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Induction of Tyrosine-α-Ketoglutarate Transaminase in Fetal Rat and Fetal Human Liver in Organ Culture

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Extract

Explants of fetal rat (13 days to term) and fetal human liver (crown to rump length = 9–20 cm) were grown in organ culture. The survival of the explants was examined by histological appearance, mitotic index, and incorporation of precursors of ribonucleic acid (RNA), deoxyribonucleic acid (DNA), and protein. Tissues adjusted to the culture system by the 24th hr and remained so throughout the duration of the experiments. A slight increase in tyrosine transaminase activity occurred in control explants during the 3-day period in culture. Eighteen-hour incubation with hydrocortisone (10^{-6} M), glucagon (5–25 μg/ml), and insulin (0.05–0.5 U/ml) increased tyrosine transaminase levels, 3.1–6.5, 2.5, and 2.3 times the control values, respectively, in 18-day to term fetal rat liver explants. Actinomycin D and cycloheximide completely inhibited the corticosteroid stimulation. Thirteen-day fetal rat liver exhibited no increase in tyrosine transaminase activity after hydrocortisone incubation for 24 hr, whereas glucagon plus dibutyryl cyclic adenosine monophosphate (AMP) did stimulate tyrosine transaminase activity twofold at this developmental stage. No increase in human fetal liver tyrosine transaminase occurred with any of the agents tested.

Speculation

These studies support the hypothesis that mammalian liver is incompetent to respond to hormone-induced enzyme synthesis before a certain developmental stage is reached.

Introduction

Tyrosine transaminase (L-tyrosine:2-oxoglutarate aminotransferase, TTA, EC. 2.6.1.5) has very low activity in fetal rat liver, but beginning with the 2nd hr after birth there is a rapid increase in activity reaching a maximum at 12 hr, as shown by Sereni *et al.* [17]. These authors were not able to induce TTA activity by hydrocortisone injected into rat fetuses *in utero*, although a later study by Yeung *et al.* [21] indicated that if fetuses close to term are exposed to corticosteroid for 10 hr or more a marked elevation of TTA activity can be observed in the liver. Greengard [3] has shown that the administration of glucagon, adrenalin, and cyclic AMP to fetal rats during the last 2 days of gestation evoked the appearance of TTA activity *in vivo*. Studies *in vitro* on fetal rat liver maintained in organ culture [19, 20] demonstrated that the activity of TTA is elevated by cyclic AMP and by hormones (glucagon and catecholamine) which stimulate formation of this nucleotide, as well as by hydrocortisone and insulin in fetal liver near term.

Kretchmer *et al.* [12], in a study of the development of the activity of the tyrosine-oxidizing system of human liver, found that adult liver has 10–30 times greater, and the liver of the full term newborn infant 3–5 times greater activity than the liver of a premature infant. No studies of the regulatory mechanisms involved in the developmental process of TTA in human liver have been presented in the literature to our knowledge. Since in the future, artificial stimulation of enzyme development during fetal life may facilitate certain metabolic adjustments of the premature and full term newborn infant to extrauterine existence, we have undertaken a comparative study of the development of TTA in fetal rat and fetal human liver maintained in organ culture with reference to the stage of development at which hormonal induction can be evoked.

Materials and Methods

Actinomycin D and cycloheximide were obtained commercially [22, 30], as were insulin and glucagon [23]; hydrocortisone [24]; triamcinolone [25]; cyclic AMP and $N^6,O^{2'}$-dibutyryl cyclic AMP [26]; and ^{14}C-orotic acid (30–50 mCi/mmole), ^{3}H-leucine (>5000 mCi/mmole), and ^{3}H-thymidine (>10,000 mCi/mmole) [27].

The culture medium was Eagle's minimum essential medium in Hank's balanced salt solution with twice the normal concentrations of glucose and bicarbonate and with 100 units penicillin and 100 μg streptomycin/ml [28].

Animals, Tissue Preparation, and Organ Culture

Adult female rats of a Sprague-Dawley strain [28] were mated for a 16-hr period, so that the predicted time of conception was ± 8 hr. At the appropriate time of gestation the mother was killed by a blow to the head, and the entire uterus was quickly removed. All of the following procedures were carried out under sterile conditions. The livers of the fetuses were immediately excised and placed in petri dishes containing the culture medium. All hepatic tissue from litters of the same gestational age was pooled and cut into pieces 1 mm per side using injection needles and were randomly placed on lens paper strips supported by stainless steel grids to allow the lower surface of the explants to be in contact with the medium [18]. Each petri dish contained two grids of 20 pieces each and 10 ml medium. The dishes were placed in high humidity chambers maintained at +37° with a circulating gas phase of 95% air + 5% CO_2. Hepatic tissue from human fetuses with a gestational age of 14–24 weeks (crown to rump length 9–20 cm) were obtained at legal therapeutic abortions. The same preparative techniques as for rat tissues were used for human tissues. The human explants, however, were maintained with a gas phase of 95% O_2 + 5% CO_2.

All explants were maintained in culture at least 40 hr before the experiments began. The condition of the explants was examined by the following means: histological appearance after embedding, sectioning, and staining with hematoxylin eosin; the mitotic index and the incorporations of radioactive precursors for protein (^{3}H-leucine), RNA (^{14}C-orotic acid), and DNA (^{3}H-thymidine). All incorporations were performed with 1-hr pulses in each culture dish, with one grid removed after 5 min and considered as a blank. The pulses used were as follows: 1 μCi/ml medium for ^{14}C-orotic acid and ^{3}H-thymidine, and 0.5 μCi/ml for ^{3}H-leucine.

We could not successfully maintain postnatal liver tissues with our system.

Analytical Methods

The activity of TTA was determined according to the procedure of Diamondstone [1]. The same substrate concentrations were used for all fetal and adult analyses (12 mM α-ketoglutarate, 48 μM pyridoxal phosphate, 2.4 mM diethyldithiocarbamate, 2.4 mM tyrosine, in 0.1 M KH_2PO_4). Approximately 40 pieces of liver were used for each enzyme assay. Enzyme activity has been expressed in units (1 μg *p*-hydroxyphenylpyruvate formed/hr at 37°). The soluble protein concentration was measured by the method of Lowry *et al.* [13] with bovine serum albumin as a standard. The activity measured with this assay was proportional to time of incubation and amount of enzyme used. Incorporations of the RNA, DNA, and protein precursors (^{14}C-orotic acid, ^{3}H-thymidine, and ^{3}H-leucine, respectively) were determined by spotting 50 μl of a homogenate containing the tissue from one culture grid in 200 μl cold 0.13 M KCl on filter paper discs by a modification of the technique of Mans and Novelli [14]. The samples were then precipitated with 5% trichloroacetic acid and rinsed twice with ethanol after which they were dried and counted in a liquid spectrophotometer [29] in vials containing 0.5 ml Hyamine [29] and 10 ml POPOP (0.01% 1,4-bis-2,4-methyl-5-phenyloxazolylbenzene) and PPO (0.4% 2,5-diphenyloxazole in toluene).

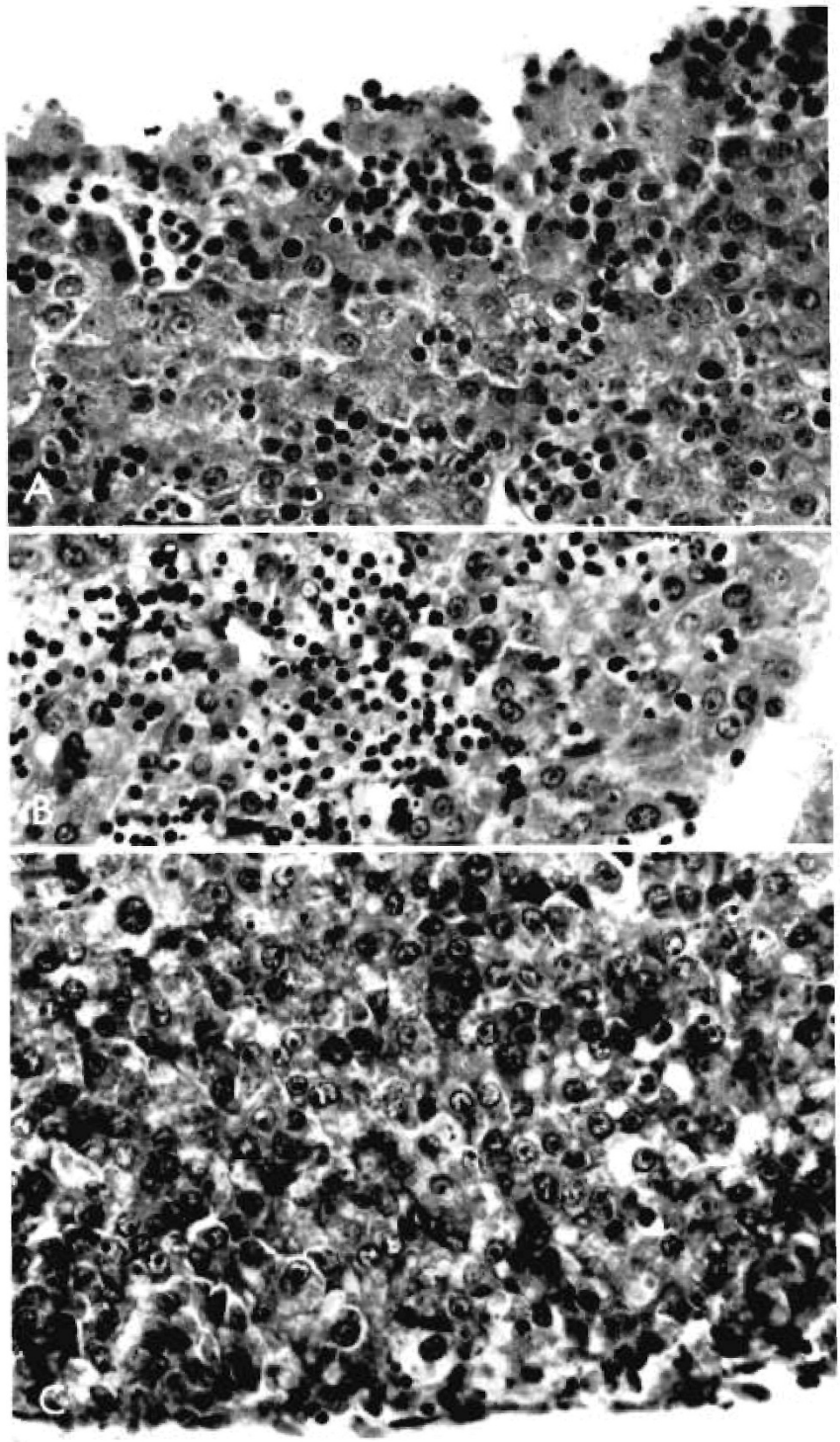

Fig. 1. Histological appearance of tissues cultured for 72 hr. *A:* Fresh preparation of term fetal rat liver. *B:* Fetal human liver in culture (crown-rump = 12 cm). *C:* Term fetal rat liver in culture. Stained with hematoxylin-eosin.

Table 1. Incorporation of ^{14}C-orotic acid, ^{3}H-leucine, and ^{3}H-thymidine into fetal rat and fetal human liver in organ culture

Liver	^{14}C-Orotic acid	^{3}H-Leucine	^{3}H-Thymidine
	cpm/mg protein		
Rat (48 hr in culture)	3480 ± 650 (12)[1]	2260 ± 600 (10)	582 ± 199 (5)
Human (39–48 hr in culture)	1020 ± 147 (12)	1393 ± 185 (15)	36 ± 20 (7)

[1] Mean ± SE, with number of observations in parentheses.

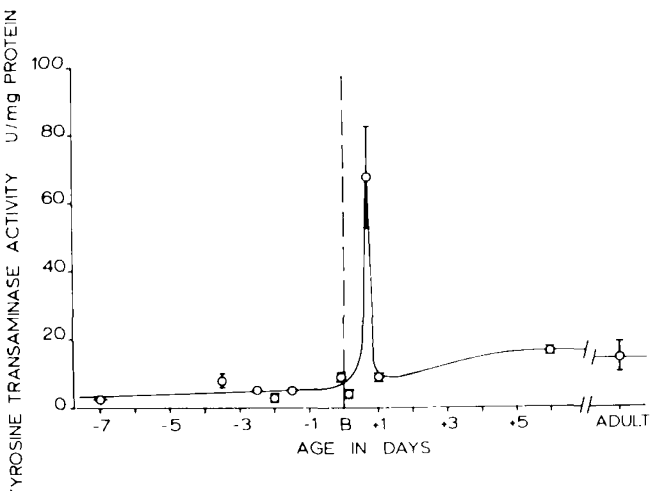

Fig. 2. Development of tyrosine transaminase activity in the rat. Each point represents the mean ± SE of at least three animals.

Results

Evaluation of the Organ Culture

The histological study of the tissues indicated that throughout a 72-hr culture period the appearance of the hepatocytes did not change although a disappearance of erythrocytes and hematopoietic cells could be noted (Fig. 1).

Incorporation of radioactive precursors for DNA, RNA, and protein showed that the tissue remained functional throughout the period of the experiments. As seen from Table 1, there was somewhat greater incorporation of ^{14}C-orotic acid, ^{3}H-leucine, and ^{3}H-thymidine into the rat tissue explants than into the human. The incorporation of ^{14}C-orotic acid and ^{3}H-leucine into the fetal rat liver explants did not vary markedly during the culture period. As seen in Table 1, very little ^{3}H-thymidine was incorporated into the fetal tissue during a 1-hr pulse; however, when the labeling time was extended to 24 hr, a mean incorporation of about 3000 cpm/mg protein was found. An examination of the fetal rat liver explants after 48 hr in culture revealed a mitotic index of 0.6%, whereas a control liver at the time of excision had a mitotic index of 1.9%.

Development in Vivo of Tyrosine Transaminase Activity

Figure 2 illustrates the normal development of TTA activity in rat liver. During fetal life the activity was very low, but soon after birth a rapid increase in TTA activity was noted. At 16 hr after birth the enzyme activity was about eight times as high as before birth but decreased again rapidly to attain within 8 hr an activity similar to that found at birth. After this transient rapid increase in activity a slow increase to about two times the fetal values was observed during the following 5 postnatal days. This activity range was also found in adult rats.

The activity of TTA in the human fetal livers at 14–24 weeks of gestation was somewhat variable and was approximately twice that found in fetal rat livers. In the liver from a 1,000-g premature infant who died at 12 hr of age, the TTA activity was approximately twice that found in the fetuses. In this case the liver sample was removed within 2 hr after death.

Induction of Tyrosine Transaminase in Organ Culture

In fetal rat liver explants in culture the TTA activity increased slightly during the culture period, as seen

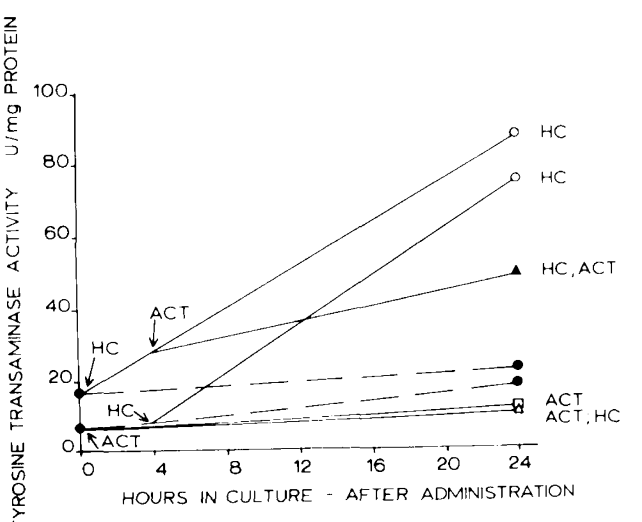

Fig. 3. Effect of actinomycin D on hydrocortisone-induced tyrosine transaminase activity in term rat liver explants. Each point represents the average of two parallel determinations. The experiments were begun after 40 hr in culture. ●: Control. ○: Hydrocortisone. □: Actinomycin D. △: Actinomycin D followed by hydrocortisone. ▲: Hydrocortisone followed by actinomycin D.

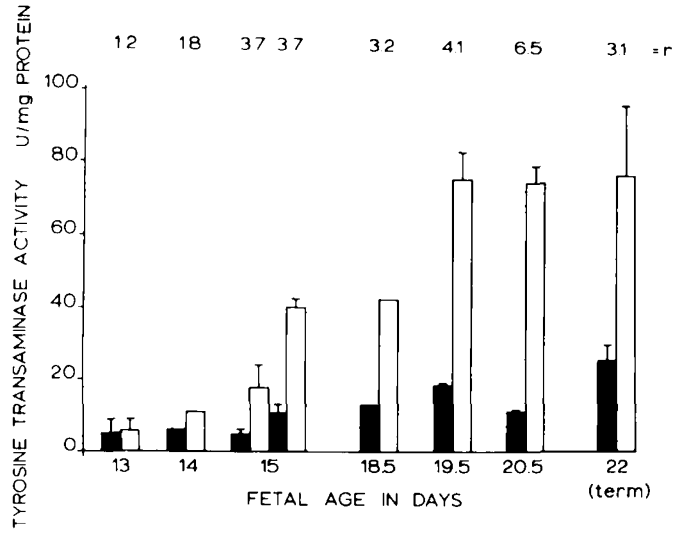

Fig. 1. Development of hydrocortisone induction of rat liver tyrosine transaminase activity in culture. Open bars: Hydrocortisone. Closed bars: Controls. Standard deviations are given for more than two determinations. Ages are ± 8 hr; r = hydrocortisone/control. All experiments began after 40 hr in culture and lasted 20–24 hr. Both sets of data at day 15 have identical r values.

Table II. Effect of developmental age on induction of TTA activity by insulin, glucagon, and cyclic AMP in fetal rat liver in organ culture[1]

	Induction of TTA activity, units/mg protein		
	13 days	15 days	22 days
Control	4.5	4.8 (3.1-6.5)	19.4
Insulin, 0.05–0.5 U/ml		8.6 (7.8–9.4)	44.8 ± 16.6
Glucagon, 5–25 μg/ml		7.6 (6.8–8.3)	50.2 ± 3.7
Glucagon, 5 μg/ml + dB-cAMP[2], 7×10^{-5} M	8.7 (8.5–8.8)		

[1] All experiments were begun after 40 hr in culture, and enzyme activity was analyzed in duplicate 18 hr later. Where two sets of data are available, the mean is followed by the range in parentheses. Standard deviation is given for more than two sets of data. Each figure at 13 days of gestation represents 15–25 pooled livers; each figure at 15 days represents 5–8 pooled livers.
[2] N^6,$O^{2'}$-Dibutyryl cyclic AMP.

in Figure 3. This pattern was also found with fetal human liver explants, *e.g.*, 7 units/mg protein at 15 hr to 10.2 units/mg protein at 40 hr in culture.

The activity of fetal rat liver TTA increased markedly after incubation of the explants with hydrocortisone (10^{-6} M), as seen in Figure 1. The activity of TTA in cultured explants increased significantly after incubation with hydrocortisone in tissues from rats of gestational age 15 days to term. No increase was evident, however, in any of the six determinations each representing 15–25 pooled livers from 13-day-old fetuses under identical conditions. The size of the rat liver underwent a fourfold increase from the 13th to the 15th day of gestation, and the color of the liver tissue changed from pale grey to red during this time.

Addition of actinomycin D (25 μg/ml) to the culture medium inhibited completely the hydrocortisone-induced increase of TTA activity in the fetal rat liver explants when added 4 hr before the steroid (Fig. 3). However, when the explants were incubated with hydrocortisone for 4 hr before the addition of actinomycin, a lesser rise in TTA activity could be noted. When cycloheximide was added (10 μg/ml) to the culture medium either 4 hr before or after the steroid, a complete inhibition of the hydrocortisone-induced increase of TTA activity was found.

In addition to hydrocortisone, incubation of term rat liver explants with glucagon (5–25 μg/ml) and insulin (0.05–0.5 unit/ml), moderately increased TTA activity. The increase after incubation with these pancreatic hormones was 2.5-fold and 2.3-fold the control values, respectively (Table II). Pooled livers in culture from 13-day-old fetuses which did not react by increased TTA activity after incubation with hydrocortisone in previous experiments exhibited a 2-fold increase in TTA activity when both glucagon and dibutyryl cyclic AMP were added to the culture medium, as seen in Table II.

No increase in TTA activity in any of the 16 human fetal livers was found with any of the agents tested: hydrocortisone (10^{-8} to 10^{-4} M), triamcinolone (10^{-4}–10^{-3} M), insulin (0.01–0.1 unit/ml), glucagon (1–24 μg/ml), cyclic AMP (10^{-3} M to 6.5×10^{-3} M), or dibutyryl cyclic AMP (7×10^{-7} M to 7×10^{-4} M). When dibutyryl cyclic AMP was administered together with hydrocortisone, there was also no effect on the TTA activity in liver explants from human fetuses, as was the case with combinations of glucagon with cyclic AMP or dibutyryl cyclic AMP.

Discussion

Some attempts were made to find optimal conditions for maintaining fetal liver explants in culture. Adding calf serum to the medium or increasing the oxygen concentration of the gas phase to 95% did not markedly increase incorporation of precursors or change the histological appearance of the explants from fetal rat

liver. With the human liver explants, however, it was found that a gas phase of 95% oxygen increased survival. The incorporation of radioactive precursors, the histological appearance, the mitotic index, and the fact that the TTA activity showed a slight increase during the culture period all provide evidence for the viability of the hepatocytes in the fetal liver explants during the experimental period. This confirms the observations made earlier by Wicks [19].

The rapid rise in TTA activity in rat liver after birth has been found by several investigators [2, 6, 17]. Greengard and Dewey [5] suggested that postnatal hypoglycemia stimulates glucagon release, which then induces liver TTA. Their finding that postnatal injection of glucose decreases the normal increase of TTA activity is in agreement with this proposal. The failure of adrenalectomized newborn rats to undergo the normal postnatal increase in TTA activity, the induction of TTA by administration of hydrocortisone to these animals [17], and, further, the observation by Franz and Knox [2] that administration of hydrocortisone to newborn rats did not elevate TTA activity until after the postnatal increase had occurred suggest that glucocorticoids apparently also participate in the immediate postnatal increase of TTA activity.

In the present study a maximal level of TTA activity was reached at about 16 hr after birth; thereafter the enzyme activity decreased rapidly to attain initial term fetal values within about 8 hr. The rapid fall in enzyme activity is consistent with a half-life of the enzyme of 3–4 hr [10], if one assumes that enzyme synthesis stops when the peak postnatal value is reached. The studies by Holten and Kenney [9] indicate that the glucagon-mediated induction of liver TTA ceases despite a continuous administration of the hormone, whereas in the case of hydrocortisone the elevated enzyme synthesis continues for as long as the steroid is present. The corticosterone concentration in postnatal rat plasma increases considerably above adult levels until 5 hr after delivery [7].

Differences have been observed in the effect of certain inducing agents of TTA as related to the developmental age of the rat. Thus, TTA can be induced *in vivo* by hydrocortisone administration only in postnatal rats [3, 17, 19] although it has been shown that the synthetic glucocorticoid, triamcinolone, markedly elevates TTA activity in term fetuses when the exposure time is greater than 10 hr [21]. Other inducing agents such as glucagon, adrenalin, and cyclic AMP act *in vivo* by elevating liver TTA activity in fetal animals [4], but also a definite developmental trend in the competence to react to these agents has been found. Greengard [3] recently presented a scheme indicating the development of the competence of the fetal rat liver to respond to glucagon and cyclic AMP *in vivo*. Her results demonstrate that the competence of liver to synthesize TTA under the influence of cyclic AMP is present 4 days before birth, but glucagon alone does not induce TTA at this developmental stage. During the next fetal day the capacity develops to raise the concentration of cyclic AMP on exposure to glucagon and adrenaline, and thus the TTA activity can be evoked by these hormones as well as by cyclic AMP.

Studies *in vitro* on rat liver explants in organ culture [19, 20] have shown that term fetal liver can be stimulated to increase synthesis of TTA by glucagon, insulin, and cyclic AMP, and that hydrocortisone is an effective inducer of TTA in cultures of liver from 17-day fetuses. Rutter [15, 16] reported that TTA activity can be stimulated by incubation of fetal rat liver explants in culture with triamcinolone at any period during the development of the rat. Thus, it seems evident from the available data that the competence to react by increased TTA activity in response to corticosteroids appears in fetal liver maintained in culture at an earlier developmental stage than *in vivo*. The results presented in this paper are consistent with this and demonstrate furthermore that fetal rat liver maintained in organ culture is not competent to respond to hydrocortisone by increasing the TTA activity before day 13–14 of gestation, although glucagon and cyclic AMP produce a stimulus at this stage. This finding is in agreement with the hypothesis presented by Kenney *et al.* [11] that the induction of TTA activity by hydrocortisone is produced by a mechanism different from that of glucagon. Furthermore, Holt and Oliver [8] have recently suggested the existence of multiple forms of rat liver TTA, each of which may be under the inductive influence of a different agent. This finding is also consistent with the developmental dissimilarities of the induction of this enzyme.

It is difficult to speculate on the reasons for the nonresponsiveness of early human fetal liver explants to any of the agents examined. We do not believe that this was due to maintenance of the fetal human liver in organ culture since all of the criteria for judging the viability of the tissue indicated that the human tissue was viable. Thus, it appears that explants of human fetal liver of 14–24 weeks' gestation in organ culture are incompetent to respond by increasing TTA activity to the external chemical stimulants used.

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35. Note: After this paper was sent to be published we received liver from an immature human fetus delivered in the hospital by spontaneous abortion at a gestational age of 28 weeks. The fetus died traumatically during breech delivery and liver tissue was immediately removed and explants were cultured according to the technique presented in this paper. Triamcinolone (1×10^{-4} M) produced a fourfold increase in TTA activity in these explants from the 28-week fetus (crown to rump length = 25 cm). The control values after 62 hr in culture were 14.7 and 17.4 units/mg protein, and after incubation with triamcinolone the values were 50.0 and 63.0 units/mg protein. The histological appearance of the hepatocytes after the incubation period was satisfactory.

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