatal development.

Presence of AFP was also demonstrated in human fetal brain. It was immunologically and electrophoretically identical to human fetal serum AFP. Developmental profile of AFP in human brain was distinct from that observed in serum compartment. It registered a peak level in brain at 20th week and declined rapidly to low levels after 24th week of gestation. It was absent (as measured by rocket assay) in the human fetal brain of subsequent ages. Unlike rodent AFP and like human AFP, it did not interact with estradiol. Brain AFP does not arise from fetal blood contamination since the blood contamination could account for traces of the AFP present in brain. Further ontogeny or AFP in brain is distinct from that observed in serum.
DEVELOPMENTAL CHANGES IN AFP AND ALBUMIN CONTENT IN SERUM OF RAT

Developmental profile of serum AFP exhibited an inverse relationship with age during postnatal life of rat. Serum AFP level declined after birth to 2.4% of the level observed in newborn rat, at 21st day of postnatal life. AFP could not be detected in sera of 28 d old rats by the present procedure. In contrast serum albumin elicited an increase by 37% during the same period. The albumin level rose in serum continuously to adult value of 37.5 mg ml⁻¹ in 9 weeks old rat.

SYNTHESIS AND SECRETION OF AFP AND ALBUMIN BY ISOLATED HEPATOCYTES IN CULTURE: CORRELATION OF SERUM AFP AND ALBUMIN PROFILES WITH THE LIVER CONTENT AND THEIR RATE OF SYNTHESIS IN THE HEPATOCYTES DURING DEVELOPMENT

AFP and albumin are synthesized in the hepatocyte and secreted into the serum. Serum level of these proteins undergoes striking change during fetoneonatal development.

Hepatocytes prepared from rat liver at various stages of development (18, 20 E and 1, 7, 14, 21, 28, 35 P and adult) by collagenase digestion procedure were maintained in short term (24 hrs) culture. Rate of synthesis and secretion of AFP and albumin were determined by following the incorporation of [¹⁴C]-leucine into immunoprecipitable AFP and albumin by the hepatocytes cultured in leucine-free medium. Hepatocytes from newborn rat synthesized AFP at a
very high rate accounting for about 85% of the total protein synthesis while albumin synthesis was only 9%.
Rate of AFP synthesis and secretion declined with the maturation of the hepatocytes. Hepatocytes from 21 d old rat demonstrated only 3% of the rate observed in fetal hepatocyte from 18 E rat. No synthesis of AFP could be demonstrated in the hepatocytes prepared from 4 week old rats. Serum AFP level and its content in liver also reduced to 1.3% and 4% respectively in 3 week old rat when compared to the values observed in 18 E fetal rats. It appears that age related change in serum AFP level and its content in liver is governed by the rate of AFP synthesis and secretion by the developing hepatocytes.

Maturation of the hepatocyte was accompanied by a marked increase in the rate of albumin synthesis, secretion and its intracellular content. Ratios of the rates of albumin to AFP synthesis in the hepatocytes from 18 E fetus and 1, 7, 14, 21, 28 d old neonatal rats were 0.05, 0.10, 0.28, 3.5 and 48 respectively. It would thus appear that an increase in serum albumin level and its intrahepatic content is due to a proportionate increase in the rate of albumin synthesis and secretion in the developing rat liver.

It may be concluded that differentiation and maturation of the hepatocyte is accompanied by a sequential inactivation of AFP and activation of albumin gene expression.
DEMONSTRATION OF AFP AND ALBUMIN SYNTHESIS IN DEVELOPING RAT BRAIN AND SKIN; CORRELATION OF TISSUE CONTENT OF AFP AND ALBUMIN WITH THEIR RATE OF SYNTHESIS

Dissociated mammalian brain cells and skin explants in primary cultures offers a useful system to investigate the biochemical events accompanying differentiation and growth of these tissues. Dissociated brain cells and skin explant cultures from newborn rat were used to answer the following questions: Is estradiol binding protein in developing rat brain and skin AFP? If so, where does this protein originate from?

Our observations, derived from $[^{14}\text{C}]$-leucine incorporation into immunoprecipitable AFP and albumin by dissociated brain cells and skin explant maintained in short term cultures, have provided evidence for the synthesis of AFP and albumin in brain and skin of newborn rat. Cycloheximide inhibited the linear incorporation of $[^{14}\text{C}]$-leucine into immunoprecipitable AFP and albumin as a function of time by these cultures. It strongly suggests that $[^{14}\text{C}]$-labelled AFP and albumin observed in brain cells and skin lysates (synthesis) and in medium (secretion) are constantly synthesized and secreted by these cultures. In addition, the intracellular content of both AFP and albumin in brain cells did not change significantly during the whole culture period. Moreover, these cells elicited a linear
accumulation (secretion) of the quantitable amounts of AFP and albumin into the medium. Total amounts of AFP and albumin secreted by brain cells were far in excess of their intracellular content at the initiation of the cultures. In view of these observations it was concluded that developing brain and skin represent an independent pool of AFP and albumin.

AFP and albumin secreted by newborn rat brain cells and skin explants cultures was immunologically, electrophoretically and with regards to molecular weights and estradiol binding properties similar to serum AFP and albumin. It may be concluded that genes for AFP and albumin in developing brain and for AFP in skin are also expressed these tissues.

Rates of synthesis of AFP and albumin during fetal-neonatal development of rat brain showed a continuous decline and their synthesis seems to be turned off after 7 d of postnatal life. High levels of these protein in fetal brain were due to their high rates of synthesis. Age related decrease in brain AFP and albumin reflected a proportionate decrease in the rates at which they are synthesized in this tissue.

Rate of synthesis of AFP in skin also decreased with increasing age and its synthesis is turned off after 14 d of postnatal life. A decrease in the skin AFP level can be accounted for by corresponding decline in the rate of its
synthesis in the developing skin. It may be concluded that intracellular levels of AFP and albumin in rat brain and AFP in rat skin reflect their rate of synthesis in those tissues. It is tempting to speculate, at this stage, that human brain also synthesize AFP.

**EXPRESSION OF AFP AND ALBUMIN GENES IN THE DEVELOPING RAT LIVER, BRAIN, AND SKIN: A COMPARATIVE STUDY**

A comparative study of the patterns of AFP synthesis during development revealed that it is turned off in rat brain, skin and liver after 7, 14, 21 d of postnatal development. The rate of AFP synthesis in newborn rat liver, brain and skin exhibited a ratio of 19:2:1. Albumin synthesis in the brain is turned off after 1 week of postnatal life while liver shows a continuous increase in the rate of albumin synthesis throughout postnatal development and attains adult basal level by about 8–9 weeks after birth. On the other hand, albumin is not synthesized in the skin. Rate of the albumin synthesis in liver; brain in 18 E, 1 P and 7 P were 1, 2 and 16. It appears that genes for AFP and albumin are expressed in developing rat liver, brain and skin and probably regulated independently depending upon the functional requirement of these proteins in the developing tissues.
HORMONAL CONTROL OF AFP AND ALBUMIN SYNTHESIS IN NEWBORN RAT LIVER, BRAIN AND SKIN

Effect of various hormones on the rate of AFP and albumin synthesis in the hepatocytes, brain cells and skin explant from newborn rats, was studied to gain some insight into ontogeny related regulation of synthesis of these proteins in rat tissues.

Dexamethasone was the most effective in inhibiting the rate of AFP synthesis and secretion in vitro cultures of hepatocytes (-45%), brain cells (-40%) and skin explant (-25%). It also caused an accelerated decrease in serum AFP level in vivo in newborn rats. dbcAMP also decreased the rate of synthesis and secretion of AFP in brain cells (-15%), hepatocytes (-7%) and skin explants (-4%). It also suppressed the serum AFP level in vivo in newborn rats supporting its in vitro effect.

Insulin exhibited a dual effect on the rate of AFP synthesis in various tissues. It increased AFP synthesis in hepatocytes (+21%) and demonstrated an opposite effect in brain cell (-15%) and skin explant (-4%). However, it did not affect serum AFP levels of newborn rats in vivo.

Neither did estradiol significantly affect the rate of AFP synthesis in various tissues in vitro nor did it change serum AFP level in vivo. The effects of dexamethasone, dbcAMP, insulin and estradiol on the rate of albumin
It appears that effects of these hormones are specific for AFP synthesis.

It appears that expression of AFP gene is a function of undifferentiated or less differentiated stem cells or embryonal cells. Different embryonal cell types with diverse ontogenic history do indeed synthesize AFP although the extent of synthesis is different. Thus expression of AFP and albumin genes during development offer a useful model for investigating molecular mechanism underlying tissue differentiation.