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Spontaneous development of cytosolic phosphoenolpyruvate carboxylase in foetal rat liver cultures

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SUMMARY

Phosphoenolpyruvate carboxylase activity rises in foetal rat liver culture after 45 h of incubation from no detectable or very low values, to high levels, very close to those usually found *in vivo* 48–72 h after birth. Both hydrocortisone and glucagon enhance *in vitro* the phosphoenolpyruvate carboxylase activity, whereas cycloheximide completely prevents this spontaneous activation.

In recent years it has been shown that cytoplasmic phosphoenolpyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) activity is absent from foetal rat liver, making its appearance only after birth[1,2]. A sharp increase of activity appears also in the liver of newborns delivered prematurely by uterine section[3]. It is commonly assumed that this is one of the key processes determining the postnatal increase of liver gluconeogenesis.

In vivo administration of glucocorticoids[4,5], adrenalin, noradrenalin or glucagon[6] to adult animals is followed by a very significant increase of this enzyme activity. The response of foetuses to the same hormones is to a certain extent different from that of adults[6].

Barnett and Wecks[7] have reported data from *in vitro* experiments, working with Reuber H35 hepatoma cells growing in monolayer cultures. They found this technique very useful to elucidate peculiarities of hormone regulation of phosphoenolpyruvate carboxylase activity. In this report we describe results obtained measuring phosphoenolpyruvate carboxylase activity of foetal rat liver organ cultures under various experimental conditions.

We observed a spontaneous activation of this enzyme activity, allowing some speculations on the mechanisms which regulate phosphoenolpyruvate carboxylase activity during the perinatal period of life.

Wistar albino rats were used. The time of conception was calculated with a maximum error of 12 h. One male and two females were put together for 24 h and midnight was presumed to be the time of impregnation.

Newborn and pregnant rats were killed by a blow on the head. Foetuses were removed from the horns under sterile conditions and placed in saline. The liver was removed as quickly as possible, cut into pieces averaging about 1 mm^3, and placed on a Millipore membrane in the tissue culture dish.

The standard medium composition was the following: Basal medium of Eagle with Hank's balanced salts, to which was added 20% horse serum, 1% L-glutamine and the antibiotics fungizone, benzylpenicillin and streptomycin, each at a final concentration of 25 μg, 100 units and 100 μg per ml. The medium was changed every 24 h. The cultures were kept in a humidified incubator at 38 °C and in an atmosphere containing 95% O_2 and 5% CO_2.

Hydrocortisone, glucagon and cycloheximide were dissolved with minimal amounts of appropriate solvents, giving a 10^{-6} M solution of hydrocortisone, a 400 μg/ml solution of glucagon and a 10^{-5} M solution of cycloheximide.

Hydrocortisone was a gift from G. Castagnaro, Farmitalia, glucagon, as hydrochloride, was from Lilly, and cycloheximide was from Sigma.

The phosphoenolpyruvate carboxylase activity was measured on sovranatant of homogenized tissue, after centrifugation at 27 000 × *g* for 15 min, according with the technique described by Chang and Lane[8], with minor modifications.

Results of enzyme activity in standard conditions are reported in Table I, where values are grouped according to the foetal age of the explants. In foetal liver no basal enzyme activity could be detected, except when foetuses very close to term were examined.

TABLE I

CHANGES OF PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY OF FOETAL RAT LIVER EXPLANTS MAINTAINED UNDER STANDARD CONDITIONS

All determinations are expressed as enzyme units/mg protein (± S.E.) with the number of determinations in parentheses. One unit of enzyme activity is defined as the incorporation of 1 pmole of $NaH^{14}CO_3$/ 15 min at 30 °C.

Gestational age (days)	*Enzyme activity*		
	*0**	*21**	*45**
18	0	35.74 ± 9.8 (5)	78.34 ± 34.8 (6)
19	0	176.42 ± 67.3 (3)	226.47 ± 72.1 (4)
20–21	0	140.80 ± 26 (5)	408.37 ± 65.3 (3)
22	7.56 ± 4.6 (5)	76.72 ± 12.5 (4)	126.90 ± 33.8 (5)

*Hours of incubation.

In this case a small but significant enzyme activity was present. A significant increase of phosphoenolpyruvate carboxylase activity was observed in each instance after *in vitro* culture. In fact, after 45 h of incubation it was possible to observe absolute values of enzyme activities very similar to those found *in vivo* after birth (Table II). The

TABLE II

POSTNATAL CHANGES OF PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY IN RAT LIVER

Enzyme activity expressed as enzyme units/mg protein. Each value is the average of at least four determinations.

Age	*1–4 h*	*24 h*	*48–96 h*	*5–7 days*	*Adults*
Enzyme activity	180.3	293.3	651.5	332.7	263.4

spontaneous activation of phosphoenolpyruvate carboxylase was completely inhibited by addition to the medium of cycloheximide (10^{-5} M), suggesting a "*de novo*" synthesis of enzyme molecules. This is in accord with more refined *in vivo* observations recently published by Philippidis and Hanson[9]. These authors were able to demonstrate with immunologic techniques that the postnatal increase of phosphoenolpyruvate carboxylase activity can be related to synthesis of new enzyme molecules. Whereas in the foetus *in vivo* glucocorticoids do not enhance phosphoenolpyruvate carboxylase activity[2], this was possible to observe *in vitro*, as shown in Table III. Moreover, our *in vitro* cultures did respond very markedly to glucagon, with a 3–14-fold increase of enzyme activity (Table IV). Finally, addition to the medium of glucose in excess does not suppress phosphoenol-

TABLE III

IN VITRO RESPONSE OF PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY TO HYDROCORTISONE (10^{-6} M)

Enzyme activity expressed as enzyme units/mg protein, values after 21 h of incubation.

Gestational age (days)	*Enzyme activity*		Δ %
	Standard medium	*Medium with hydrocortisone*	
18	41.44	95.53	+ 130
19	310.84	563.34	+ 78
19	109.20*	334.98	+ 207
19	116.15	139.46	+ 20
21	76.68	77.7	+ 1
21	231.05	600.95	+ 160
22	52.84	98.95	+ 87

* Average of two control values.

TABLE IV

IN VITRO RESPONSE OF PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY TO GLUCAGON (400 μg/ml)

Enzyme activity expressed as enzyme units/mg protein.

Gestational age (days)	*Enzyme activity*				Δ %	
	*21**		*45**		*21**	*45**
	Standard medium	*+ glucagon*	*Standard medium*	*+ glucagon*		
19	109.2**	445.5	160.2**	1998.6	+309	+1148
22	52.8	379.2	158.4	1018.3	+618	+ 543

* Hours of incubation.

** Average of two control values.

TABLE V

INFLUENCE OF HIGH GLUCOSE CONCENTRATION (5 mg/ml) ON PHOSPHOENOLPYRUVATE CARBOXYLASE ACTIVITY ON FOETAL RAT LIVER CULTURES

Enzyme activity expressed as enzyme units/mg protein.

Gestational age (days)	*Enzyme activity*				Δ %	
	*21**		*45**		*21**	*45**
	Standard medium	*+glucose*	*Standard medium*	*+glucose*		
19	109.2**	71.7	160.2**	409.8	− 34	+ 156
22	52.84	111.7	158.4	484.4	+ 110	+ 206

* Hours of incubation.
** Average of two control values.

pyruvate carboxylase activity, as it was observed for tyrosine aminotransferase, when *in vitro* cultures of hepatoma cells were used[10]. As a matter of fact, although no significant variations were observed after 21 h of incubation, after 45 h the enzyme activity was greater in cultures with an excess of glucose than in the control ones (Table V).

These data on spontaneous development of phosphoenolpyruvate carboxylase activity in foetal rat liver tissue cultures are essentially in agreement with our previous report on tyrosine aminotransferase (EC 2.6.1.5) activity in the same *in vitro* system[11]. In both cases a sharp activation of enzyme activity occurs, which is apparently independent of hormone stimulation. Furthermore, in both cases we found an unequivocal *in vitro* response to glucocorticoids. The reason why the passage from the "*in vivo*" to the "*in vitro*" milieu entails an activation of enzyme activity and a greater responsiveness to glucocorticoid stimulation is still unknown. Work is now in progress to check the importance of some peculiarities of our standard *in vitro* conditions.

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