Biochem. J. (1979) **180**, 153–160
Printed in Great Britain

Hepatocyte Differentiation in Culture

APPEARANCE OF TYROSINE AMINOTRANSFERASE

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(*Received* 18 *August* 1978)

Liver of rat foetuses from 14 to 19 days of gestation and cultured hepatocytes derived from foetuses of 14 or 15 days gestation show a limited capacity to transaminate tyrosine. This low tyrosine transamination activity can be ascribed to aspartate aminotransferase. Definitive tyrosine aminotransferase can be demonstrated in 1-day-old cultures of hepatocytes taken from 19-day foetuses, but not from 15-day foetuses. However, after 3 days of culture hepatocytes from 15-day foetuses are able to synthesize tyrosine aminotransferase. Induction studies reveal that dexamethasone is capable of increasing tyrosine aminotransferase activity once it is detectable in culture.

Tyrosine aminotransferase (EC 2.6.1.5), an enzyme that is subject to hormonal controls, has been extensively studied as a model for the control of gene expression (Kenney, 1970; Greengard, 1970; Sereni & Sereni, 1971). From a developmental viewpoint it is considered to be an enzyme which appears postnatally (Greengard, 1970). However, since it can be induced prematurely by administration of glucagon or triamcinalone *in utero* (Greengard, 1969; Yeung *et al.*, 1967) or by premature delivery of the foetus (Holt & Oliver, 1968) as well as by culturing explants of foetal liver (Sereni & Sereni, 1970), it must be concluded that foetal hepatocytes are capable of tyrosine aminotransferase synthesis. However, enzyme synthesis is suppressed in the foetus by a mechansim which is not yet defined. The available evidence indicates that a permissive environment, possibly a specific hormonal status, is a prerequisite for tyrosine aminotransferase production. The overriding factor which then dictates whether a given hepatocyte synthesizes the enzyme must be an event that renders the hepatocyte capable of utilizing that part of its genome that codes for the enzyme. This study was undertaken to determine (i) the developmental stage at which foetal rat hepatocytes undergo this putative differentiation event and (ii) whether this event can be shown to occur in culture.

Materials and Methods

Materials

Animals. Wistar albino rats were used. These animals have a gestation period of 22 days. Gestational age was determined from the time of detection of spermatozoa in the vaginal tract of females and is accurate to ± 8h.

Chemicals. L-[3,5-^{3}H]Tyrosine was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. L-Tyrosine and sodium diethyl dithiocarbamate were from Merck, Darmstadt, Germany. Pyridoxal 5-phosphate, 2-oxoglutarate, dithiothreitol, NADH and 2,5-diphenyloxazole were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Collagenase (grade II) was from Boehringer Mannheim, Mt. Waverley, Victoria, Australia. 2,4-Dinitrophenylhydrazine and L-aspartate were from Ajax Chemicals, Sydney, Australia; hydroxyapatite was supplied by Bio-Rad Laboratories, Richmond, CA, U.S.A., CM-Sephadex (C-50) by Pharmacia Fine Chemicals, Uppsala, Sweden, and diaminobenzoic acid by Eastman Kodak Co., Rochester, NY, U.S.A. Cell culture reagents, Fungizone, penicillin/streptomycin and glutamine were obtained from Grand Island Biological Co., Grand Island, NY, U.S.A. Eagle's Minimal Essential Medium and foetal calf serum were from Flow Laboratories, Annandale, N.S.W., Australia, and horse serum was from Microbiological Media Makers of Australia, Melbourne, Australia.

Methods

Hepatocyte isolation. Foetal rats were delivered by Caesarian section, livers were removed aseptically and rinsed free of blood in balanced salts solution (Hanks & Wallace, 1949) and dried on sterile paper tissues. Livers from 17-, 18-, 19- and 20-day-foetal

rats were chopped on a Mickle Chopper (Mickle Laboratories Engineering Co., Gomshall, Surrey, U.K.) set at 0.1mm by using two traverses, one perpendicular to the other. The pieces were then placed in balanced salts solution containing 0.5mg of collagenase/ml. About 500mg of liver/ml of collagenase solution was used. The flask was then gassed with O_2/CO_2 (19:1) and incubated with shaking at 37°C for 15min. An equal volume of balanced salts solution was then added to this mixture, which was filtered through cheesecloth. The cells were pelleted and washed twice in balanced salts solution by centrifugation at 50*g* for 2min. Finally the cells were suspended (approx. 0.5ml of packed cells/10ml of medium) in modified Eagle's minimal essential medium supplemented with 10% foetal calf serum, glutamine (2.4mM final concentration), Fungizone (28μg/ml) and penicillin/streptomycin (57units/ml and 570μg/ml respectively). Cells were then plated in 90mm-diameter plastic culture dishes (Sterillin Products, Teddington, Middx., U.K.) previously coated with collagen, in a volume of 9ml. Hepatocytes from 15- and 16-day-foetal rats were initially prepared by collagenase treatment. However, very poor cell yields were obtained. Subsequently these livers were disaggregated by dispersion by using a Pasteur pipette and then processed in the same manner as for collagenase-disaggregated liver from 17–20-day-foetal rats. This method provides a good yield of cells with enzyme activities and rates of total protein, albumin and transferrin synthesis similar to those of collagenase-isolated hepatocytes. The isolation of 17-, 18- and 19-day-foetal hepatocytes was also attempted by using Pasteur-pipette dispersion. This gave poor yields of cells, although the characteristics of the cells which attached to the collagen substrate overnight were similar to those isolated by using collagenase. Optimum yields of cells could thus be obtained by using collagenase for 17-, 18- and 19-day-foetal liver and by Pasteur-pipette dispersion for 15- and 16-day-foetal liver.

Culture conditions. Cultures were given fresh medium 24h after plating and every 48h thereafter. The initial removal of medium results in the removal of most of the erythropoietic cells which do not attach to the substrate, leaving behind a population consisting primarily of liver parenchymal cells. Cultures were maintained at 37°C in a water-saturated atmosphere of 5% CO_2 in air.

Preparation of extracts. (*a*) Whole liver. Liver tissue was homogenized in 0.1M-potassium phosphate buffer, pH7.4, containing pyridoxal 5-phosphate (0.3mM), dithiothreitol (1mM), 2-oxoglutarate (0.5mM) and EDTA (1mM). Where foetal rats were used, livers from a number of litters were pooled.

(*b*) Cultured cells. Cells were harvested by using a Teflon scraper and suspended in balanced salts solution. A cellular pellet was obtained by centrifugation at 1600*g* for 2min in a bench centrifuge. The pellet was resuspended in the buffer (0.7ml) used in (*a*).

Homogenates and cell suspensions were sonicated in a Branson model L5 75 sonifier (Branson Instruments, Danbury, CT, U.S.A.) at 2A for 15s (microtip). A sample of the sonicated preparation was taken for determinations of DNA content. Sonicated preparations were centrifuged in a Beckman model L5-65 ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.) at 300000$g_{max.}$ for 20min in the SW50.1 rotor at 4°C. For smaller volumes (less than 200μl) of extract, samples were centrifuged in a Beckman Airfuge at approx. 210kPa for 12min in the A-100 rotor.

Preparation of immune antisera. Rabbit antiserum to rat tyrosine aminotransferase was obtained by immunization of rabbits with enzyme purified by the method of Valeriote *et al.* (1969). Rabbits received 0.5mg of purified enzyme mixed with 1ml of Freund's adjuvant (complete) every 2 weeks for 6 weeks and were bled on the seventh week. A further 0.5mg of purified enzyme was injected on the seventh week and the rabbits were bled again after 1 week. The purity of the enzyme preparation was checked by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and the specificity of the antiserum was verified by immunoelectrophoresis.

Antibody treatment. Rabbit antibody to tyrosine aminotransferase, diluted in Tris/HCl buffer (0.1M, pH7.4), was added to an equal volume of the test solution. The mixture was incubated at 37°C for 30min, then centrifuged in an International B-20 refrigerated centrifuge (International Equipment Co., Boston, MA, U.S.A.) at 9000*g* for 40min at 4°C. The supernatant was assayed for tyrosine aminotransferase.

Chromatography. (*a*) Hydroxyapatite. Hydroxyapatite gel was equilibrated overnight in 0.1M-potassium phosphate buffer, pH6.9, then poured into columns (1cm×10cm). Liver cell supernatant was pumped on to the column with 15ml of 0.1M-phosphate, pH6.9, and eluted with a gravity-mixed linear gradient (70ml) from 0.1M- to 0.5M-phosphate, pH6.9. The enzyme was protected by the addition of dithiothreitol to the eluting buffers. The phosphate was pumped through the column at a flow rate of 1.4ml/min by using a peristaltic pump (Pharmacia). Fractions (1ml) were collected and assayed for aminotransferase activity.

(*b*) CM-Sephadex. CM-Sephadex C-50 (Pharmacia) was equilibrated overnight in 0.05M-potassium phosphate buffer, pH6.5, then poured into columns (0.7cm×3cm). Sonicated preparations in 0.05M-potassium phosphate buffer, pH6.5 (1:1, w/v), were incubated at 0°C for 45min. Extracts (100–200μl) were run into the column, then washed with 1ml of 0.05M-potassium phosphate buffer,

pH6.5, and left for 15min. Subsequently five 1ml fractions were eluted with 0.04M-KCl in 0.05M-potassium phosphate buffer, pH6.5. Then five 1ml fractions were eluted with 0.33M-KCl in 0.05M-potassium phosphate buffer, pH6.5. Each fraction was then assayed for aspartate aminotransferase, tyrosine aminotransferase and protein. Aspartate aminotransferase is eluted primarily in the low-salt (0.04M-KCl) buffer fractions and tyrosine aminotransferase in the high-salt (0.33M-KCl) fractions. In some experiments the eluates were concentrated 2-fold by using Millipore concentrators (Millipore Corp., Bedford, MA, U.S.A.).

Protein determination. Protein was measured by the method of Lowry *et al.* (1951), with bovine serum albumin (Sigma) as a standard.

DNA determination. DNA was measured by the method of Hinegardner (1971). Samples of sonicated cell preparations (25–50μl) were dried overnight at 50°C, then redissolved in 100μl of diaminobenzoic acid in water (0.4g/ml). This mixture was heated at 50°C for 40min, then 1M-HCl (1.5ml) was added with mixing. The fluorescence was measured with a fluorimeter (G. K. Turner and Associates, Palo Alto, CA, U.S.A.) with type 405 primary filter and combined 2A-12 and 65A secondary filters. Highly polymerized calf thymus DNA (Sigma) was used as a standard.

Enzme assays. (*a*) Aspartate aminotransferase. Aspartate aminotransferase activity was assayed by using a modification of the method of Sizer & Jenkins (1962). The reaction mixture contained 66μmol of potassium phosphate, pH7.4, 2.2μg of NADH (0.3μmol), 6.7μmol of 2-oxoglutarate, 50μl of malate dehydrogenase preparation and soluble aspartate aminotransferase in a volume of 1ml. The malate dehydrogenase preparation consisted of 20μl of enzyme (Boehringer Mannheim; 25mg/ml)/ml of 1% bovine serum albumin. The A_{340} of the mixture was measured to ensure that oxidation of NADH was not occurring in the absence of aspartate. The assay was initiated by addition of 33μmol of L-aspartate and the activity determined by measuring the decrease in A_{340} at 37°C. The assay was linear with time for 5min and with respect to enzyme concentration. One unit of enzyme activity is that which oxidizes 1μmol of NADH/min at 37°C.

(*b*) Tyrosine aminotransferase. This was measured by a modification of the methods of Miller & Thompson (1972) and Lees & Weiner (1975). The initial mixture contained 2-oxoglutarate (5mM), pyridoxal 5-phosphate (0.13mM), diethyl dithiocarbamate (53mM) and L-tyrosine (3mM), in 0.1M-potassium phosphate buffer, pH7.4. L-[3,5-^{3}H]-Tyrosine (approx. 1μCi) was added to 1ml of this mixture. A sample (100μl) was added to 10ml of a scintillant solution consisting of naphthalene (180g) and 2,5-diphenyloxazole (4g) per litre of dioxan to determine the radioactivity. The reaction mixture (100μl) was preincubated at 37°C in a shaking water bath and the reaction started by addition of enzyme. 2,4-Dinitrophenylhydrazine (15mM) in H_2SO_4 (1M) was used to stop the reaction after 60min. Blanks were prepared by addition of 2,4-dinitrophenylhydrazine before the addition of enzyme. The product was extracted into 1ml of toluene/ethyl acetate (10:1, v/v) and the solution centrifuged at 1600*g* for 15min. The organic phase (0.7ml) was added to 0.3M-HCl (0.5ml), mixed and centrifuged as above. The radioactivity of the organic phase was determined in an Isocap 300 liquid-scintillation counter (Nuclear–Chicago) by using a scintillant containing 2,5-diphenyloxazole (6g) and ethanol (0.5ml) per litre of toluene. The counting efficiency was 40%. The assay was linear with time for 90min of incubation and with respect to enzyme concentration. Tyrosine aminotransferase was calculated as nmol of *p*-hydroxyphenylpyruvate/h per μg of DNA.

Results

Table 1 shows the activity of tyrosine aminotransferase in 1-day-old cultures of liver cells obtained from rat foetuses varying from 14 days to 20 days gestation. The enzyme activity in cultures supplemented with the steroid analogue dexamethasone is also presented. The data indicate that tyrosine

Table 1. *Tyrosine aminotransferase activity in cultured hepatocytes obtained from foetuses of various gestational ages*

Livers from rat foetuses at various gestational ages were cultured for 1 day in normal medium (control) or medium supplemented with dexamethasone (20μM). Cells were prepared and cultured by the procedures described in the Materials and Methods section. The results are the means of tyrosine aminotransferase activity determined in two independent cultures for each age group. The separate values agreed to within 5% for foetuses less than 18 days of age, whereas for older foetuses the agreement was within 15%. A unit of tyrosine aminotransferase activity is defined as the amount of enzyme that produces 1nmol of *p*-hydroxyphenylpyruvate/h at 37°C.

Foetal age (days)	Tyrosine aminotransferase activity (units/μg of DNA)	
	Control	+ Dexamethasone
14	0.05	0.05
15	0.06	0.07
16	0.07	0.06
17	0.06	0.15
18	0.12	0.18
19	0.26	0.56
20	0.37	0.51

aminotransferase is present in the livers of the entire age group, though its activity is very low in cultures from 17-day foetuses or earlier animals. However, the enzyme is only inducible in livers obtained from 17-day foetuses or from older animals.

When assays were performed on freshly isolated foetal rat liver for a range of gestational ages, the activity of tyrosine aminotransferase was comparable with that found in 1-day-cultured liver cells taken from foetuses of the same age. Fig. 1 is a summary of results obtained when liver tyrosine aminotransferase of extracts derived from 15-day and 19-day foetuses and from adult rats are chromatographed on hydroxyapatite by the procedure described in the Materials and Methods section. The profiles reveal that a peak of activity from both foetal age groups corresponds to form III (according to the terminology of Iwasaki & Pitot, 1971). The apparent activity that is eluted before the peak is due to the blank value of the radiochemical assay, which was not subtracted. The adult enzyme is resolved into three forms, II, III and IV. The elution profile of aspartate aminotransferase was also determined for the foetal extracts, and the major peak was seen to co-migrate with peak III. When extracts from 15-day-foetal liver cultured for 3 days and a similar culture exposed to dexamethasone were analysed (Figs. 2*a* and 2*b*), it was apparent that form IV of the enzyme appears. The presence of dexamethasone enhances the activity of this form, whereas the activity of form III remains unaltered.

The result of treating extracts from 15-day-foetal and 1-day-postnatal liver with antiserum raised against purified rat liver tyrosine aminotransferase is shown in Table 2. Although the antiserum appreciably lowers the activity of assayable tyrosine aminotransferase in extracts from postnatal liver, it does

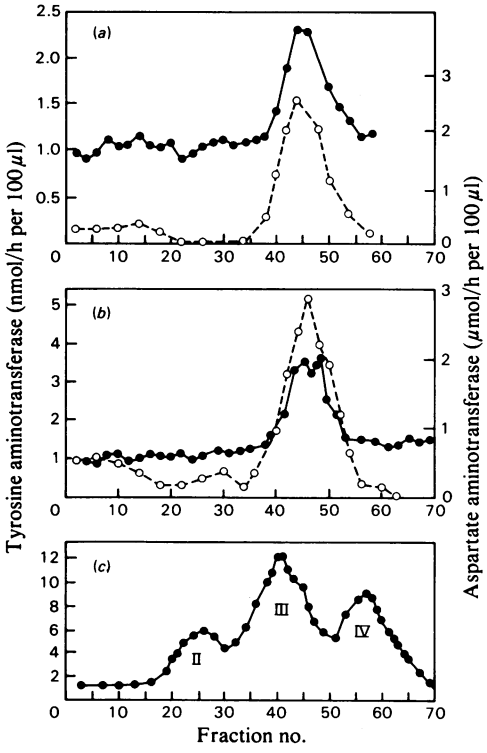

Fig. 1. *Hydroxyapatite chromatography of liver extracts from (a) 15-day and (b) 19-day foetuses and (c) adult rat*

Liver from 15-day and 19-day foetuses and from adult rats was homogenized in 1 vol. of buffer, sonicated and centrifuged as described in the Materials and Methods section. Supernatant (400 μl) was applied to hydroxyapatite (4.5 g) by using 15 ml of 0.1 M-phosphate buffer, pH 6.9, containing dithiothreitol. Enzyme was eluted with a gradient from 0.1 M- to 0.5 M-phosphate, pH 6.9. Fractions (1 ml) were assayed for tyrosine aminotransferase and aspartate aminotransferase. Tyrosine aminotransferase activity (●) is expressed as nmol of *p*-hydroxyphenylpyruvate formed/h per 100 μl of fraction. Aspartate aminotransferase activity (○) is expressed as nmol of NADH oxidized/h per 100 μl of fraction.

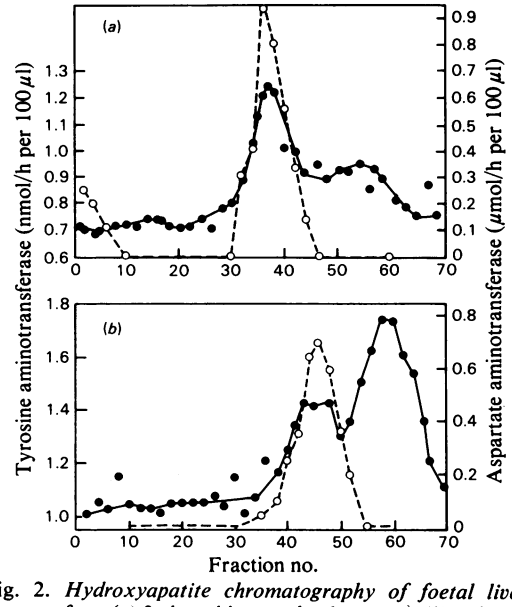

Fig. 2. *Hydroxyapatite chromatography of foetal liver extracts from (a) 3-day-old control cultures and (b) cultures exposed to dexamethasone*

Extracts were prepared from liver of 15-day foetuses after 3 days culture. See Fig. 1 legend for other details. Dexamethasone (20 μM) in propylene glycol was added to the medium 18 h before assay. Control cultures received an equal volume of propylene glycol (100 μl).

Table 2. *Effect of antibody on tyrosine aminotransferase from 15-day-foetal liver, 15-day-foetal liver cultured for 3 days and 1-day-postnatal liver*

Livers were homogenized in 1 vol. of buffer, sonicated and centrifuged as described in the Materials and Methods section. Supernatant was treated with either antibody or an equal volume of 0.1 M-Tris/HCl buffer, pH 7.4, incubated for 30 min at 37°C, then centrifuged at 10000*g* for 40 min. The final supernatant was assayed for tyrosine aminotransferase activity, which is expressed as nmol of *p*-hydroxyphenylpyruvate formed/h per μg of DNA, and presented as the means ± S.E.M. obtained from the numbers of determinations shown in parentheses. The decrease in activity after antibody treatment is expressed as a percentage of the activity in the untreated preparation.

Source of extract	Tyrosine aminotransferase activity (nmol/h per μg of DNA) Control	+Antibody	Difference (%)
15-day-foetal liver	0.036 ± 0.003 (4)	0.040 ± 0.005 (4)	+11
15-day-foetal liver cultured for 3 days	0.85 ± 0.23 (3)	0.32 ± 0.07 (3)	−62
1-day-postnatal liver	3.49 ± 1.13 (3)	0.34 ± 0.12 (3)	−90

Table 3. *Effect of aspartate on tyrosine aminotransferase activity in 15-day-foetal liver and adult liver*

Supernatants were prepared from 15-day-foetal (1 vol. of buffer) and from adult liver (8 vol. of buffer) as described in the Materials and Methods section, then assayed for tyrosine aminotransferase in the presence and absence of L-aspartate. For aspartate-treated assays, L-aspartate (3 mM) was added to the tyrosine aminotransferase assay mixture before the addition of enzyme. Aspartate was added to the control assays after the addition of 2,4-dinitrophenylhydrazine and before the toluene/ethyl acetate extraction. Tyrosine aminotransferase activity is expressed as nmol of *p*-hydroxyphenylpyruvate formed/h per μg of DNA, and presented as the means ± S.E.M. obtained from four determinations.

Age	Tyrosine aminotransferase activity (nmol/h per μg of DNA) − Aspartate	+ Aspartate	Decrease in activity (%)
15-day foetal	0.074 ± 0.02	0.016 ± 0.001	78
Adult	5.96 ± 0.94	5.31 ± 0.85	11

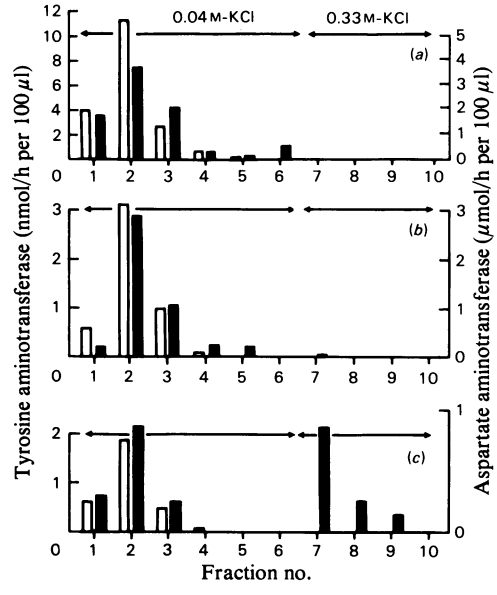

Fig. 3. *CM-Sephadex chromatography of extracts from (a) 15-day- and (b) 19-day-foetal rat liver and (c) 3-day-old cultured hepatocytes from 15-day-foetal rats*

Extracts were prepared in 0.05 M-potassium phosphate buffer, pH 6.5, and chromatographed as described in the Materials and Methods section. Tyrosine aminotransferase activity (filled bars) is expressed as nmol of *p*-hydroxyphenylpyruvate formed/h per 100 μl of fraction. Aspartate aminotransferase (open bars) is expressed as μmol of NADH oxidized/h per 100 μl of fraction.

not react with similarly prepared extracts from foetal liver. However, the addition of antiserum to extracts of hepatocytes cultured from 15-day-foetal rats for 3 days appreciably lowers the amount of tyrosine aminotransferase.

Table 3 summarizes the results obtained from experiments designed to test the effect of adding aspartate (equimolar relative to tyrosine) on the activity of tyrosine aminotransferase in extracts derived from 15-day-foetal and adult rat liver. The data show that whereas the activity in the adult is marginally altered by the presence of aspartate in the assay (11% decrease), the activity measured in extracts from 15-day-foetal liver is decreased by 78%.

By using CM-Sephadex chromatography it is possible to separate the tyrosine aminotransferase activity from the aspartate aminotransferase activity. Fig. 3(*c*) shows the tyrosine aminotransferase and aspartate aminotransferase profiles obtained when

extracts from hepatocytes cultured from 15-day-foetal rats for 3 days are chromatographed. These data confirm what was suggested by the experiments involving hydroxyapatite chromatography and antibody treatment of these extracts, i.e. that after 3 days in culture definitive tyrosine aminotransferase is synthesized by hepatocytes derived from 15-day-foetal rat liver. This technique confirms that more than 99% of the tyrosine aminotransferase activity in 15-day- and 19-day-foetal rat liver (Figs. 3*a* and 3*b*) is aspartate aminotransferase activity. About 99% of the tyrosine aminotransferase activity from foetal extracts is recovered in the aspartate aminotransferase fractions (peak I, fractions 1–3), whereas less than 1% of the activity appears in the tyrosine aminotransferase fractions (peak II, fractions 7–9). When peak-I enzyme is assayed in the presence of equimolar aspartate for transamination of tyrosine, the activity is decreased by more than 90%. Under similar conditions the activity in peak II is decreased by only 8%.

Table 4 summarizes the data obtained when authentic tyrosine aminotransferase (separated from aspartate aminotransferase by CM-Sephadex chromatography) is assayed in extracts derived from 15-day- and 19-day-foetal liver before culture and after culture for 1 day and 3 days. With both age groups no tyrosine aminotransferase can be detected initially. After 1 day of culture hepatocytes from 15-day foetuses still do not display any enzyme activity. This result was obtained in spite of doubling the volume of extract applied to the column as well as doubling the concentration of the eluate (see the Materials and Methods section). In contrast, significant amounts of enzyme appear in 1-day-old cultures derived from 19-day-foetal rats. By 3 days both sets of cultures accumulate an appreciable amount of enzyme. In 1-day-old cultures derived from 19-day foetuses, the presence of dexamethasone at 0.2nM greatly enhances the activity of the enzyme (9-fold induction). In contrast the steroid fails to cause appearance of the enzyme in 1-day-old culture derived from 15-day foetuses. When both sets of cultures are 3 days old the untreated cultures contain tyrosine aminotransferase and the activity can be markedly increased when the cultures are treated with dexamethasone.

Table 4. *Tyrosine aminotransferase activity in hepatocytes derived from 15-day- and 19-day-foetal liver on culture days 0, 1 and 3 in the absence and presence of dexamethasone*

Day-0 values are for freshly isolated livers or freshly prepared cells. Extracts are prepared and chromatographed on CM-Sephadex as described in the Materials and Methods section. Dexamethasone was used at a concentration of 0.2nM. Data are presented as the means ± S.E.M. and numbers of determinations are given in parentheses. N.D. indicates not detectable by assay used.

Foetal age	Time in culture (days)	Tyrosine aminotransferase activity (units/mg of protein)	
		Control	+ Dexamethasone
15 days	0	N.D. (8)	—
	1	N.D. (8)	N.D. (8)
	3	58 ± 6 (4)	160 ± 32 (4)
19 days	0	N.D. (4)	—
	1	38 ± 3	328 ± 22 (4)
	3	64 ± 18 (4)	368 ± 74 (4)

Table 5. *Tyrosine aminotransferase activity in aspartate aminotransferase-free extracts from 1-day-old cultured hepatocytes*

Tyrosine aminotransferase activity is expressed as units of activity/mg of protein. Hepatocytes were prepared and cultured as described in the Materials and Methods section. Data are presented as means ± S.E.M. for the numbers of determinations shown in parentheses. N.D. indicates not detectable by assay used.

Foetal age (days)	Tyrosine aminotransferase activity (units/mg of protein)
15	N.D. (8)
16	17.2 ± 3.4 (4)
17	26.6 ± 4.0 (7)
18	28.4 ± 0.6 (4)
19	38 ± 3 (4)

Table 5 summarizes the data obtained when tyrosine aminotransferase is assayed after aspartate aminotransferase is removed from extracts of 1-day-old cultured hepatocytes derived from foetal rats of 15, 16, 17, 18 and 19 days gestation. The data reveal that by 17 days gestation the liver of the foetus has acquired the ability to synthesize tyrosine aminotransferase in culture.

Discussion

Previous experiments (Yeoh & Morgan, 1974) have indicated that the liver of rat foetuses of 13–14 days gestation is incapable of synthesizing transferrin, although significant albumin synthesis was detected. Transferrin synthesis could be demonstrated by 15–16 days gestation. There are several enzymes, notably tyrosine aminotransferase (Greengard, 1970) and phosphoenolpyruvate carboxylase (Oliver, 1974), which appear in rat liver postnatally. At even later ages, at about weaning, glucokinase accumulates in the liver (Walker & Holland, 1965). These observations suggest that liver cells may acquire the ability to synthesize various proteins in a sequential manner during development such that a lineage of hepatocytes could be identified (see Holtzer *et al.*, 1975). Equally possible is the proposal that the first hepatocytes in

the liver diverticulum, which evaginates from the embryonic gut at about 10.5 days gestation, are fully differentiated cells capable of synthesizing the entire range of liver proteins. The developmental stage at which specific liver proteins are synthesized, and in what quantity, may then be controlled by environmental factors such as hormones, cyclic nucleotides or other factors.

For tyrosine aminotransferase, some evidence suggests that the normal postnatal appearance of the enzyme does not coincide with the emergence of hepatocytes capable of making the enzyme. Spontaneous development of tyrosine aminotransferase activity occurs in rat foetal liver explants (Sereni & Sereni, 1970); premature delivery (Holt & Oliver, 1968, 1969) and administration of glucagon or triamcinalone *in utero* (Yeung *et al.*, 1967; Greengard, 1969) results in the precocious appearance of the enzyme. Two conclusions can be drawn from these findings: firstly, the liver of the near-term foetus has acquired the capacity to synthesize tyrosine aminotransferase; and secondly, the environment *in utero* does not permit synthesis of the enzyme. Therefore in order to establish at which stage the foetal liver becomes capable of tyrosine aminotransferase synthesis, it is necessary to maintain the cells in an environment that is conducive to synthesis of the enzyme. In the present study, this is achieved by the culture of foetal hepatocytes. To maximize tyrosine aminotransferase activity, medium was also supplemented with dexamethasone. Hepatocytes derived from foetuses of various gestational ages were then cultured under these conditions to determine the developmental stage at which synthesis of the enzyme could occur.

Surprisingly, this survey revealed that at all ages examined, i.e. between 14 and 20 days gestation, there was detectable enzyme activity (Table 1). Although the rate of conversion of tyrosine into *p*-hydroxyphenylpyruvate was extremely low in extracts of cultured hepatocytes from 14-, 15- and 16-day-old foetal rats, it was nevertheless linear with incubation time and significant compared with assay blanks. To establish that this activity was not due to an artifact of cell culture, fresh livers of comparable age were assayed and similar activities were found for the respective ages. In addition, where there was a low activity, it was not increased by culturing the cells in dexamethasone. In view of this, and the finding by Ohisalo & Pispa (1976) that in rat liver enzymes which transaminate tyrosine can be isoelectrically focused into four fractions, one of which corresponds to L-aspartate–2-oxoglutarate aminotransferase (EC 2.6.1.1), the possibility that the transamination of tyrosine observed in early-foetal liver was due to aspartate aminotransferase was investigated.

When extracts from 15-day-foetal liver were chromatographed on hydroxyapatite a single peak of activity was obtained which contained both tyrosine aminotransferase and aspartate aminotransferase. Treatment with antibody against adult-rat liver tyrosine aminotransferase did not decrease tyrosine transamination. The activity showed a marked preference for aspartate as substrate, since aspartate added at equimolar concentration with tyrosine markedly diminished tyrosine transamination. These facts strongly suggest that the transamination of tyrosine by early-foetal liver is due to aspartate aminotransferase.

In further experiments, it was shown that two activities were resolved by chromatography on CM-Sephadex. Competition experiments using aspartate revealed that one activity is due to aspartate aminotransferase and the other to tyrosine aminotransferase. The lack of effect of dexamethasone on aspartate aminotransferase and on its capacity for promoting tyrosine transamination, in contrast with the pronounced increase of the specific tyrosine aminotransferase peak, gives further support to this proposition.

Collectively, these data show that tyrosine aminotransferase is not present in foetal liver extracts from 15- or 19-day-foetal rats. By the chromatographic method, cultured hepatocytes were reassessed for their ability to synthesize tyrosine aminotransferase. This study revealed that although neither 15- nor 19-day-foetal rat liver contains tyrosine aminotransferase, 19-day-foetal liver, but not 15-day-foetal liver, is capable of enzyme synthesis after culture for 1 day. Tyrosine aminotransferase activity is detectable in the 15-day-foetal liver cultures only after 3 days in culture. In both series of cultures the acquisition of ability to make the enzyme coincides with the ability to respond to dexamethasone in terms of increased enzyme production. It is noteworthy that without the background of tyrosine transamination by aspartate aminotransferase, dexamethasone is effective at concentrations as low as 0.2nM.

Assay of hepatocytes from foetuses of various gestational ages cultured for 1 day reveals that by day 16 the foetal hepatocyte has acquired the capacity to synthesize the enzyme. This event could occur in the foetal liver at about day 17 if hepatocytes continue to develop in culture as they would *in vivo*. It is necessary to incubate the cultures for 18h before harvesting the cells in order to allow hepatocytes to recover from the isolation procedure and attach to the substratum. Shorter culture periods result in poorer yields of cells, with less than optimum activities of liver enzymes and ATP compared with fresh liver (G. Bulanyi, G. C. T. Yeoh & I. T. Oliver, unpublished work).

It is proposed from the results of this study that early-foetal liver cells of about 15 days gestation are

not capable of tyrosine aminotransferase synthesis. Transamination of tyrosine, which was detected in both cultured foetal liver and fresh livers, is due to aspartate aminotransferase. The foetal liver cells become competent with respect to tyrosine aminotransferase synthesis at around 16 days gestation. These hepatocytes will produce the enzyme when placed in the culture environment. In contrast, hepatocytes from earlier foetuses, such as 15 days gestation, will not produce the enzyme under identical culture conditions. However, after 3 days in culture, these cells will synthesize the enzyme and thus undergo the differentiative event which occurs *in vivo* between day 15 and day 16 of gestation. It is of interest to find that when basal amounts of the enzyme could not be detected, its activity could not be induced by the addition of steroid to the culture medium. Once the cells acquire the enzyme, its activity can be markedly increased by dexamethasone. This is analogous to the induction of tryptophan pyrrolase in mammalian liver by cortisone. The hormone is ineffective until the cells are able to produce the enzyme (Knox & Greengard, 1965).

Since synthesis of albumin and transferrin could be demonstrated in cultures identical with those used in this study (Yeoh *et al.*, 1977), the data lend support to the notion that liver cells may acquire the ability to synthesize various proteins in a sequential manner during development. Thus it should be possible to demonstrate that a lineage of liver cells exists (Holtzer *et al.*, 1975).

We wish to record the excellent technical assistance of Mr. M. McGrath and Ms. J. Wassenburg. Rabbit antiserum against rat tyrosine aminotransferase was generously provided by Mr. J. W. Sadleir. Special thanks are due to Mr. W. V. Smith and Mr. J. T. Firman for help with initial problems associated with the tissue-culture facilities. This work was supported by research grants from the Australia Research Grants Committee, the National Health and Medical Research Council of Australia and the University of Western Australia. This work was performed while G. C. T. Y. was the recipient of a C.J. Martin Postdoctoral Fellowship from the National Health and Medical Research Council of Australia.

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