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Induction of Phosphopyruvate Carboxylase in Neonatal Rat Liver by Adenosine 3′,5′-Cyclic Monophosphate*

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ABSTRACT: The activity of cytoplasmic phosphopyruvate carboxylase (guanosine triphosphate:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) normally develops in liver following birth or premature delivery of the fetus. The enzyme activity can be precociously induced *in utero* by intraperitoneal injection of 3′,5′-cyclic adenosine monophosphate to fetuses, the effect is largely prevented by simultaneous injection of actinomycin D and approximately doubled by simultaneous caffeine injection. The postnatal development of enzyme activity demonstrable in prematurely delivered rats is increased by injection of 3′,5′-cyclic adenosine monophosphate and the effect is potentiated by caffeine. N^6-2′-*O*-Dibutyryl 3′,5′-cyclic adenosine monophosphate is more potent than the parent compound in enzyme induction in both fetuses and premature animals but 5′-adenosine monophosphate, 2′,3′-cyclic adenosine monophosphate, 3′,5′-cyclic thymidine monophosphate, 3′,5′-cyclic guanosine monophosphate, and 3′,5′-cyclic inosine monophosphate are ineffective. No effect of dibutyryl 3′,5′-cyclic adenosine monophosphate is found on fetal liver activities of fructose diphosphatase and pyruvate kinase. Repression of phosphopyruvate carboxylase synthesis in premature rat liver is achieved by injection of insulin, ergotamine tartrate, or prostaglandin PGE_1. Activation of hepatic phosphopyruvate carboxylase by 3′,5′-cyclic adenosine monophosphate *in vitro* could not be demonstrated. The results are discussed in terms of mechanisms of enzyme induction in tissue differentiation.

Previous work has shown that the activity of cytoplasmic phosphopyruvate carboxylase (GTP[1]:oxaloacetate carboxy-lyase transphosphorylating, EC 4.1.1.32) develops postnatally in the rat liver (Ballard and Hanson, 1967; Yeung *et al.*, 1967) and that premature delivery of fetuses by uterine section also results in rapid appearance of the enzyme (Yeung and Oliver, 1967). Administration of the glucocorticoid analog, triamcinolone, to fetal rats *in utero* does not lead to development of the enzyme (Yeung *et al.*, 1967). In a more recent study Yeung and Oliver (1968) have shown that the administration of glucagon, adrenalin, and noradrenalin to fetal rats results in development of the carboxylase activity *in utero*. In addition, glucagon injection to surgically delivered rats potentiates the postnatal production of the enzyme. As these hormones have been shown to promote the production of 3′,5′-cyclic AMP in liver (Sutherland and Robinson, 1966), Yeung and Oliver (1968) have suggested that the cyclic nucleotide may function in the system as an effector molecule bringing about derepression of the synthetic system for cytoplasmic phosphopyruvate carboxylase. In the present paper further evidence, both direct and indirect, is presented to support the hypothesis that 3′,5′-cyclic AMP is involved in the initiation of cyto-

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plasmic phosphopyruvate carboxylase synthesis in neonatal rat liver.

Experimental Procedure

Chemicals. Phosphoenolpyruvate (sodium salt), ITP, ADP, 3′,5′-cyclic IMP, 3′,5′-cyclic GMP, 3′,5′-cyclic TMP, 3′,5′-cyclic UMP, and N^6-2′-*O*-dibutyryl 3′,5′-cyclic AMP were obtained from C. F. Boehringer und Soehne, GmbH Mannheim, Germany. Some preparations of dibutyryl 3′,5′-cyclic AMP were prepared by Dr. W. Segal by the method of Falbriard *et al.* (1967). IDP, oxaloacetic acid, NADH, NADP, fructose 1,6-diphosphate, 3′,5′-cyclic AMP, 5′-AMP, 2′,3′-cyclic AMP, and ergotamine tartrate were from Sigma Chemical Co., St. Louis, Mo, U. S.; insulin (B. P.) from Boots Pure Drug Co. Ltd., Nottingham, England; caffeine from British Drug Houses, Dorset, England. Prostaglandin PGE_1 was from Professor S. Bergstrom, Karolinska Institute, Stockholm, Sweden. Glucose phosphate isomerase (EC 5.3.1.9), glucose 6-phosphate dehydrogenase (EC 1.1.1.49), malate dehydrogenase (EC 1.1.1.37), and pyruvate kinase (EC 2.7.1.40) were from C. F. Boehringer und Soehne; lactate dehydrogenase (EC 1.1.1.27), type III, glucose oxidase (EC 1.1.3.4), and peroxidase (EC 1.11.1.7) were from Sigma Chemical Co. $Na_2C^{14}O_3$ (36 Ci/mole) was from the Radiochemical Centre, Amersham Buckshire, England.

Animals. Rats of the Wistar albino strain of *Rattus norwegicus* were used. Fetal ages were established from a fetal age–weight curve on the colony (Yeung *et al.*, 1967). Surgical procedures, premature deliveries by uterine section, and postnatal care of animals were as previously described (Yeung *et al.*, 1967; Yeung and Oliver, 1967).

Assay of Blood Glucose. Blood was obtained by jugular puncture and glucose determined by the glucose oxidase procedure (Huggett and Nixon, 1957).

Enzyme Assays. Phosphopyruvate carboxylase was routinely assayed in liver extracts (Yeung and Oliver, 1967) by the method of Chang and Lane 1966). ^{14}C was counted in a liquid scintillation spectrometer as previously described (Yeung and Oliver, 1967). Enzyme activity is defined as micromoles of oxaloacetate formed per milligram protein per hour at 37°.

Fructose diphosphatase (EC 3.1.3.11) and pyruvate kinase were assayed in liver extracts as described by Yeung *et al.* (1967). Enzyme activities are defined as micromoles of NADP reduced (fructose diphosphatase) or NADH oxidized (pyruvate kinase) per milligram of protein per hour at 37°.

Determination of Protein. Protein concentrations were determined by the method of Lowry *et al.* (1951) after 50-fold dilution of liver extracts in 2% (w/v) Na_2CO_3 in 0.1 N NaOH. Bovine serum albumin was used as a standard.

Results

The intraperitoneal injection of 3′,5′-cyclic AMP into fetal rats *in utero* results in the appearance of cytoplasmic phosphopyruvate carboxylase activity which is still detectable 48 hr after injection. Actinomycin D prevents the induction of activity by 3′,5′-cyclic AMP but caffeine injection potentiates the effect (Table I). The following nucleotides, injected at the same dose as 3′,5′-cyclic AMP, did not result in the development of enzyme activity in fetal rats: 5′-AMP, 2′,3′-cyclic AMP, 3′,5′-cyclic GMP, 3′,5′-cyclic IMP, 3′,5′-cyclic TMP, 3′,5′-cyclic UMP, and 3′,5′-cyclic CMP. The low activities shown in "control" animals are not induced by the operative procedure (Yeung *et al.*, 1967) and have been attributed to interfetal transfer of injected material *via* the placentas and uterine circulation (Yeung and Oliver, 1968). The apparent increased response in larger and therefore older fetuses is probably due to the increased proportion of parenchymal cells in the fetal liver (Oliver *et al.*, 1963) over the particular period of gestation (19–22 days) that was investigated.[2]

In premature rats, injection of 3′,5′-cyclic AMP results in increased production of phosphopyruvate carboxylase and, while caffeine alone is without effect, it potentiates the effect of cyclic AMP when injected at the time of delivery. 5′-AMP has no effect on the postnatal development of enzyme activity (Table II). In adult rats, the injection of 3′,5′-cyclic AMP and caffeine at the same dose (per unit body weight) as in neonatal rats elevated the phosphopyruvate carboxylase activity from 1.82 to 2.99 μmoles per mg of protein per hr. Caffeine alone did not significantly raise the activity.

The treatment of premature rats with 3′,5′-cyclic AMP prevents the transient phase of neonatal hypoglycaemia shown by litter-mate controls receiving saline only and it potentiates the development of enzyme activity (Table III).

The administration of N^6-2′-*O*-dibutyryl 3′,5′-cyclic AMP to fetal and premature rats also results in induction of phosphopyruvate carboxylase activity and it is somewhat more potent than the parent compound (Tables IV and V). It should be noted that this compound has no effect on the activities of fructose diphosphatase and pyruvate kinase in the fetal rat liver (Table VI).

Insulin injection in premature rats at delivery leads to prolongation of the postnatal hypoglycaemia and to repression of the normal postnatal synthesis of phosphopyruvate carboxylase (Table VII). Small doses of ergotamine tartrate have similar effects on enzyme production but the later the drug is given after

[2] The percentage volume of fetal liver occupied by nonparenchymal cells falls rapidly over this period and since the percentage of nucleated cells appearing in the nuclear fraction of standard homogenates decreases in a parallel fashion a constant proportion of such cells (60–70%) are broken by homogenization of liver (D. M. Robertson and I. T. Oliver, unpublished experiments). Accordingly, as the relative numbers of such cells decrease, the yield of nonparenchymal protein would decrease in extracts of older fetal livers, and the specific activity of induced phosphopyruvate carboxylase would be expected to show an apparent rise if the induction by 3′,5′-cyclic AMP is restricted to the parenchymal cell.

TABLE I: Induction of Phosphopyruvate Carboxylase *in Utero* by 3′,5′-Cyclic AMP and the Effect of Actinomycin D and Caffeine.[a]

Treatment	Av Wt of Fetuses When Assayed (g)	Time after Injection (hr)	Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate formed/mg of protein per hr) Control	Test
3′,5′-Cyclic AMP (0.14 μmole)	2.0	3.5	0.020	0.120
	2.7	3.5	0.018	0.115
	3.0	3.5	0.000	0.252
	4.1	3.5	0.040	0.301
	4.7	3.5	0.040	0.312
	5.0	3.5	0.120	0.480
	5.4	3.5	0.110	0.502
3′,5′-Cyclic AMP (0.14 μmole)	2.8	24		0.095
	3.3	24		0.092
	1.8	48		0.036
3′,5′-Cyclic AMP (0.14 μmole) + actinomycin D (2.5 μg)	4.9	3.5	0.045	0.070
	5.2	3.5	0.089	0.090
3′,5′-Cyclic AMP (0.14 μmole)	4.2	4	0.020	0.120
Cyclic AMP + caffeine (1.1 mg)	4.2	4	0.015	0.250

[a] Rat fetuses in one horn of the uterus were injected intraperitoneally with the compounds dissolved in 0.15 M NaCl (test) and animals in the other horn received the same volume of 0.15 M NaCl (control). The fetuses were left *in utero* for the times stated and then removed and killed. The enzyme activity was determined in pooled liver extracts derived from four animals. Each horizontal row represents data obtained from a single litter. In the experiments with caffeine fetuses of approximately equal weight were selected for comparison.

TABLE II: The Effect of 3′,5′-Cyclic AMP, 5′-AMP, and Caffeine on the Induction of Phosphopyruvate Carboxylase in Premature Rats.[a]

Experiment No.	Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate/mg of protein per hr) 1	2	3	4	5	6
Control	0.32	0.34	0.35	0.29	0.32	0.29
Caffeine (1.1 mg)	0.32	0.36	0.36			
3′,5′-Cyclic AMP (0.14 μmole)	0.41	0.42	0.39	0.57	0.48	0.54
Cyclic AMP (0.14 μmole + caffeine (1.1 mg)	0.60	0.80	0.71	0.72		
5′-AMP (0.14 μmole)					0.31	0.30
No. of animals in each group	2	2	2	2	3	3

[a] In each experiment a litter of animals was delivered by uterine section and injected intraperitoneally in groups as shown with 0.15 M NaCl (control) or the test compounds in the amounts shown (dose per animal). Animals receiving 3′,5′-cyclic AMP and 5′-AMP were injected at 0 and 2 hr after delivery, those receiving saline and caffeine at zero time only. All animals were maintained in the humidicrib at 37° for 4 hr, killed, and enzyme activity was assayed in pooled liver extracts from each group.

delivery the less is its effect. Prostaglandin PGE_1 was also tested at doses ranging from 0.01 μg/animal but had no significant effect on postnatal enzyme development except at a dose of 0.25 μg. At this dose the mean repression was only 16% compared with controls but the difference of the means is significant at the 5% level ($t = 2.183$, $df = 12$, and $p < 0.05$; Table VIII). Repeated subcutaneous injections of prostaglan-

TABLE III: Effect of 3′,5′-Cyclic AMP on Blood Glucose and Phosphopyruvate Carboxylase in Premature Rat Liver.[a]

Treatment	Dose/Fetus (μmole)	hr after Delivery	Blood Glucose (mg/100 ml)		Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate/mg of protein per hr)	
			Control	Test	Control	Test
3′,5′-Cyclic AMP	0.14	0	51			
	0.14	2	20	54	0.18	0.30
		3.5	29	54	0.27	0.52
		5	41	53	0.42	0.79
3′,5′-Cyclic AMP	0.14	0	48			
	0.14	2	18	58	0.15	0.27
		3.5	29	60	0.24	0.44
		5	43	60	0.40	0.72

[a] Results are presented from two experiments with a litter of 14 animals in each case. In each experiment the animals were delivered by uterine section. Control animals (six) received an intraperitoneal injection of 0.15 M NaCl and test animals (six) received an injection of 3′,5′-cyclic AMP in 0.15 M NaCl at 0 and 2 hr after delivery. Blood was obtained from both the remaining animals by jugular puncture and pooled for glucose determination. At the time stated blood was pooled from two animals in each group for glucose assay and the livers were pooled and extracted for enzyme assay.

TABLE IV: Induction of Phosphopyruvate Carboxylase in Fetal Rat Liver by N^6-2′-*O*-Dibutyryl 3′,5′-Cyclic AMP.[a]

Treatment	Av Wt of Fetuses (g)	hr after Injection	Phosphopyruvate Carboxylase Act. (μmoles of Oxaloacetate/mg of protein per hr)	
			Control	Test
N^6-2′-*O*-Dibutyryl cyclic AMP (0.14 μmole)	2.0	3.5	0.0	0.202
	2.3	3.5	0.0	0.221
	3.1	3.0	0.04	0.412
	3.7	2.75	0.03	0.492
	4.2	2.75	0.02	0.520
	5.2	2.75	0.12	0.598

[a] Details of experiment as in Table I. Control animals received 0.15 M NaCl, test animals dibutyryl cyclic AMP. Pooled liver extracts were obtained from four fetuses for enzyme assay.

din (0.25 μg each hour after delivery until 1 hr before sacrifice) did not yield more significant results.

Experiments designed to test for activation of phosphopyruvate carboxylase by 3′,5′-cyclic AMP in the presence and absence of caffeine yielded negative results in both adult and fetal liver extracts (Table IX). A recent suggestion that cyclic AMP may promote release of nascent protein from polyribosomes (Khairallah and Pitot, 1967) led to the second experiment of Table IX in which homogenates of both fetal and adult liver were preincubated with cyclic AMP in the presence and absence of caffeine. No increase in enzyme activity was subsequently detected.

Discussion

In a previous paper it was reported that glucagon, adrenalin, and noradrenalin induce the appearance of phosphopyruvate carboxylase activity in fetal rat liver (Yeung and Oliver, 1968). This result suggests the participation of 3′,5′-cyclic AMP in the synthetic or inductive system for phosphopyruvate carboxylase in the neonatal rat liver since each of these hormones has been shown to promote the production of the cyclic nucleotide in tissues (see Sutherland and Robinson, 1966). In the present study, it has been shown that administration of 3′,5′-cyclic AMP to fetal rats *in utero* results in the induction of the enzyme, and treatment of premature rats with the cyclic nucleotide leads to increased enzyme production (see Tables I and II). Shrago *et al.* (1963) have found the activity of phosphopyruvate carboxylase to be elevated in the liver of adult rats treated with glucagon, and in the present work similar elevation in the adult rat liver was ob-

tained with 3′,5′-cyclic AMP. Exton *et al.* (1966), studying the effect of glucagon, adrenalin, and 3′,5′-cyclic AMP on gluconeogenesis in the perfused rat liver, have presented evidence which they interpret as the activation of some step (or steps) between pyruvate and phosphoenolpyruvate by these agents. Such an effect could be explained by the elevated synthesis of phosphopyruvate carboxylase.

Yeung and Oliver (1968) showed that actinomycin D completely blocks the postnatal development of enzyme activity resulting from premature delivery and *de novo* synthesis of the enzyme thus appears to occur. Table I shows that actinomycin D largely blocks the enzyme-inductive effect of 3′,5′-cyclic AMP in fetal rats. Since actinomycin inhibits the synthesis of both r- and mRNA in rat liver (Harel *et al.*, 1964), the results suggest that 3′,5′-cyclic AMP specifically derepresses one or both of these processes essential for phosphopyruvate carboxylase synthesis. It seems unlikely that preformed polysomes, coded for synthesis of the carboxylase, are activated or derepressed by the cyclic nucleotide.

Milman and Yurowitzki (1967) reported the activation of pyruvate kinase in loach embryo extracts by 3′,5′-cyclic AMP. No activation of phosphopyruvate carboxylase in fetal or adult rat liver extracts was obtained in a similar study reported here (see Table IX). The incubation of liver polysomes with 3′,5′-cyclic AMP was also reported to result in release of nascent protein (Khairallah and Pitot, 1967) but no effect on the carboxylase activity in rat liver homogenates was obtained under similar conditions (see Table IX).

The induction of enzyme synthesis in neonatal rat liver is highly specific to 3′,5′-cyclic AMP since the following analogs were without effect: 2′,3′-cyclic AMP, 5′-AMP, 3′,5′-cyclic GMP, 3′,5′-cyclic IMP, 3′,5′-cyclic TMP, and 3′,5′-cyclic UMP.

Henion *et al.* (1967) demonstrated that various esterified derivatives of 3′,5′-cyclic AMP are more potent in promoting glycogenolysis than the parent compound. There is massive induction of phosphopyruvate carboxylase activity in both fetal and premature rat liver following injection of dibutyryl 3′,5′-cyclic AMP. When the results of Tables IV and V are corrected to the same time after injection, the response to the dibutyryl ester is 1.6 times greater than to cyclic AMP in fetal rat liver and 3 times greater in premature animals. For this reason the effect of dibutyryl 3′,5′-cyclic AMP on two other enzymes in fetal rat liver was tested but neither fructose diphosphatase nor pyruvate kinase activities were altered by injection of the nucleotide (Table VI). This constitutes further evidence for specificity of action of 3′,5′-cyclic AMP in the induction of phosphopyruvate carboxylase.

In tissues, a specific phosphodiesterase hydrolyzes 3′,5′-cyclic AMP in the 3′ position to form 5′-adenosine monophosphate (Sutherland and Rall, 1958; Drummond and Perrott-Yee, 1961; Butcher and Sutherland, 1962). The increased potency of the dibutyryl ester may be due to elevated resistance to phosphodiesterase action or to greater permeability of cell membranes to the derivative. As 3′,5′-cyclic AMP is hydrolyzed in tissues, the concomitant administration of phosphodiesterase inhibitors should potentiate the effect of the cyclic nucleotide. Berthet *et al.* (1957) showed that caffeine and theophylline increased the activation of phosphorylase by 3′,5′-cyclic AMP in various cell-free systems from liver, and it was subsequently shown that methylxanthines inhibit the purified phosphodiesterase (Butcher and Sutherland, 1962). Northrop and Parks (1964) further demonstrated potentiation of the hyperglycaemic effects of 3′,5′-cyclic AMP in intact animals by simultaneous administration of theophylline. In Tables I and II it is shown that the induction of phosphopyruvate carboxylase activity by 3′,5′-cyclic AMP is greater when it is administered together with caffeine than when given alone.

TABLE V: Effect of N^6-2′-*O*-Dibutyryl 3′,5′-Cyclic AMP on Induction of Phosphopyruvate Carboxylase in Premature Rat Liver.[a]

	Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate/mg of protein per hr)		
Expt No.	1	2	3
Control (0.9% NaCl)	0.29	0.24	0.24
Test (dibutyryl cyclic AMP) (0.14 μmole)	0.51	0.79	0.68
No. of animals in each group	4	4	4

[a] Details of experiments as in Table II. Animals receiving dibutyryl cyclic AMP were injected at 0 and 2 hr after delivery. All animals were killed 3.5 hr after delivery, enzyme activities were determined on pooled liver extracts.

The inductive effects of injected 3′,5′-cyclic AMP in perinatal rats must be considered in relation to the physiological situation in the new-born animal. Greengard and Dewey (1967) suggested that postnatal hypoglycaemia caused release of glucagon from the pancreas and that the hormone then initiates enzyme-inductive events in the liver which result in the development of tyrosine aminotransferase (EC 2.6.1.5). In adult animals it has been shown that hypoglycaemia stimulates the release of glucagon (Unger and Eisentraut, 1964), adrenalin, and noradrenalin (Goldfein, 1966). It is logical to assume that postnatal hypoglycaemia promotes a similar hormonal flux in the new-born rat, since the animals recover rapidly from the intense hypoglycaemic phase that follows delivery (Yeung and Oliver, 1968). Ergotamine tartrate inhibits the adrenalin-potentiated production of 3′,5′-cyclic AMP in liver homogenates (Berthet *et al.*, 1957) and when the drug is administered to neonatal rats *in utero*, the subsequent activity of phosphopyruvate carboxylase resulting from delivery is much less than normal. When the drug is given at increasing times after birth its repressive

TABLE VI: Effect of Dibutyryl 3′,5′-Cyclic AMP on Enzymic Activities in the Fetal Rat Liver.[a]

Treatment	Av Wt of Fetuses (g)	Fructose Diphosphatase (μmoles of NADP reduced/mg of protein per hr)		Pyruvate Kinase (μmoles of NADH oxidized/mg of protein per hr)		Phosphopyruvate Carboxylase (μmoles of oxaloacetate formed/mg of protein per hr)	
		Control	Test	Control	Test	Control	Test
N^6-2′*O*-Dibutyrylcyclic AMP (0.14 μmole)	2.8	0.20	0.21	3.10	3.05	0.00	0.35
	3.9	0.34	0.32	4.20	4.10	0.01	0.48
	5.0	1.12	1.08	4.00	4.05	0.00	0.42

[a] Details of experiments as in Table I. Pooled livers from four animals in each group were extracted and assayed for all three enzymes in suitable aliquots.

TABLE VII: Repression of Phosphopyruvate Carboxylase Synthesis in Premature Rat Liver by Insulin.[a]

Treatment (dose/animal)	Time after Delivery (hr)	Blood Glucose (mg/100 ml)		Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate/mg of protein per hr)	
		Control	Test	Control	Test
Insulin (2 munits)	0	59			
	1.5	38	18		
	4.5	52	23	0.33	0.12
	0	60			
	1.0	40	17		
	4.0	63	22	0.31	0.17
	5			0.50	0.28
	5			0.48	0.35

[a] Experiments carried out as in Tables II and III. Control animals received 0.15 M NaCl, test animals received insulin diluted in 0.15 M NaCl, all injections made at delivery only. Animals were sacrificed in pairs for blood glucose assay and the last pairs were used also for enzyme assay in pooled liver extracts. In the last two experiments groups of four livers were pooled for enzyme assay.

effect becomes less. The quantitative data from these experiments (Table VIII) indicate that adrenalin is of approximately equal importance to glucagon in the postnatal induction of the enzyme.

Bergstrom (1967) has recently reviewed the activities of a new group of hormones, the prostaglandins. Prostaglandin PGE_1 strongly antagonizes the stimulatory effects of a number of hormones on the release of glycerol and free fatty acids from adipose tissue preparations *in vitro*. Increased lipolysis in these systems results from the addition of 3′,5′-cyclic AMP or its dibutyryl ester but prostaglandin is not antagonistic. Thus, Bergstrom (1967) has suggested that prostaglandin inhibits adenyl cyclase activity. If adrenalin and glucagon are indirectly responsible for the postnatal appearance of phosphopyruvate carboxylase activity through the activation of adenyl cyclase and subsequent 3′,5′-cyclic AMP production, the administration of prostaglandin to new-born rats might be expected to repress the appearance of enzyme. The results obtained in such experiments (Table VIII) indicate a small repressive effect with a dose of 0.25 μg/fetus. With higher and lower doses there was no significant effect. While this paradoxical effect cannot be explained at present, it is not unique as Bergstrom has also described certain anomalous results with high doses of prostaglandin. Hansson and Samuelsson (1965) showed that ^3H-labeled prostaglandin PGE_1 is concentrated in liver and kidney of the mouse even within 2 min of intravenous injection. Intraperitoneal and subcutaneous injections were made in this study and it was assumed that rapid accumulation of the hormone by the liver would also occur.

Butcher *et al.* (1966) showed that insulin lowers the intracellular level of 3′,5′-cyclic AMP in adipose tissue incubated with adrenalin, adrenalin plus caffeine, or glucagon. If 3′,5′-cyclic AMP is the primary inducer of phosphopyruvate carboxylase activity, insulin injection should reduce the postnatal development of the enzyme, since Exton *et al.* (1966) have demonstrated

TABLE VIII: Repression of Phosphopyruvate Carboxylase Synthesis in Premature Rats by Ergotamine Tartrate and Prostaglandin.[a]

Treatment (dose/animal)	Time of Injection after Delivery (hr)	Phosphopyruvate Carboxylase Act. (μmoles of oxaloacetate/mg of protein per hr)		Time of Enzyme Assay (hr after delivery)
		Control	Test	
Ergotamine tartrate (3 μg)	−0.5	0.27	0.09	3
		0.25	0.14	
		0.24	0.15	
		0.28	0.20	
	−0.5	0.28	0.18	3
	0	0.29	0.20	
	2.0	0.38	0.35	
Prostaglandin PGE_1 (0.25 μg)	0 Mean =	0.38 ± 0.02 (7)	0.32 ± 0.04 (7)	4
		$t = 2.183$, $df = 12$, $p < 0.05$		

[a] In experiments with ergotamine tartrate, fetal rats were sometimes injected with the drug 0.5 hr before delivery, in other experiments ergotamine tartrate or prostaglandin was injected intraperitoneally in saline solution into premature rats at the times shown after delivery. All control animals received 0.15 M NaCl. Enzyme activity was assayed in pooled liver extracts from four animals in each group with the exception of the second experiment with ergotamine where two animals constituted a group. The figures in parentheses for prostaglandin represent the number of separate determinations in different litters and the mean ±1 SE is given for each group.

TABLE IX: Effect of 3′,5′-Cyclic AMP on Phosphopyruvate Carboxylase Activity *in Vitro*.[a]

Experiment	Incubation Medium	Phosphopyruvate Carboxylase Act.	
		Fetal Liver	Adult Liver
1. Activation test	Complete	0.00	2.25
	Complete + 1 mM 3′,5′-cyclic AMP	0.00	2.21
	Complete + 1 mM cyclic AMP + 5 mM caffeine	0.00	2.12
2. Enzyme release	Complete	0.00	0.81
	Complete + 1 mM cyclic AMP	0.00	0.85
	Complete + 5 mM caffeine	0.00	0.79
	Complete + 1 mM cyclic AMP + 5 mM caffeine	0.00	0.80

[a] In expt 1, the enzyme was prepared from liver extracts by zone centrifugation and assayed by the method of Yeung and Oliver (1967) with the indicated additions. Enzyme activities expressed as micromoles of phosphopyruvate formed per hour per milligram of protein at 37°. In expt 2, liver homogenates (Yeung and Oliver, 1967) were preincubated for 15 min at 37° in the medium of Khairallah and Pitot (1967) in the presence of the additions given. Aliquots were then removed and immediately assayed for enzyme activity by the method of Chang and Lane (1966). Enzyme activities expressed as micromoles of oxaloacetate per hour per milligram of homogenate protein at 37°.

inhibitory effects of insulin on gluconeogenesis in perfused rat liver. Table VII shows that administration of insulin, although it prolongs the postnatal hypoglycaemic phase, represses the development of enzyme activity in new-born rats. In previous work it has been shown that glucose injection to new-born rats prevents postnatal hypoglycaemia and reduces the amount of enzyme that is found (Yeung and Oliver, 1968). These data are consistent with the view that glucose or its metabolites have no direct repressive effect on the system and that postnatal hypoglycaemia triggers an indirect mechanism, the hormone-mediated production of 3′,5′-cyclic AMP discussed above. Fructose and mannose, which do not relieve postnatal hypoglycaemia, strongly repress postnatal synthesis of phosphopyruvate carboxylase (Yeung and Oliver, 1968), but these sugars stimulate the release of insulin from isolated perfused pancreas *in vitro* (Grodsky *et al.*, 1963). Thus, their

effects are explicable in terms of the insulin antagonism to the action of adrenalin. An explanation has still to be sought for repression of enzyme synthesis following galactose injection as this sugar does not relieve hypoglycaemia (Yeung and Oliver, 1968) nor stimulate insulin release (Grodsky *et al.*, 1963), but high levels of blood galactose may act like glucose in suppressing the release of glucagon. Shrago *et al.* (1963) showed that following pancreatectomy, or administration of alloxan or mannoheptulose in adult animals, the activity of hepatic phosphopyruvate carboxylase is always higher than in control rats. In these situations there is probably a low circulating insulin level and these results are thus consistent with the repression of enzyme synthesis by insulin.

Direct analytical data on 3′,5′-cyclic AMP levels in rat liver during the immediate postnatal period is lacking at this time, but indirect evidence indicates that an increase in concentration takes place. In new-born rats, hepatic glycogen phosphorylase activity increases sharply from the late fetal level during the first 4 hr after birth (Ballard and Oliver, 1963). The 3′,5′-cyclic AMP mechanism is the only one known to result in activation of liver phosphorylase. Thus, it is suggested that the physiological hypoglycaemic phase in the new-born rat, occurring as a result of a large increase in muscular activity of the animal and the abrupt termination of the maternal glucose supply, results in the release of glucagon and adrenalin. These hormones potentiate production of 3′,5′-cyclic AMP in the liver which brings about the eventual activation of glycogen phosphorylase and the derepression of phosphopyruvate carboxylase synthesis. Liver glycogen is mobilized (Shelley, 1961) and as the gluconeogenic chain of enzymes is completed (Yeung *et al.*, 1967; Ballard and Hanson, 1967; Yeung and Oliver, 1967) active synthesis of glucose follows. It should be noted that the carboxylase activity induced *in utero* by a single injection of 3′,5′-cyclic AMP persists in the liver for at least 2 days and this fact suggests that the chemical induction is identical with the physiological inductive mechanism postulated above. Further work is in progress on the kinetics of induction in the fetal liver.

The data presented in this paper indicate that one of the functions of 3′,5′-cyclic AMP in mammalian systems is the derepression of synthesis of a specific enzyme. The extremely low concentrations at which 3′,5′-cyclic AMP occurs in tissues (Turtle and Kipnis, 1967) is in accord with one criterion required of an inducer, derepressor, or effector molecule in the familiar bacterial models of inducible systems. Such compounds should be biologically active at very low concentrations. Derepression of tissue-specific enzyme synthesis by 3′,5′-cyclic AMP may be the model for a general mechanism of hormonal potentiation of protein synthesis and a mechanism of tissue differentiation. A large number of chemically dissimilar hormones potentiate the production of 3′,5′-cyclic AMP in different tissues (see Sutherland and Robinson (1966) and Bergstrom (1967) for summaries) and adenyl cyclase has been shown in some tissues to be associated with the cell membrane (Davoren and Sutherland, 1963; Sutherland and Robinson, 1966). Hence, a particular hormone may exert its ultimate intracellular effect through the agency of 3′,5′-cyclic AMP even without penetration to the intracellular environment.

It is further likely that there is hormone-potentiated production of other cyclic ribonucleotides and deoxyribonucleotides which function as effectors in a similar fashion to cyclic AMP. Such cyclic nucleotides might escape detection by conventional techniques of tissue analysis due to their low concentrations. In this respect it is of interest that 3′,5′-cyclic GMP as well as 3′,5′-cyclic AMP has been identified in mammalian urine (Butcher and Sutherland, 1962; Ashman *et al.*, 1963). Hechter *et al.* (1967) have reported that 3′,5′-cyclic AMP stimulates RNA synthesis, the incorporation of amino acids into protein, and other biosynthetic processes involving lipid and glycogen synthesis in the rat uterus *in vitro*. Although the effects were not exclusive to 3′,5′-cyclic AMP, similar results being obtained with a wide range of nucleotides, it was suggested that 3′,5′-cyclic AMP, or perhaps a set of nucleotides, are involved in estradiol action in the uterus by the activation of metabolic processes operative at the gene and other intracellular loci.

Konijn *et al.* (1967) have tentatively identified 3′,5′-cyclic AMP as the chemical messenger acrasin, a substance that promotes aggregation of myxamoebae during the life cycle of the cellular slime mould. The aggregation phenomenon precedes morphogenetic activity that culminates in the production of fruiting bodies. This finding, together with the evidence presented in this paper, emphasizes the potential importance of cyclic nucleotides in biochemical mechanisms of morphogenesis and cellular differentiation.

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