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, N\(^6\), O\(^2\), dibutyryl 3',5'-cyclic adenosine monophosphoric acid (dbcAMP), dexamethasone, estrone (E\(_1\)), estradiol-17\(\beta\) (E\(_2\)), diethyl-stilboesterol (DES) sodium dodecyl sulphate (SDS) and \(\beta\)-mercaptoethanol were procured from Sigma Chemical Company, St. Louis, MO, USA. Agarose, commassie brilliant blue, 4-(2-hydroxyethyl)-1-piperazine-N'-2-ethane sulphonic 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 -10B 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 reagent 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 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 μmol/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-Cl, 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 lyophillized. 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 bled 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-HAF*.

**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 [\(^3\)H]-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 [\(^3\)H]-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 E2 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 CYANOGEN 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 scrapping 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 $-20^\circ$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$^\circ$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 Na₂HPO₄, 0.22 mM KH₂PO₄, 5.5 mM
glucose, 59.0 mM sucrose, pH 7.2). Each brain was care­
fully cleaned of pia matter and blood vessels, washed
twice with isotonic buffer and minced to about 1-2 cubic
mm pieces with fine scissor. 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 μg ml⁻¹ penicillin, 100 μg ml⁻¹ streptomycin,
and 0.25 μg 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 corium side down, and freed of the subcutaneous tissues by scratching 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/ml 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 $^{14}\text{C}]$-LEUCINE INTO AFP AND ALBUMIN BY RAT BRAIN CELLS, HEPATOCYTES, AND SKIN EXPLANT CULTURES

Incorporation of $^{14}\text{C}]$-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.