Changes in the Activities of the Enzymes of Urea Synthesis Caused by Dexamethasone and Dibutyryladenine 3':5'-Cyclic Monophosphate in Foetal Rat Liver Maintained in Organ Culture

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(Received 9 April 1976)

Liver explants from 19-day foetal rats were maintained in organ culture, in a defined medium, for up to 48 h. Both 6-*N*,2'-*O*-dibutyryl cyclic AMP, in the presence of theophylline, and dexamethasone caused an increase in the activities of carbamoyl phosphate synthase, argininosuccinate synthetase, argininosuccinate lyase and arginase. These increases could be abolished by simultaneously incubating the explants with cycloheximide. No change in the activity of ornithine transcarbamoylase was found with either hormone. Previous work has shown that injection of corticosteroids into 19.5-day foetal rats *in utero* did not cause an increase in the arginine synthetase system. Present results suggest that this lack of effect is not due to any incompetence of the foetal rat liver at this stage to respond to this agent. The observations on ornithine transcarbamoylase activity suggest that this enzyme is induced in the liver of the perinatal rat by neither corticosteroids nor hormones acting via cyclic AMP, and it may be that all the enzymes of the urea cycle are induced physiologically by an agent or agents as yet unidentified.

The enzymes of urea synthesis are carbamoyl phosphate synthase (EC 2.7.2.5), ornithine transcarbamoylase (EC 2.1.3.3), argininosuccinate synthetase (EC 6.3.4.5), argininosuccinate lyase (EC 4.3.2.1) and arginase (EC 3.5.3.1). These enzymes have low activity in the liver of foetal rats, but during the perinatal period a rapid increase occurs (Kennan & Cohen, 1959; Räihä & Suihkonen, 1968; Miller & Chu, 1970).

Schwartz (1972) found that explants of foetal rat liver in organ culture gave an increase in the activity of the arginine synthetase system when incubated with the synthetic corticosteroid triamcinolone, 6-*N*,2'-*O*-dibutyryl cyclic AMP or glucagon, but when the steroid or glucagon was injected into the 19.5-day foetal rats *in utero* no increase in enzyme activity occurred. As the arginine synthetase system is composed of both argininosuccinate synthetase and argininosuccinate lyase (Ratner, 1955), we have determined the effect of the corticosteroid, dexamethasone, and dibutyryl cyclic AMP on the separate enzymes and extended the observations to the other three enzymes of the urea cycle, by using the method of organ culture described by Räihä *et al.* (1971) and Schwartz (1972).

Our findings show that carbamoyl phosphate syn-

thase, argininosuccinate synthetase, argininosuccinate lyase and arginase can be increased in activity in explants of foetal liver by both dexamethasone and dibutyryl cyclic AMP in the presence of theophylline, but that no change occurs in the activity of ornithine transcarbamoylase after treatment with either agent.

Experimental

Materials

Cycloheximide was purchased from Nutritional Biochemicals Corp., Cleveland, OH, U.S.A.; *N*-acetylglutamic acid, L-ornithine, L-aspartic acid and L-arginine were from Calbiochem, Los Angeles, CA, U.S.A.; [*carbamoyl*-¹⁴C]citrulline (specific radioactivity 32 mCi/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K.; dexamethasone, carbamoyl phosphate, argininosuccinic acid, cetyltrimethylammonium bromide, ornithine transcarbamoylase, argininosuccinate lyase, arginase, ATP and dibutyryl cyclic AMP were from Sigma Biochemicals, St. Louis, MO, U.S.A. The culture medium, Eagle's minimum essential medium in Hanks balanced salt solution containing twice the normal concentrations of bicarbonate, glucose and L-glutamine with 100 i.u. of penicillin and 100 µg of streptomycin per ml, was purchased from Orion Oy/Ab, Helsinki, Finland.

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Organ culture

Adult female rats of the Sprague-Dawley strain (Orion Oy/Ab, Helsinki) were mated over a 12h period. On the 19th day of gestation the mothers were killed and the foetuses removed under sterile conditions. The foetal livers were excised and placed immediately in a 9cm Petri dish containing culture medium. With the aid of a binocular microscope, cubes of liver of volume about 1 mm^3 were cut with no. 21 gauge injection needles. After 50–60 cubes had been cut they were transferred to lens tissue supported on two stainless-steel grids in a 6cm Petri dish containing 14ml of culture medium. The amount of medium was adjusted so that the liver pieces were at the gas/liquid interface. The Petri dish was immediately placed in an incubator, kept at 37°C , and ventilated with $\text{O}_2 + \text{CO}_2$ (95:5). By this procedure, 16 dishes could be prepared in 2h, and from the moment the livers were removed from the foetuses they were in contact with culture medium. The viability of explants made by this method has been established by Schwartz (1972).

Experimental agents were dissolved in culture medium and pipetted into the Petri dish before the cubes of liver were added. Thus the liver cubes were in contact with the hormones immediately they were placed on the grids. The preincubation procedure previously used (Räihä *et al.*, 1971; Schwartz, 1972) was omitted.

At the end of 24 or 48h incubation, material from four grids was pooled and homogenized in 0.2ml of 0.1% cetyltrimethylammonium bromide (Brown & Cohen, 1959). The homogenate was centrifuged at $7500g$ for 15min at 4°C , and the supernatant was used for the measurement of enzyme activity and protein concentration.

Analytical methods

Carbamoyl phosphate synthase and ornithine transcarbamoylase activities were measured by the procedures of Brown & Cohen (1959). Arginase was measured by the colorimetric method of Rowe *et al.* (1970) and argininosuccinate lyase by the method of Tomlinson & Westall (1964). In all procedures the final volume was adjusted to $100\mu\text{l}$ for incubation at 37°C and the reactions were stopped with $50\mu\text{l}$ of 20% (w/v) HClO_4 . Citrulline or urea was measured in $100\mu\text{l}$ samples by the method of Coulombe & Favreau (1963). In the measurement of citrulline, a stable colour was obtained with a molar extinction coefficient about 60% that of the urea derivative. The non-enzymic reaction with carbamoyl phosphate and ornithine was negligibly low.

Argininosuccinate synthetase was assayed by the procedure of Schimke (1964), but without the ATP-regeneration system.

Protein was determined by the method of Lowry *et al.* (1951), with albumin as a standard. All enzyme activity units are $\mu\text{mol/h}$, and specific activities are units/mg of protein.

Results and Discussion

Soluble protein of liver cultures

Table 1 shows the protein, soluble in 0.1% cetyltrimethylammonium bromide, that remains in the liver explants after 24 or 48h of incubation. There is a loss on incubation which is not affected by either dexamethasone or dibutyryl cyclic AMP. Although the protein soluble in cetyltrimethylammonium bromide cannot be compared directly with the soluble protein obtained in a KCl homogenate (see following paragraph), as some proteins are precipitated whereas others become soluble (Brown & Cohen, 1959), the values are probably not very different. If this is the case, then Table 1 shows that only 50% of the soluble protein is lost from the explants made by our procedure. The explants also lose erythrocytes; the pieces of liver become paler as the incubation proceeds.

MacDonnell *et al.* (1975) have questioned the suitability of the organ-culture technique for studying the induction of hepatic enzymes during late foetal life. Using similar conditions for the culture of liver from foetal rats, in the absence of serum from the culture medium they observed a loss of over 80% of the protein soluble in KCl from the explants after 48h incubation. Moreover, they were unable to observe any increase in aspartate aminotransferase

Table 1. Protein, soluble in 0.1% cetyltrimethylammonium bromide, from explants of foetal rat liver

Explants were made as described in the text. The amount of liver used was adjusted so that each grid received an equal quantity. A liver sample was harvested immediately for the zero-time value and the rest were incubated as described for 24 or 48h. Material from four grids was pooled and homogenized in 0.1% cetyltrimethylammonium bromide, and after centrifuging, the protein in the supernatant was measured. The final concentration for dexamethasone was $4.6\mu\text{M}$, and for dibutyryl cyclic AMP 0.1 mM , with 0.5 mM -theophylline. The Table gives the means $\pm$ S.E.M. in mg of protein per grid. The values in parentheses are the numbers of separate experiments.

Additions Time of culture (h)	Protein			
	...	0	24	48
None	0.66 ± 0.10 (6)	0.41 ± 0.05 (8)	0.30 ± 0.03 (7)	
Dexameth- asone		0.40 ± 0.03 (6)	0.33 ± 0.07 (5)	
Dibutyryl cyclic AMP		0.46 ± 0.03 (5)	0.36 ± 0.05 (5)	

Table 2. *Effect of dexamethasone or dibutyryl cyclic AMP on the enzymes of the urea cycle in explants from liver of foetal rats*

Explants were made as described in the text. After 0, 24 or 48 h incubation the explants were homogenized in 0.1% cetyltrimethylammonium bromide and after centrifuging the homogenate, the supernatant was used for the determination of enzyme activity. The concentrations of dexamethasone, dibutyryl cyclic AMP and theophylline were as shown in Table 1. The values given are the means $\pm$ S.E.M., with the number of separate experiments in parentheses. *P* values were calculated by Student's *t* test; n.s., *P* > 0.05.

Enzyme	Additions	Enzyme activity (units/mg of protein)						
		Time of culture	...	0	24	<i>P</i>	48	<i>P</i>
Argininosuccinate synthetase	None		0.06 ± 0.01 (6)	0.11 ± 0.01 (7)			0.14 ± 0.02 (6)	
	Dexamethasone			0.27 ± 0.06 (5)	<0.02		0.62 ± 0.03 (4)	<0.001
	Dibutyryl cyclic AMP			0.34 ± 0.03 (4)	<0.001		0.52 ± 0.06 (3)	<0.001
Argininosuccinate lyase	None		0.63 ± 0.07 (8)	0.76 ± 0.05 (7)			1.04 ± 0.09 (7)	
	Dexamethasone			1.25 ± 0.05 (5)	<0.001		2.21 ± 0.10 (4)	<0.001
	Dibutyryl cyclic AMP			1.61 ± 0.16 (4)	<0.001		2.20 ± 0.15 (4)	<0.001
Arginase	None		82.3 ± 6.5 (7)	53.8 ± 6.3 (8)			51.4 ± 8.7 (7)	
	Dexamethasone			112.0 ± 8.9 (6)	<0.02		143.0 ± 15.5 (6)	<0.001
	Dibutyryl cyclic AMP			142.5 ± 33.2 (4)	<0.005		173.7 ± 40.1 (3)	<0.005
Carbamoyl phosphate synthase	None		0.34 ± 0.03 (6)	0.16 ± 0.02 (6)			0.16 ± 0.01 (5)	
	Dexamethasone			0.40 ± 0.01 (6)	<0.001		0.51 ± 0.02 (6)	<0.001
	Dibutyryl cyclic AMP			0.37 ± 0.03 (3)	<0.005		0.52 ± 0.15 (3)	<0.025
Ornithine trans-carbamoylase	None		48.9 ± 5.2 (7)	65.1 ± 7.1 (7)			87.4 ± 15.3 (7)	
	Dexamethasone			56.6 ± 3.7 (7)	n.s.		80.6 ± 18.0 (7)	n.s.
	Dibutyryl cyclic AMP			50.0 ± 7.2 (3)	n.s.		52.7 ± 11.3 (3)	n.s.

activity in explants treated with cortisol, although Monder & Coufalik (1972a,b) reported an increase in this activity when explants were incubated with 2 μ M-cortisol. The large loss of protein and the absence of a response to cortisol suggests that the liver pieces used by MacDonnell *et al.* (1975) were not viable, even though microscopic evidence suggested otherwise.

Argininosuccinate synthetase and argininosuccinate lyase

Table 2 gives the specific activities of the enzymes of the urea cycle and the effect of dexamethasone and dibutyryl cyclic AMP.

Argininosuccinate synthetase increases in specific activity, in the absence of hormonal agents, after incubation of the explants for 24 and 48 h. However, this increase is not due to induction of enzyme; when the activity of the argininosuccinate synthetase is expressed in units per grid (taking into account the decrease in soluble protein), no increase is noted. In contrast, when dibutyryl cyclic AMP in the presence of theophylline, or dexamethasone is included in the culture medium, increases in specific activity and in total enzyme units occur both after 24 and 48 h of incubation. These increases in activity are highly significant statistically and can be abolished by incubating the explants simultaneously with 10 μ g of cycloheximide/ml and the hormonal

agents. Argininosuccinate lyase shows similar behaviour, increasing in activity under the influence of either hormone.

The argininosuccinate synthetase is more responsive than the lyase, increasing in specific activity some 3.5 times more than the control, whereas the lyase only increases some two times. These observations are consistent with Schwartz's (1972) finding that both dibutyryl cyclic AMP and triamcinolone could increase the activity of the arginine synthetase system in liver explants from foetal rats. The same study showed that explants retain their ability to incorporate amino acids and orotic acid into trichloroacetic acid-precipitable material of the tissue as well as showing a response to hormones by the arginine synthetase system.

Arginase and carbamoyl phosphate synthase

The specific activities of arginase and carbamoyl phosphate synthase decrease in explants when incubated for 24 and 48 h in culture medium without any additions. With the addition of dibutyryl cyclic AMP or dexamethasone, the specific activity of both arginase and carbamoyl phosphate synthase increase compared with the controls after 24 and 48 h of incubation (Table 2). These increases can be abolished by incubating the explants simultaneously with cycloheximide and the hormonal agents.

Ornithine transcarbamoylase

In contrast with the other four enzymes of the urea cycle, ornithine transcarbamoylase was not increased in activity by either dexamethasone or dibutyryl cyclic AMP. No statistically significant increase in activity was observed with either hormone even after 48 h incubation.

Comparison of effects in vivo and in vitro

The increases in activity of argininosuccinate synthetase, argininosuccinate lyase and arginase obtained by incubating explants of foetal liver with dexamethasone are in contrast with what happens when steroid is injected into foetal rats *in utero*. Thus Greengard *et al.* (1970) found no increase in arginase activity in the livers of foetal rats after injection of cortisol *in utero*, and Schwartz (1972) could not induce the arginine synthetase system with triamcinolone under similar conditions. The lack of response *in utero* could be due to some inhibitor in the foetus that is not present during organ culture. Holt & Oliver (1968) and Ghisalberti (1976) have shown that the corticosterone concentration in the plasma of 18–19-day foetal rats is high, but no induction of argininosuccinate synthetase, argininosuccinate lyase, arginase or carbamoyl phosphate synthase takes place at this stage (Räihä & Suihkonen, 1968). One possibility is that high concentrations of insulin, known to be present in the plasma of foetal rats (Felix *et al.*, 1968; Girard *et al.*, 1973; Ghisalberti, 1976), could inhibit the induction of enzymes *in vivo* by steroid. Smith *et al.* (1975) showed that cortisol could increase the production of phosphatidylcholine in foetal rabbit lung cells maintained in monolayer culture, and this increase could be abolished by simultaneously incubating the cells with cortisol and insulin. We have shown (Räihä & Edkins, 1976) that activity increases in argininosuccinate synthetase and argininosuccinate lyase caused by dexamethasone in organ culture can be inhibited by insulin.

Induction of the enzymes of the urea cycle in vivo

The physiological trigger for the induction of the enzymes of the urea cycle remains to be identified. Table 2 shows that ornithine transcarbamoylase is affected by neither steroid nor dibutyryl cyclic AMP, although *in vivo* this enzyme is induced at the

same time as the other enzymes (Räihä & Suihkonen, 1968). It appears that ornithine transcarbamoylase is induced by some other agent or agents. Although in the explants four enzymes of the urea cycle are responsible to both steroid and cyclic AMP, the same (unknown) stimulus for the induction of ornithine transcarbamoylase may be responsible physiologically for the simultaneous induction of all the enzymes of the urea cycle.

This study was supported by the Sigrid Juselius Foundation, Helsinki.

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