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Influence of Glucagon, 6-*N*,2′-*O*-Dibutyryladenosine 3′:5′-Cyclic Monophosphate and Triamcinolone on the Arginine Synthetase System in Perinatal Rat Liver

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1. The administration of triamcinolone (19–190 μg/animal) to postnatal rats increased the arginine synthetase system activity 1.2–2.5-fold above control values 24h after exposure to the hormone. Cortisol (hydrocortisone), however, increased the arginine synthetase system activity only when larger (190 μg/animal) or repeated daily doses were given. Glucagon (100 μg/animal) stimulated arginine synthetase system activity only after the second postnatal day. None of these agents increased the activity in 19.5–21.5-day foetuses after intrauterine administration. 2. The viability of foetal rat liver explants maintained in organ culture for up to 54h was validated both by ultramicroscopic examination and by incorporation of radioactive leucine and orotic acid. 3. In organ cultures of foetal rat liver explants (18.5 days to term), triamcinolone (20 μg/ml of medium) evoked a 2.8–4.3-fold increase after 24h of incubation. This increase was completely inhibited by actinomycin D (25 μg/ml) or cycloheximide (10 μg/ml). Cortisol (5–50 μg/ml) or glucagon (0.067–67 μg/ml) also increased the arginine synthetase system activity above the respective control values, but there was no increase in activity with insulin (0.05–0.25 i.u./ml). 4. Maximum concentrations of glucagon (67 μg/ml), dibutyryl cyclic AMP (6-*N*,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate) (0.1 mM) and triamcinolone (20 μg/ml) incubated for 24h with foetal rat liver explants each produced between a two- and three-fold increase in the activity of the arginine synthetase system. Combinations of maximum amounts of glucagon and the cyclic nucleotide did not produce a greater effect than either agent alone. However, the combination of dibutyryl cyclic AMP with triamcinolone appeared to produce somewhat less than additive effects. 5. The effects of the cyclic nucleotide and triamcinolone were evident after 12h of incubation and increased steadily throughout the 24h of observation. This time-course of increased enzyme activity is very much slower than that reported for the induction of other enzymes in explant cultures of foetal rat liver.

The arginine synthetase system, composed of argininosuccinate synthetase [1-citrulline–L-aspartate ligase (adenosine 5′-phosphate), EC 6.3.4.5] and argininosuccinate lyase (L-argininosuccinate arginine-lyase, EC 4.3.2.1), catalyses the synthesis of arginine from citrulline (Ratner, 1955). In urea biosynthesis the arginine synthetase system contains the rate-limiting step, namely argininosuccinate synthetase (Kennan & Cohen, 1959; Kekomäki *et al.*, 1970), as the maximum arginine synthetase system activity is always fivefold less than the argininosuccinate lyase activity during the development of the rat (Räihä & Suihkonen, 1968). During development the arginine synthetase system in the rat undergoes a fivefold increase in activity after normal delivery. Corticosteroids are capable of increasing the activities of the system in adult (Freedland & Sodikoff, 1962), and postnatal rats (Räihä & Suihkonen, 1968). In addition, injection of glucagon into adult rats has been found to increase activities of the system (McLean & Novello, 1965).

Studies on the induction of rat liver tyrosine transaminase (L-tyrosine–2-oxoglutarate aminotransferase, EC 2.6.1.5) *in vivo* indicate that the competence of the liver to respond to hormonal inducers may be present in the late foetal liver, but can be elicited only under specific environmental conditions (Greengard, 1969*a*,*b*). However, in explant cultures of foetal rat liver, Wicks (1968) has provided an isolated liver system in which induction by hormones can be observed easily.

The present paper reports a development study on the effects of pharmacological agents on the arginine synthetase system. The response of the arginine synthetase system activity to administration *in vivo* of

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steroid, glucagon and insulin and to incubation of foetal liver *in vitro* with steroid, glucagon and dibutyryl cyclic AMP (6-*N*,2′-*O*-dibutyryladenosine 3′:5′-cyclic monophosphate) were examined.

An abstract of a portion of this study has been reported at the American Institute of Biological Sciences Meeting, 23–29 August 1970.

Experimental

Materials

Actinomycin D was a gift of Merck, Sharp and Dohme, Rahway, N.J., U.S.A. Cycloheximide was purchased from Nutritional Biochemicals, Cleveland, Ohio, U.S.A. Insulin was obtained from Norvo Industri, Copenhagen, Denmark; glucagon from Eli Lilly, Indianapolis, Ind., U.S.A.; hydrocortisone acetate (cortisol) from Merck, Sharp and Dohme; triamcinolone acetonide (Kenacort) from Squibb, Stockholm, Sweden; dibutyryl cyclic AMP from Calbiochem, Los Angeles, Calif., U.S.A. [1-^{14}C]-Leucine (specific radioactivity 62mCi/mmol) and [5-^{3}H]orotic acid (specific radioactivity 15Ci/mmol) were purchased from Amersham/Searle, Des Plaines, Ill., U.S.A. [*ureido*-^{14}C]Citrulline (specific radioactivity 3.7mCi/mmol) was obtained from New England Nuclear Corp., Boston, Mass., U.S.A. The culture medium, Eagle's minimum essential medium in Hanks balanced salt solution with twice the normal concentrations of bicarbonate, glucose and L-glutamine and with 100 units of penicillin and 100μg of streptomycin added per ml, was purchased from Grand Island Biological Co., Grand Island, N.Y., U.S.A.

Studies in vivo

Adult female rats of a Sprague–Dawley strain (Orion Oy/Ab) were mated for a 16h period, so that the time of conception was known to have occurred within this confined period. At the appropriate time of gestation (i.e. 19.5 or 20.5 days) the mother was lightly anaesthetized with ether and a laparotomy was performed. Injections into the peritoneal cavities of all foetuses in the larger horn were performed through the uterine wall. Animals in the other horn served as saline-injected controls. The uterus was replaced and the incision closed. The entire time for the operation was less than 5min. After 24h the mother was killed by a blow to the head and immediately the foetuses were removed and their livers excised.

Postnatal studies were performed by an intraperitoneal injection of triamcinolone, cortisol, glucagon, or insulin, all in 0.9% NaCl, to one half of the members of one litter; the remaining littermates were given only 0.9% NaCl. All injections *in vivo* were in volume of 0.075ml and the period between the administration of the agent and the excision of the liver was 24h, unless specified otherwise. All animals remained with their mother during the experimental period.

Organ culture

A complete description of the organ culture system has been reported (Räihä *et al.*, 1971). As this system was used in both laboratories during this investigation and small differences in the experimental conditions exist, the experiments performed in Helsinki are referred to as series A and those in Cleveland as series B.

In series A, adult female rats of a Sprague–Dawley strain (Orion Oy/Ab, Helsinki) were mated for a 16h period. The mother was killed at the appropriate time and the foetal livers were removed under sterile conditions. In series B, adult Holtzman–Sprague–Dawley strain (Holtzmann, Wis., U.S.A.) rats were mated for a 24h period, after which vaginal smears were taken and those animals found to have sperm present were considered to be at day 1 of gestation. All mothers were killed at 19.5 day of gestation (2.5 days before birth) and the foetal livers were removed under sterile conditions. Each experiment was performed with livers pooled from foetuses of the same litter. The livers were cut into 1mm cubes and placed on stainless-steel grids, which supported the tissue between the liquid medium and gas phase, air+CO_2 (95:5) (series A) or O_2+CO_2 (95:5) (series B). All explants were maintained in culture at least 24h before experiments began. The pH of the medium did not decrease below 7.4 throughout the duration of the experiments.

Analytical methods

Arginine synthetase system activity. The maximum arginine synthetase system activity from experiments *in vivo* was measured by the method of Räihä & Suihkonen (1968). Freshly excised liver was homogenized (1:10, w/v) and after centrifugation at 4000*g* a sample of the supernatant solution was incubated in a phosphate-buffered medium (pH7.8) containing [*ureido*-^{14}C]citrulline (15000 c.p.m./sample), L-aspartate, ATP, $MgSO_4$ (all 3.6mM) and an excess of arginase. After incubation, the samples were transferred to sealed vessels into which urease was injected to cleave the [^{14}C]urea. The $^{14}CO_2$ was quantitatively recovered in Hyamine (Packard Instrument, La Grange, Ill., U.S.A.) and counted in a Packard Tri-Carb scintillation counter. All analyses were performed in duplicate and enzyme activity is expressed per g wet wt. of liver.

Slight modifications were made for the analysis of

the system activity from foetal liver explants in culture. For this procedure 75 explants from three grids (one grid from each of three dishes) were pooled and quickly frozen. The remaining 75 explants were treated in an identical manner. Thus local culture effects were equally distributed. Duplicate determinations were made from each pooled sample, which was homogenized in 0.160ml of 0.1% cetyltrimethylammonium bromide (Eastman Organic Chemicals, Rochester, N.Y., U.S.A.), then centrifuged for 30min at 4°C and 14000*g* and 0.050ml of the supernatant fraction was analysed in the usual manner. Preliminary results showed that enzyme activity per unit volume of the 4000*g* and 14000*g* supernatant fractions were equal.

Thus specific enzyme activity calculated for the protein content of the whole homogenate is the same with either fraction. The method of Lowry *et al.* (1951) was used for all protein determinations. In series A, the specific enzyme activity is expressed as the system activity/g of protein of the whole homogenate. In series B, protein determinations were made on the 14000*g* supernatant fraction and the specific activity is expressed as the system activity/g of protein of the 14000*g* supernatant.

Glucose 6-phosphatase activity. The activity of glucose 6-phosphatase (EC 3.1.3.9) was measured in the direction of glucose formation by release of P_i as described by de Duve *et al.* (1955), in which 0.030ml of whole tissue homogenate was added to a reaction mixture to yield final concentrations of 7mM-histidine, 1mM-EDTA and 0.04M-glucose 6-phosphate. This mixture was incubated at 37°C after which the trichloroacetic acid-supernatant fraction was analysed for P_i by the method of Fiske & SubbaRow (1925). In experiments in which both the arginine synthetase system and glucose 6-phosphatase were measured, 100 explants from four grids (one from each of four dishes) were pooled and quickly frozen. Homogenates were prepared in 0.20ml of 0.1% cetyltrimethylammonium bromide, of which 0.030ml was used for each glucose 6-phosphatase activity determination. The remaining 0.140ml was centrifuged at 14000*g* at 4°C for 30min and the supernatant fraction was used for the arginine synthetase system activity and protein determinations as described above. The activity of glucose 6-phosphatase is expressed as μmol of P_i released/10min per mg of protein (of the 14000*g* supernatant fraction).

Both system activity and glucose 6-phosphatase activity are expressed in terms of the protein in the 14000*g* supernatant fraction. The system activity could also be expressed in terms of glucose 6-phosphatase activity, e.g. nmol of urea formed/30min per μmol of P_i formed per 10min. Statistical significance is expressed as *P* values calculated from Student's *t* test.

Incorporation of [^{14}C]leucine and [^{3}H]orotic acid

After preincubation, pulses of either 1μCi of [1-^{14}C]leucine or 75μCi of [5-^{3}H]orotic acid were added to the culture dishes. After 7min, one grid from each dish was removed. The remaining grid was removed after an additional 60, 120, or 180min. The explants were immediately removed and quickly frozen. The tissues were then homogenized in 0.10ml of 0.1M-KCl and samples were used for the determination of protein, total radioactivity or trichloroacetic acid-precipitable radioactivity, as described by Räihä *et al.* (1971), in which samples are spotted on filter-paper discs, precipitated with cold 5% trichloroacetic acid, rinsed with ethanol and counted directly in Bray's (1960) solution in a Nuclear–Chicago scintillation counter. The 7min sample from each dish served as an internal blank for each determination.

Electron-microscopic procedures

The explants were fixed sequentially in formaldehyde–glutaraldehyde and in osmium tetroxide, then were dehydrated in ethanol and embedded in Araldite. Sections were stained with uranyl acetate and lead citrate and examined in an AEI EM-6B electron microscope. Suitable controls from freshly excised foetal liver were also examined.

Results

Development of the arginine synthetase system in vivo

Fig. 1 shows the normal development of the arginine synthetase system in the rat during the perinatal period. The system activity remained low during foetal development, but within the first two postnatal days underwent an eightfold increase. This pattern of development is consistent with those reported by Kennan & Cohen (1959) and Räihä & Suihkonen (1968).

Induction of the arginine synthetase system in vivo

Steroid effects. The effects of exogenous corticosteroid administered during the perinatal period were studied to evaluate their stimulatory effect as a function of the developmental age of the rat. The dose, 19–190μg of triamcinolone or cortisol/animal, corresponds on a weight basis to the doses given to adult rats to increase the activity of the arginine synthetase system (McLean & Gurney, 1963). There is a statistically significant increase in the values in all postnatal animals 24h after administration of triamcinolone *in vivo* (Table 1). The same general pattern of response was exhibited when cortisol was administered in place of triamcinolone, although the

effects were less pronounced in postnatal animals. No stimulation could be elicited after steroid administration to foetal rats *in utero*.

Glucagon and insulin effects. As glucagon increases the arginine synthetase system activity in adult rats (McLean & Novello, 1965) and as the early hypoglycaemic response in neonatal animals (Dawkins, 1963) occurs at the same time as the postnatal rise in system activity, the effects of glucagon administration during the perinatal period were examined. The dose used corresponded on a weight basis to that used in adult rats by McLean & Novello (1965). After exposure to this hormone for 24h, there was no increase in activity apparent ($P>0.05$) *in utero*. An effect was observed on 5-day-old animals, whereas no effect was observed with 1-day-old animals (Table 2).

The effects of pharmacological doses of insulin (4i.u./animal) on rats during the perinatal period was examined, but the results were inconclusive. All animals injected *in utero* died. The results from postnatal animals were variable and therefore no conclusions can be made as to the effects of insulin on arginine synthetase system activity during the perinatal period.

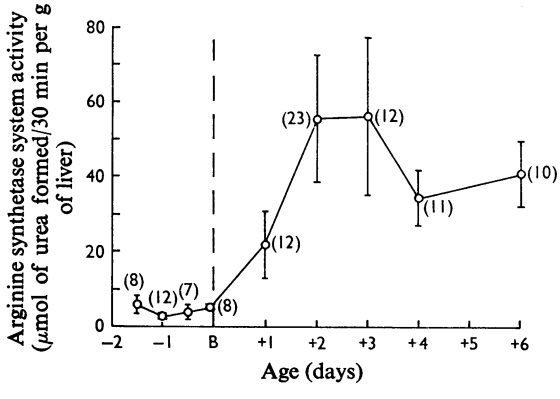

Fig. 1. *Development of arginine synthetase system activity in perinatal rat liver*

Each point represents the mean value observed for the number of animals indicated in parentheses. The time of conception was known within ±8h. The standard deviations are indicated by vertical lines.

Induction of the arginine synthetase system in vitro

Evaluation of the culture system. The biochemical viability of the cultured foetal liver explants was assessed by the incorporation of [1-^{14}C]leucine or [5-^{3}H]orotic acid into trichloroacetic acid-precipitable material of the tissue. After 24h in culture the incorporation of [1-^{14}C]leucine (into protein) continually increased during the 3h pulse period (Fig. 2*a*). The total radioactivity/mg of protein also increased throughout the 3h pulse period. After 54h in culture, the incorporation of [1-^{14}C]leucine progressively increased during the entire pulse period (Fig. 2*b*). The values at 1h are identical with those after 24h in culture, although incorporation at 2 and 3h was slightly greater than that after 24h in culture. The total radioactivity/mg of protein also increased after each hour of pulse (Fig. 2*b*), but the absolute values were greater than those observed after 24h in culture.

Table 1. *Effect of triamcinolone on the arginine synthetase system during development*

Each experiment was performed on one litter of animals. At the time indicated the animals were injected with triamcinolone in 0.075ml of saline; the controls received only the saline. All animals remained with their mother during the experimental period. The animals were killed 24h after administration of the agent and the activity of the system was determined on fresh liver samples. Each value represents one animal and activities are expressed as the mean ± S.D. (number of animals). *P* values were calculated by Student's *t* test; n.s., >0.05.

Age (days)	Dose of triamcinolone (μg/animal)	Arginine synthetase system activity (μmol of urea formed/30min per g of liver) Control	Experimental	*P*
−2.5*	190	7.2± 1.1 (5)	9.6± 2.4 (6)	n.s.
−1.5*	19	5.1± 0.7 (4)	5.8± 1.9 (6)	n.s.
+1.0	19	37.9± 4.1 (3)	61.4± 9.6 (4)	<0.005
	190	29.6± 3.2 (3)	73.4± 3.3 (4)	<0.001
+1.5	190	32.5± 9.5 (2)	70.8±16.9 (5)	<0.01
+2.0	100	48.2±10.2 (3)	68.9± 1.3 (3)	<0.02
+5.0	190	40.3± 9.1 (4)	79.4±14.0 (4)	<0.005

* *In utero.*

Table 2. *Effect of glucagon on the arginine synthetase system during development*

The experiments were performed in the same way as those described in the text of Table 1. All experimental animals were injected with 100 μg of glucagon. Each value represents one animal and activities are expressed as the mean ± S.D. (number of animals).

Age (days)	Arginine synthetase system activity (μmol of urea formed/30 min per g of liver) Control	Experimental	*P*
−2.5*	2.4± 0.4 (4)	2.3±0.5 (6)	n.s.
+1.0	24.7± 3.9 (6)	21.8±4.5 (6)	n.s.
+2.0	48.4±10.2 (3)	57.7±6.5 (3)	n.s.
+5.0	41.2± 9.1 (6)	53.0±4.3 (7)	<0.01

* *In utero.*

The incorporation of [5-^{3}H]orotic acid into trichloroacetic acid-precipitable material (mainly RNA) from explants after 30h in culture was greater at each successive hour of pulse (Fig. 3). The intercept on the *x*-axis of the trichloroacetic acid-precipitated radioactivity is at approx. 20–30min, which suggests that there may be a lag period. The total radioactivity/mg of protein at 2h was twice that at 1h, and remained at this value at the third hour (Fig. 3).

The total protein/grid decreased during incubation. The total mg of protein/grid (n=9) at 24, 30 and 54h in culture was 0.94±0.03, 0.89±0.03 and 0.69±0.03 respectively. The protein content in samples at 30h was not significantly less than in the 24h samples ($P \approx 0.08$). However, after 54h the protein content was significantly less than after 30h ($P<0.001$).

Cultured liver explants have also been examined ultramicroscopically. A detailed evaluation of the cultured tissue will not be given here, but a few of the more general findings from this study must be considered. The general appearance of the cultured tissues was similar to the fresh controls. The explants generally showed mitochondria of normal shape, size and distribution. The rough endoplasmic reticulum also appeared in normal arrangement. Although in some cells of the explants there was mitochondrial swelling, dilation of the endoplasmic reticulum and more vacuoles (lysosome formation?), the cellular and nuclear membranes were undisturbed, and it is fair to state that the ultrastructural details of explants after 36h in culture were similar to those in uncultured controls.

In order to validate the expression of arginine synthetase system activity on a protein basis, the increase in the system activity was expressed on the basis of a non-induced enzyme, glucose 6-phosphatase. The glucose 6-phosphatase activity/mg of protein after 24h incubation with triamcinolone was identical with that of the controls, whereas the system activity/mg of protein measured in the same tissues increased 2.73-fold (Table 3). When the system activity was expressed relative to that of glucose 6-phosphatase, the activity of the triamcinolone-incubated samples was increased by 2.77-fold (Table 3). In addition the protein content/grid was the same in cultures incubated for 24h with 20 μg of triamcinolone/ml (0.82 ± 0.06 mg) as in control cultures (0.82 ± 0.07 mg) (14 experiments).

Effects of triamcinolone. In order to examine the direct effects of corticosteroids on the isolated foetal liver, experiments were performed on foetal rat liver explants maintained in organ culture. The system activity increased well above control values in every

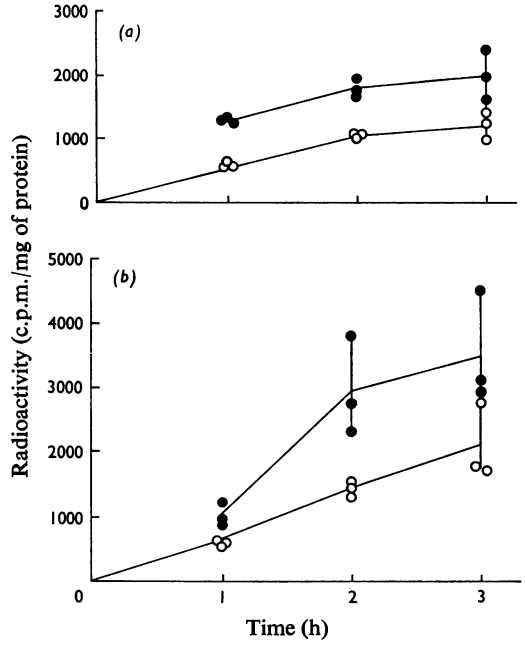

Fig. 2. *Incorporation of [^{14}C]leucine into foetal rat liver explants in organ culture*

After 24h (*a*) or 54h (*b*) of preincubation, 1.0 μCi of [1-^{14}C]leucine was added to each culture dish. At 7min one grid was removed from each dish to serve as a blank. The range of the 7min blank values was 19–90c.p.m./mg of protein and 150–550c.p.m./mg of protein for the precipitable and total radioactivity, respectively. The remaining tissues were removed at 67, 127 or 187min and were analysed for total (●) or trichloroacetic acid-precipitable (○) radioactivity. Each point represents one determination.

experiment in which either triamcinolone or cortisol (5–50 μg/ml) was added. When organ cultures of foetal liver explants were incubated with 20 μg of triamcinolone/ml of medium the system activity increased threefold (Table 4). It is difficult to decide on a maximum dose from the results in Table 4, in that the values do not allow differentiation between effects of 0.2 and 20 μg/ml. However, it appeared to require 18 h for an effect of 0.2 μg/ml to become visible (Fig. 4*a*), whereas incubation with 20 μg/ml indicated that 12 h of exposure was sufficient to produce a significant effect ($P<0.01$) (Fig. 4*b*). Therefore a concentration of 20 μg/ml was used for further experiments. An increase in the system activity well above the control value (24.2, 27.6 μmol of urea formed/30 min per g of liver protein) was also produced after incubation of 20 μg of triamcinolone/ml with explants from 18.5-day foetuses (103, 103 μmol of urea formed/30 min per g of liver protein).

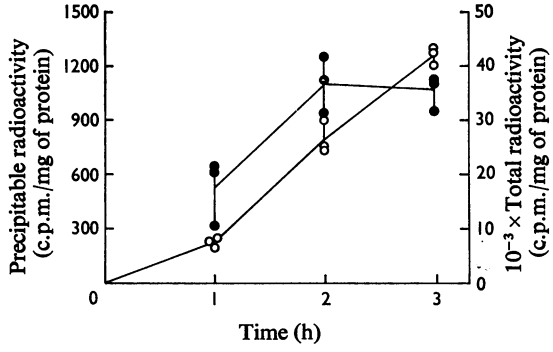

Fig. 3. *Incorporation of [5-^{3}H]orotic acid into foetal rat liver explants in organ culture*

After 30 h of preincubation, 75 μCi of [5-^{3}H]orotic acid was added to each culture dish. At 7 min one grid was removed to serve as a blank. The range of the 7 min blank values was 0–16 c.p.m./mg of protein and 6800–13 000 c.p.m./mg of protein for the precipitable and total radioactivity, respectively. The remaining tissues were removed at 67, 127, or 187 min and were analysed for total (●) or trichloroacetic acid-precipitable (○) radioactivity. Each point represents one determination.

The rise in the system activity is probably attributable to an increased production of enzyme protein. When cycloheximide (10 μg/ml) was added 4 h before the steroid, the rise in the system activity was prevented (Table 5). The same result was also observed when cycloheximide was added 4 h after the steroid (Table 5). The addition of actinomycin D (25 μg/ml) to the medium also prevented the rise in system activity when added 4 h before or 4 h after the steroid (Table 5). It is possible that changes in protein concentration of the explants after incubation with steroid or antibiotics could influence the observed specific activity of the system. However, any such changes in protein content could have had only a minor effect on the values, because the greatest difference in protein content between experimental and control explants was 29%.

Effects of glucagon and dibutyryl cyclic AMP. Both glucagon (67 μg/ml) and dibutyryl cyclic AMP (0.1 mM) produced a twofold increase in arginine synthetase system activity in explants of foetal liver (Table 6). The inclusion of 0.5 mM-theophylline increased the response to 0.1 mM-dibutyryl cyclic AMP ($P<0.02$), but not to either high concentrations of glucagon or triamcinolone (Table 6). The response to 6.7 μg of glucagon/ml was significantly less than the response to 67 μg/ml ($P<0.05$), in the presence of theophylline. In an experiment in which the dose response to dibutyryl cyclic AMP in the presence of 0.5 mM-theophylline was examined, 0.1 mM-dibutyryl cyclic AMP increased system activities from 22.6 ± 1.2 (2) to 76.9 ± 3.3 (2) μmol of urea formed/30 min per g of protein. The response to 1 mM-dibutyryl cyclic AMP [63.6 ± 2.6 (2) μmol of urea formed/30 min per g of protein] was significantly less than

Table 3. *Effect of triamcinolone on the arginine synthetase system in foetal rat liver explants in culture*

Explants were prepared from 19.5-day foetuses. After 24–28 h of preincubation, agents were added to the medium. The explants were removed from the cultures 24 h later and 100 explants were pooled for each duplicate determination of system activity or glucose 6-phosphatase activity, as described in the text. Each value represents the mean ± S.E.M. of six duplicate determinations.

Final concentration of triamcinolone (μg/ml)	Arginine synthetase system activity (μmol of urea formed/30 min per g of protein) I	Glucose 6-phosphatase activity (mmol of P_i formed/10 min per g of protein) II	Ratio I/II
A 0.0	34.1 ± 2.0	1.52 ± 0.13	24.2 ± 4.4
B 20.0	92.8 ± 5.6	1.47 ± 0.13	67.1 ± 9.0
Ratio B/A	2.73	0.97	2.77

Table 4. *Effect of triamcinolone on the arginine synthetase system in foetal rat liver explants in culture*

Explants in series A were prepared from 21-day to term foetuses. Explants in series B were prepared from 19.5-day foetuses. After 24–28h of preincubation, triamcinolone was added to the medium. The explants were removed from the cultures 24h later and analysed for system activity, as described in the text. Each value represents the mean ± S.E.M. (the number of duplicate determinations).

Series	Final concentration of triamcinolone (μg/ml)	Arginine synthetase system activity (μmol of urea formed/30min per g of protein)	
		Control	Experimental
A	0.2	32.1 ± 3.7 (6)	89.3 ± 12.8 (6)
	20.0	28.0 ± 3.1 (15)	87.1 ± 5.7 (15)
B	20.0	34.1 ± 2.1 (6)	93.5 ± 5.1 (6)

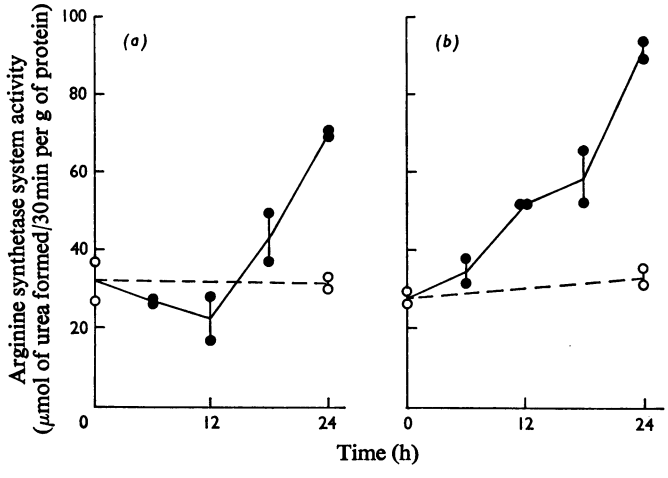

Fig. 4. *Time-course of induction of arginine synthetase system activity by triamcinolone*

(*a*) Explants were prepared from term foetuses (series A). After 24h of preincubation triamcinolone was added (time 0) to yield a final concentration of 0.20μg/ml. (*b*) Explants were prepared from 19.5-day foetuses (series B). After 24h of preincubation triamcinolone was added (time 0) to yield a final concentration of 20μg/ml. At the appropriate times explants were removed and were pooled for each determination of arginine synthetase system activity, as explained in the text. Each point represents one duplicate determination. ○, Control; ●, triamcinolone incubated.

that to 0.1mM ($P<0.05$). Similarly, the response to 0.01mM-dibutyryl cyclic AMP [48.3 ± 8.6 (2) μmol of urea formed/30min per g of protein] was also significantly less than that to 0.1mM ($P<0.05$). Thus in order to obtain maximum effects with dibutyryl cyclic AMP, experiments were done with 0.1mM-dibutyryl cyclic AMP in the presence of 0.5mM-theophylline.

The maximum response to 0.1mM-dibutyryl cyclic AMP was the same as the maximum response to glucagon at a concentration of 67μg/ml. Further, the response to the combination of 0.1mM-dibutyryl cyclic AMP, plus glucagon, 67μg/ml, was statistically the same as that to either agent alone (Table 6).

On the other hand, the combination of dibutyryl cyclic AMP with triamcinolone produced an effect greater than that of either agent alone ($P<0.05$) (Table 6). The time-course of effects produced by dibutyryl cyclic AMP and triamcinolone separately and in combination is presented in Fig. 5. The arginine synthetase system activity of the control samples remained constant throughout the experimental period, although there was greater variation in the zero time samples than in later controls. The response to both dibutyryl cyclic AMP and triamcinolone increased progressively. With either agent, the increase after 12h was small, but statistically significant when compared with the 12h

Table 5. *Effect of cycloheximide or actinomycin D on triamcinolone-induced arginine synthetase system activity*

Explants were prepared from 21-day foetuses in experiments with cycloheximide or term-foetuses in experiments with actinomycin D. After 26–31 h of preincubation additions were made to the medium to yield final concentrations of 20 μg of triamcinolone/ml, 10 μg of cycloheximide/ml or 25 μg of actinomycin D/ml. After 50–55 h in culture the explants were removed and system activity was determined, as described in the text. Each value represents the mean ± S.E.M. (number of duplicate determinations).

Agent	Total time of exposure (h)	Arginine synthetase system activity (μmol of urea formed/30 min per g of protein)
Control	—	22.5 ± 1.4 (4)
Triamcinolone	20–24	78.0 ± 11.7 (4)
Cycloheximide	20–24	24.6 ± 3.8 (4)
Triamcinolone + cycloheximide	24 / 20	28.2 ± 4.5 (2)
Cycloheximide + triamcinolone	24 / 20	10.2 ± 0.2 (2)
Control	—	31.5 ± 4.2 (4)
Triamcinolone	20–24	79.6 ± 8.3 (4)
Actinomycin D	20–24	9.6 ± 3.3 (4)
Triamcinolone + actinomycin D	24 / 20	18.5 ± 3.5 (2)
Actinomycin D + triamcinolone	24 / 20	8.2 ± 3.2 (2)

controls ($P \approx 0.02$ for dibutyryl cyclic AMP; $P < 0.05$ for triamcinolone).

The response to combinations of dibutyryl cyclic AMP plus steroid was greater than that to either agent alone at all times examined. This effect was greater than the control values, as early as 12 h ($P < 0.05$), and produced a 4.4-fold increase after 24 h (Table 6).

When insulin (0.05–0.25 i.u./ml) was incubated for 24 h with explants [25.5 ± 3.8 (4) μmol of urea formed/30 min per g of protein] no increase above control values [27.8 ± 1.5 (2) μmol of urea formed/30 min per g of protein] was evident.

Discussion

The viability of the explants in this culture system appears to be maintained, as suggested by all of the criteria employed, both biochemical and morphological. The ultrastructural appearance of the cultured tissue is similar to the freshly excised controls and shows no signs of the cellular necrosis seen, for example, in studies of mouse liver incubated *in vitro* (Trump *et al.*, 1965).

Incorporation of [1-^{14}C]leucine and [5-^{3}H]orotic acid into trichloroacetic acid-precipitable material (protein and RNA, respectively) also attest to the viability of the system. In studies with this type of system, Wicks (1968) and Räihä *et al.* (1971) examined protein and RNA synthesis with 1 h pulses; however, it would seem that assessing the rate of incorporation over a 3 h period is a more rigorous criterion for the viability of the biosynthetic machinery of the tissue. The slight difference in the rate of precipitable [1-^{14}C]leucine accumulation after different preincubation times may be due to the decrease in total protein of the explants. This may simply represent loss of preformed erythrocytes (Wicks, 1968) or may be due to the wash-out of plasma proteins (Kekomäki *et al.*, 1971). In addition, enzyme induction might be considered as another criterion of viability.

The results of the present study suggest that either corticosteroids or glucagon are capable of inducing an increase in the arginine synthetase system in the postnatal rat *in vivo* but neither has this effect *in utero*. (The term 'induction' here implies the progressive differential accumulation of enzymes.) However, both of these agents can elicit the inductive response from isolated foetal liver explants in culture.

The stimulation of arginine synthetase system activity after corticosteroid administration *in vivo* (Table 1) is consistent with earlier reports of corticosteroid stimulation of the urea cycle enzymes in older rats. Freedland & Sodikoff (1962) first reported a 280% stimulation of the arginine synthetase system by cortisol in adult rats. Schimke (1963) and McLean & Gurney (1963) found, respectively, 140 and 120% increases in the arginine synthetase system activity in adult rats after cortisol administration. Räihä & Suihkonen (1968) have reported the inability of adrenalectomized newborn rats to undergo the

Table 6. *Effect of combinations of glucagon, dibutyryl cyclic AMP and triamcinolone on the arginine synthetase system in foetal rat liver explants in culture in the presence of theophylline*

The experiments were performed in the same way as those described in the text of Table 3. The final concentration of theophylline was 0.5 mM. Each value represents the mean ± S.E.M. with the number of duplicate determinations in parentheses.

Agent	Final concentration	Arginine synthetase system activity (μmol of urea formed/30 min per g of protein) With theophylline	Without theophylline
Control	—	30.4 ± 2.6 (12)	31.9 ± 2.1 (13)
Glucagon	6.7 μg/ml	57.3 ± 5.7 (6)	61.8 ± 6.2 (2)
	67.0 μg/ml	69.9 ± 4.0 (8)	65.0 ± 4.0 (2)
Dibutyryl cyclic AMP	0.1 mM	78.7 ± 6.2 (12)	58.8 ± 5.2 (2)
Triamcinolone	20 μg/ml	94.0 ± 9.1 (6)	93.5 ± 5.1 (6)
Glucagon + dibutyryl cyclic AMP	67 μg/ml + 0.1 mM	75.0 ± 0.2 (2)	—
Dibutyryl cyclic AMP + triamcinolone	0.1 mM + 20 μg/ml	129.1 ± 13.2 (6)	—

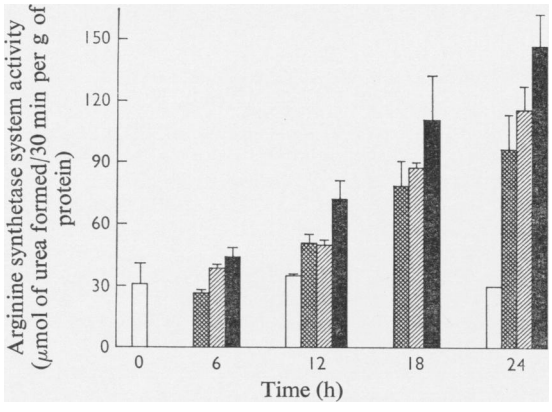

Fig. 5. *Time-course of induction of arginine synthetase system activity by dibutyryl cyclic AMP and triamcinolone*

Explants were prepared from two litters of 19.5-day foetuses. After 24 h of preincubation, either dibutyryl cyclic AMP, triamcinolone or both were added to yield final concentrations of 0.1 mM (dibutyryl cyclic AMP) or 20 μg/ml (triamcinolone). All experiments were done with a final concentration of 0.5 mM-theophylline. At the appropriate times, explants were removed from culture and pooled for determination of arginine synthetase system activity, as explained in the text. Each value represents the mean of two duplicate determinations; the vertical bar indicates the standard error. □, Control; ▩, dibutyryl cyclic AMP; ▨, triamcinolone; ■, dibutyryl cyclic AMP plus triamcinolone.

normal postnatal increase in system activity, and the prevention of this effect of adrenalectomy by injection of triamcinolone.

In addition, the effects of glucagon administration to postnatal rats *in vivo* (Table 2) are consistent with the report of McLean & Novello (1965), that glucagon increased the activity of the system in adult rats *in vivo*.

The lack of stimulation *in utero* by steroid or glucagon (Tables 1 and 2) suggests that either the liver is incapable of responding at this stage of development or that some suppressive phenomenon is present *in utero*. The first of these possibilities seems unlikely, as both steroid and glucagon increase the activity of the system after incubation with foetal liver explants.

In this report evidence has been presented demonstrating that both glucagon and glucocorticoids can increase the arginine synthetase system in foetal rat liver in organ culture. The ability of triamcinolone to increase the system activity in explants *in vitro* conforms to the general phenomenon of enzyme induction (in cultured tissue), i.e. this increased enzyme activity is blocked by inhibitors of protein and RNA synthesis. These effects were evident in experiments with cycloheximide and actinomycin D (Table 5). The results from these experiments also provide some preliminary information about the time-course of the steroid-mediated increase in enzyme activity. In other experiments, it was observed that the increased activity was not present before the 18th hour with a dose of 0.2 μg of triamcinolone/ml (Fig. 4*a*). Although 0.2 μg/ml of triamcinolone appears to produce maximum effects after 24 h of exposure, the rate of increase may be dependent upon concentration, as studies with 20 μg/ml produced significant increases after 12 h of exposure (Fig. 4*b*).

The increase in arginine synthetase system activity after steroid incubation with explants from 18.5-day foetuses suggests that younger foetal liver is capable

of responding to steroid in a way similar to tyrosine transaminase activity (Räihä *et al.*, 1971).

The results available thus far are compatible with the hypothesis that cyclic AMP mediates the action of glucagon, but not of glucocorticoids, in a way similar to the interpretations applied to observations on the hormonal induction of tyrosine transaminase (Wicks, 1969; Granner *et al.*, 1968). The evidence for cyclic AMP-mediation of glucagon action can be summarized as follows: first, incubation of foetal liver explants with dibutyryl cyclic AMP for 24h produced more than a twofold increase in system activity; secondly, the maximum effect of dibutyryl cyclic AMP was not less than the maximum effect of glucagon; and thirdly, the effect of a combination of maximum amounts of dibutyryl cyclic AMP and glucagon was not greater than the effect of dibutyryl cyclic AMP alone. By contrast, the effect of a combination of maximum amounts of dibutyryl cyclic AMP and triamcinolone was greater than that of dibutyryl cyclic AMP alone and on the average appeared to be somewhat less than the sum of the effects of the two agents individually (Table 6). These results differ from the observations of Wicks (1969) who reported that maximum doses of cortisol and dibutyryl cyclic AMP produced considerably greater than additive effects on the accumulation of tyrosine transaminase.

There are a number of other differences between the effects of hormonal agents on the activity of the arginine synthetase system and on the induction of tyrosine transaminase in foetal rat liver explants: first, insulin produced no effect on arginine synthetase system activity in contrast with its inductive effects on tyrosine transaminase (Wicks, 1969); secondly, although the effects of cortisol and dibutyryl cyclic AMP on tyrosine transaminase become visible after 1–2h of incubation (Wicks, 1969), the effects of triamcinolone and dibutyryl cyclic AMP on arginine synthetase system activity require between 6 and 12h to become apparent; and thirdly, the effects of both triamcinolone and dibutyryl cyclic AMP on arginine synthetase system activity progress steadily for at least 24h as does the effect of cortisol on tyrosine transaminase accumulation (Wicks, 1968), but the action of dibutyryl cyclic AMP on the latter enzyme appears to decrease after 6h (Wicks, 1969). It is possible that the smaller effect of 24h of exposure to 1mM-dibutyryl cyclic AMP compared with that of 0.1mM-dibutyryl cyclic AMP observed here was the result of an earlier maximum response at the higher concentration followed by a subsequent decrease in the activity of the system. The explanation of these peculiar effects of dibutyryl cyclic AMP is not apparent at this time.

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