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Factors Affecting the Premature Induction of Phosphopyruvate Carboxylase in Neonatal Rat Liver

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1. Phosphopyruvate carboxylase activity rapidly appears in the liver of prematurely delivered rats and development of activity is prevented by injection of actinomycin D just before delivery. 2. The activity is considerably decreased by puromycin and amino acid analogues and thus appears to be due to enzyme synthesis. 3. Newborn or premature animals show a transient intense phase of hypoglycaemia after delivery. 4. When the hypoglycaemic phase is prevented by glucose injection little phosphopyruvate carboxylase activity appears in the liver, but galactose, mannose and fructose, which have no effect on the blood glucose concentration, also repress enzyme development. 5. Lactate, pyruvate and glycerol injections repress the premature development of phosphopyruvate carboxylase. 6. Injections of glucagon, adrenalin and noradrenalin into the rat foetus *in utero* result in development of phosphopyruvate carboxylase activity. 7. These findings are discussed in relation to the mechanism of initiation of enzyme synthesis in neonatal rat liver.

In recent papers it has been shown that cytoplasmic phosphopyruvate carboxylase (EC4.1.1.32) activity is absent from foetal rat liver, making its appearance only after birth (Ballard & Hanson, 1967; Yeung, Stanley & Oliver, 1967). Large amounts of activity rapidly appear in the liver of foetuses delivered prematurely by uterine section and this process results in overall activity of the gluconeogenesis system in the liver (Yeung & Oliver, 1967*b*).

Several hepatic enzymes can be apparently induced by premature delivery, including tryptophan oxygenase (EC 1.13.1.12) (Nemeth, 1959, 1963) and tyrosine aminotransferase (EC 2.6.1.5) (Holt & Oliver, 1968) in rats and glucose 6-phosphatase (EC 3.1.3.9) (Dawkins, 1961) and tyrosine aminotransferase (Litwack & Nemeth, 1965) in rabbits. However, Yeung & Oliver (1967*b*) showed that the increase in glucose 6-phosphatase activity after premature delivery of the rat was comparable with the normal rate of increase measured in late-foetal rats *in utero*.

The mechanism of these effects is obviously relevant to the problem of biochemical differentiation during foetal maturation, and in this paper various factors that influence the premature induction of phosphopyruvate carboxylase are examined.

MATERIALS AND METHODS

Chemicals. Phosphoenolpyruvate (sodium salt), ITP and ADP were obtained from C. F. Boehringer und Soehne G.m.b.H. (Mannheim, Germany); IDP, oxaloacetic acid, NADH and DL-*p*-fluorophenylalanine were from Sigma Chemical Co. (St Louis, Mo., U.S.A.); D-glucose, D-galactose, D-mannose, 2-deoxy-D-glucose, D-ribose, pyruvic acid and lactic acid were from British Drug Houses Ltd. (Poole, Dorset); D-fructose (glucose-free) and glycerol were from General Chemical and Pharmaceutical Co. Ltd. (Sudbury, Middx.); puromycin was from Nutritional Biochemical Corp. (Cleveland, Ohio, U.S.A.); L-ethionine was from Calbiochem (Los Angeles, Calif., U.S.A.); actinomycin D was from Merck, Sharp and Dohme Inc. (West Point, Pa., U.S.A.); glucagon (U.S.P., for injection) was from Eli Lilly and Co. (Indianapolis, Ind., U.S.A.); adrenalin (B.P.) was from Farmer Hill (Pty.) Ltd. (Sydney, N.S.W., Australia); noradrenalin was from Winthrop Laboratories Sterling Pharmaceutical (Pty.) Ltd. (Sydney, N.S.W., Australia); $Na_2{}^{14}CO_3$ (36mc/m-mole) was from The Radiochemical Centre (Amersham, Bucks.). Malate dehydrogenase (EC 1.1.1.37) and pyruvate kinase (EC 2.7.1.40) were obtained from C. F. Boehringer und Soehne G.m.b.H.; 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. All drugs and ethionine were dissolved in 0·9% NaCl; *p*-fluorophenylalanine was suspended in 0·9% NaCl–0·06% pluronic acid detergent by ultrasonic treatment. Glucagon was dissolved in 1·6% glycerol–0·2% phenol in water, and diluted for use with the same vehicle. D-Galactose was assayed by the glucose oxidase procedure (Huggett & Nixon, 1957) and contained less than 0·02% of glucose. Lactic acid and pyruvic acid were neutralized with 0·2M-imidazole buffer before use.

Animals. Wistar albino rats were used. Foetal ages were established from a foetal age–weight curve established on the animal colony as described by Yeung *et al.* (1967).

Premature animals (of 20·5–22 days gestational age) were

delivered by uterine section as described by Yeung & Oliver (1967*b*).

The animals were maintained in a humidicrib at 37° in an atmosphere enriched with O_2+CO_2 (95:5). All injections of carbohydrates, hormones and drugs, with the exception of *p*-fluorophenylalanine, were given intraperitoneally from an Agla micrometer syringe (Burroughs Wellcome and Co., Beckenham, Kent) equipped with polythene canula tubing and 30-gauge needle. The suspension of *p*-fluorophenylalanine was injected from a 26-gauge needle.

Intraperitoneal injections into foetal animals *in utero* were made through the uterine wall as described by Yeung *et al.* (1967). All animals were killed by cervical fracture and the livers dissected out and weighed.

Assay of blood glucose. Blood was obtained from the animals by jugular puncture and glucose determined by the glucose oxidase procedure of Huggett & Nixon (1957).

Assay of phosphopyruvate carboxylase. Livers were removed from the animals and washed in chilled 0·9% NaCl. Liver extracts were prepared exactly as described by Yeung & Oliver (1967*b*) and enzyme activity was assayed after the zonal-centrifugation procedure described by Yeung & Oliver (1967*b*). In some experiments the unfractionated liver extracts were assayed by the method of Chang & Lane (1966).

In enzyme assays involving spectrophotometry, a Beckman model DU spectrophotometer fitted with a Gilford Instruments (Oberlin, Ohio, U.S.A.) model 2000 multiple-sample extinction recorder was used.

Assay of ^{14}C. In using the assay of Chang & Lane (1966), 300 μl. of the ^{14}C-labelled sample, 10 ml. of Diotol (Herberg, 1960) and 100 μl. of Hyamine hydroxide (1 M in methanol solution) were pipetted into vials and counted in a Nuclear–Chicago Corp. model 6860 (Mk 1) automatic liquid-scintillation spectrometer. Channels-ratio data were obtained for quenching corrections. The efficiency of the counter for ^{14}C was 80%. All samples were counted to at least 1000 counts above the background.

Determination of protein. Protein concentrations were determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Liver extracts were diluted 50-fold in 2% (w/v) Na_2CO_3–0·1 N-NaOH for assay.

RESULTS

The appearance of phosphopyruvate carboxylase activity in rat liver after premature delivery can be largely prevented by puromycin and actinomycin, and the amino acid analogues ethionine and *p*-fluorophenylalanine partially inhibit the process. Actinomycin D given to foetuses 1 hr. before delivery of the animals by uterine section completely

Table 1. *Effects of actinomycin D, puromycin and amino acid analogues on the induction of phosphopyruvate carboxylase in liver of premature rats*

Animals were prematurely delivered and immediately injected with 0·9% NaCl (control) or the inhibitors (test) in 0·9% NaCl. They were maintained for 5 hr. in a humidicrib at 37° and then killed and the enzyme was assayed in liver extracts as described in Yeung & Oliver (1967*b*). Livers were pooled in groups of at least three for assay. Enzyme activities are expressed as μmoles of phosphoenolpyruvate formed/hr./mg. of protein.

Inhibitor	Dose (μg./animal)	Phosphopyruvate carboxylase activity: Control	Phosphopyruvate carboxylase activity: Test	Repression (%)
Puromycin	175	1·02	0·0	100
	350	1·19	0·36	70
	350	1·45	0·62	58
	700	1·16	0·57	51
	700	1·48	0·47	68
	700	1·17	0·33	72
	700	1·51	0·52	66
Actinomycin D	3·5	1·16	0·21	82
	3·5	1·52	0·24	84
	3·5	1·58	0·28	82
	3·5	1·32	0·14	89
	3·5	—	0*	100
DL-*p*-Fluorophenylalanine	500	1·04	0·65	38
	500	1·25	0·52	58
	500	1·53	0·80	48
L-Ethionine	500	1·15	0·92	20
	500	1·05	0·54	49
	500	1·07	0·48	56
	500	1·32	0·53	60

*Foetuses injected *in utero* 1 hr. before delivery.

Table 2. *Blood glucose concentrations in 'newborn' rats*

Littermate premature and normal animals were maintained without feeding in a humidicrib at 37°. Blood samples from paired animals were obtained at the times indicated by jugular puncture and blood glucose was specifically determined by the glucose oxidase procedure.

Time after delivery (hr.)	Blood glucose concentration (mg./100ml. of blood)	
	Premature	Normal birth
0	93	71
1	34	20
2	18	14
3	33	20
4	76	56
5	73	60

prevents the normal appearance of enzyme activity (Table 1).

Both normally delivered and surgically delivered rat foetuses rapidly become hypoglycaemic and then spontaneously recover in the absence of food (Table 2). The administration of a glucose regime that prevents the hypoglycaemic phase of surgically delivered rats also results in a marked repression of the development of phosphopyruvate carboxylase activity. Fructose, galactose and mannose bring about similar repression when administered in a similar regime, though the first two hexoses have no effect on the hypoglycaemic phase. The C_3 compounds lactate, pyruvate and glycerol bring about similar repression, but 2-deoxyglucose and ribose have no effect on the enzyme development (Table 3).

In experiments not reported in detail here, ethanol injection (10 and 50 μg.) at delivery was also without effect. An environment of 100% oxygen also had no effect on the extent or rate of enzyme development after surgical delivery of premature foetuses.

Table 4 shows that glucose injection some time after delivery almost completely terminates further enzyme synthesis (Expt. 1). Expt. 2 shows that there is no additive effect of glucose and puromycin in repression of enzyme development. Expt. 3 shows that actinomycin D injection 2hr. after delivery completely terminates further development of enzyme activity. When actinomycin D is used to prevent further enzyme synthesis the preformed enzyme is slowly degraded (Expt. 4).

Table 5 shows that the intrauterine administration of glucagon, adrenalin and noradrenalin results in the rapid appearance of phosphopyruvate carboxylase activity in foetal rat liver. This response can be elicited with glucagon from at least day 19 of gestation, and with adrenalin it is demonstrated from day 16 of gestation. However, the effect seems to be greater in older foetuses. The activity found in control animals is not due to the operative procedure (Yeung *et al.* 1967) and must be ascribed to induced activity brought about by hormonal transfer, probably via the placentas and maternal blood from test to control foetuses. A similar situation occurs in the induction of tyrosine aminotransferase in foetal rats by triamcinolone (Yeung *et al.* 1967), and in such experiments the control animals cannot be considered as independent from the test animals. For this reason the data of Table 5 were statistically analysed by the paired-difference t test and were found to be highly significant ($P < 0.001$). The use of this procedure is further discussed by Holt & Oliver (1968). The P value is similar when the data are analysed as independent control and test values. Glucagon at 2·5 μg./foetus is equally effective for enzyme induction.

Table 6 shows that glucagon injection into premature rats increases the amount of phosphopyruvate carboxylase that is formed, but that the injection of glucose together with glucagon only marginally diminishes this effect.

DISCUSSION

The rapid development of phosphopyruvate carboxylase activity after premature delivery is due to enzyme synthesis, since the administration of actinomycin D and puromycin at the time of surgical delivery represses the development by 70–80%, and the injection of actinomycin D 1hr. before delivery of the foetus leads to complete repression of activity. Amino acid analogues given in the same way to premature rats on delivery also result in the development of lower activities than found in control animals (see Table 1). Since actinomycin D inhibits all DNA-directed RNA synthesis (see Reich & Goldberg, 1964), including that of ribosomal and messenger RNA in rat liver (Harel, Harel, Boer, Imbenotte & Carpeni, 1964), the results suggest that the entire process of enzyme synthesis, including the formation of enzyme-specific polysomes, is initiated immediately at delivery (see also Holt & Oliver, 1968).

The foetal administration of the fluorinated glucocorticoid triamcinolone fails to induce phosphopyruvate carboxylase activity *in utero* (Yeung *et al.* 1967). This fact is taken to mean that corticosteroids are not involved in the induction of the carboxylase during neonatal foetal development, though several reports have appeared on corticosteroid induction in the adult rat (Shrago, Lardy, Nordlie & Foster, 1963; Ashmore, Wagle & Uete, 1964; Foster, Ray & Lardy, 1966).

In neonatal rats normal or surgical delivery results in an acute transient phase of hypoglycaemia

Table 3. *Effects of carbohydrate administration on blood glucose concentration and induction of phosphopyruvate carboxylase in premature rats*

Littermate animals were delivered by uterine section and maintained in the humidicrib at 37°. At the times after delivery indicated, intraperitoneal injections of the carbohydrates were made at the given doses to half the animals (test) and the other animals received the same volume of 0·9% NaCl (control). One animal in each group was killed at the times indicated for blood glucose determination, the blood was obtained by jugular puncture. At 5 hr. after delivery the remaining animals in each group were killed and phosphopyruvate carboxylase activity was determined in pooled liver extracts by the method of Chang & Lane (1966) or fractionated and assayed by the method of Yeung & Oliver (1967*b*). At least two animals were used in each group for enzymic determination. A total of six experiments with glucose and two experiments with each of the other carbohydrates gave similar results to those listed.

Carbohydrate	Dose (mg./animal)	Time after delivery (hr.)	Blood glucose concentration (mg./100 ml.)		Phosphopyruvate carboxylase activity	
			Control	Test	Control	Test
Glucose	25	0	78	—		
	12·5	2·25	15	121		
	12·5	4·0	45	—		
	—	5·0	56	182	1·37†	0·29†
Galactose	25	0	62	—		
	12·5	2·0	21	19		
	12·5	3·5	39	35		
	—	5·0	49	48	1·42†	0·55†
Fructose	25	0	79	—		
	12·5	2·0	39	35		
	12·5	3·5	—	—		
	—	5·0	68	64	1·25†	0·45†
Mannose	25	0	*	*		
	12·5	2·0	*	*		
	12·5	3·5	*	*		
	—	5·0	*	*	0·54‡	0·18‡
Ribose	25	0	48	—		
	12·5	1·5	23	25		
	12·5	3·5	34	37		
	—	5·0	42	45	1·27†	1·24†
2-Deoxyglucose	25	0	*	*		
	12·5	2	*	*		
	12·5	4	*	*		
	—	5	*	*	1·24†	1·19†
Glycerol	25	0				
	12·5	1				
	12·5	3				
	—	5			0·51‡	0·26‡
Lactate	6·25	0				
	2·5	1				
	2·5	3				
	—	5			0·42‡	0·20‡
Pyruvate	2·5	0				
	2·5	2·0				
	2·5	3·5				
	—	5·0			0·38‡	0·12‡

*Injected sugars interfered with blood glucose determination.

†Assayed by method of Yeung & Oliver (1967*b*) and expressed as μmoles of phosphoenolpyruvate formed/hr./mg. of protein.

‡Assayed by method of Chang & Lane (1966) and expressed as μmoles of oxaloacetate formed/hr./mg. of protein.

Table 4. *Repression of phosphopyruvate carboxylase synthesis in liver of premature rats*

Littermate animals were delivered by uterine section and maintained in the humidicrib at 37°. At the times stated the materials were administered by intraperitoneal injection and the animals maintained in the humidicrib until they were killed. The enzyme was assayed in pooled liver extracts of three animals by the method of Chang & Lane (1966). Enzyme activities are expressed as μmoles of oxaloacetate formed/hr./mg. of protein.

Expt. no.	Treatment	Time of treatment (hr. after delivery)	Time of assay (hr. after delivery)	Phospho-pyruvate carboxylase activity
1	None	—	2·5	0·25
	0·9% NaCl	2·5	5·0	0·48
	Glucose (25 mg.)	2·5	5·0	0·27
2	None	—	5·0	0·33
	0·9% NaCl	5·0	7·0	0·50
	Puromycin (700 μg.)	5·0	7·0	0·40
	Puromycin + glucose (25 mg.)	5·0	7·0	0·39
3	None	—	2·0	0·20
	Actinomycin D (4·4 μg.)	2·0	4·0	0·22
	0·9% NaCl	2·0	4·0	0·43
4	None	—	5·0	0·40
	Actinomycin D (4·4 μg.)	0	5·0	0·05
	Actinomycin D (4·4 μg.)	5·0	10·0	0·21
	0·9% NaCl	5·0	10·0	0·70

(see Table 2), but blood glucose concentrations return almost to normal by the fifth hour after delivery. Gluconeogenesis, though absent from foetal rat liver, develops rapidly after birth (Ballard & Oliver, 1963, 1965; Yeung & Oliver, 1967*a*, *b*) and there is a linear correlation between the activity of phosphopyruvate carboxylase and the rate of glucose synthesis (Yeung & Oliver, 1967*b*). The postnatal initiation of gluconeogenesis undoubtedly contributes to the correction of the postnatal hypoglycaemia, though liver glycogen stores, which are mobilized during this period (Shelley, 1961), must also contribute. When the hypoglycaemia is prevented by glucose injection, an approximately 70% decrease in the development of phosphopyruvate carboxylase is observed. The injection of glucose 2·5 hr. after delivery, when the lowest concentrations of endogenous blood glucose are reached, leads to termination of enzyme production (Tables 3 and 4). The rate of enzyme production can be similarly decelerated by puromycin, but when glucose is given together with puromycin the repression is no greater than that obtained with puromycin alone (Table 4). Thus the glucose effect is not due to inhibition of the enzyme, but appears to be due to repression of synthesis. Actinomycin D virtually terminates enzyme production even when given a few hours after delivery, which suggests that messenger-RNA production is continuous throughout the period. When actinomycin is used to block enzyme synthesis at the fifth hour after delivery, the activity of the enzyme declines about 50% over the next 5 hr. (Table 4). This result suggests that the half-life of the enzyme is relatively short.

The injection of glucagon into foetal rats *in utero* results in the production of high activities of phosphopyruvate carboxylase in 5 hr. (Table 5). In addition, glucagon injection into surgically delivered rats potentiates the production of the enzyme, but the concomitant injection of glucose has only a marginal effect on the glucagon-potentiated activity (Table 6). This finding indicates that glucose or a metabolic product does not act as a direct repressor, but exerts its effects in an indirect fashion, perhaps through mediation of the release of endogenous glucagon, normally promoted by the postnatal hypoglycaemia. Greengard & Dewey (1967) reported the induction of tyrosine aminotransferase in foetal rat liver after injection of glucagon *in utero*. When glucose was injected into normal postnatal animals the development of tyrosine aminotransferase, which normally occurs after birth, was considerably diminished. As a result of these observations, Greengard & Dewey (1967) postulated that postnatal hypoglycaemia promotes release of glucagon from the pancreas and that this then induces the production of the liver enzyme. Holt & Oliver (1968) confirmed these results, but also showed that galactose, mannose and fructose decrease tyrosine aminotransferase production.

These sugars also mimic the repressive effect of glucose on phosphopyruvate carboxylase development, but do not normalize the blood glucose

Table 5. *Induction of phosphopyruvate carboxylase in foetal rats* in utero *by hormones*

Rat foetuses in one horn of the uterus were injected intraperitoneally with the hormones shown (test), and animals in the other horn received 0·9% NaCl (control). They were left *in utero* for 5 hr. and then removed and killed. The enzyme activity was determined in pooled liver extracts from at least four animals by the method of Chang & Lane (1966) and expressed as μmoles of oxaloacetate formed/hr./mg. of protein.

Hormone	Dose (μg./foetus)	Mean wt. of foetuses (g.)	Phosphopyruvate carboxylase activity: Control	Test
Glucagon	25	3·0	0·028	0·212
	25	3·0	0·020	0·182
	25	4·0	0·030	0·352
	25	4·0	0·029	0·304
	25	4·2	0·0	0·331
	25	4·5	0·112	0·303
	25	4·8	0·153	0·482
	25	5·2	0·060	0·332
			Mean 0·054	Mean 0·250
			$P < 0·001$	
Adrenalin	5	0·35	0·043	0·051
	5	1·00	0·045	0·098
	5	1·90	0·006	0·191
	5	1·98	0·053	0·218
	5	1·98	0·015	0·192
	10	3·10	0·025	0·213
	10	3·10	0·021	0·220
	10	3·20	0·011	0·382
	10	3·70	0·040	0·220
	10	3·80	0·035	0·323
	10	4·00	0·057	0·323
	10	4·5	0·054	0·305
	10	5·2	0·101	0·512
			Mean 0·039	Mean 0·325
			$P < 0·001$	
Noradrenalin	10	2·9	0·020	0·142
	20	4·5	0·019	0·229

Table 6. *Effects of glucagon and glucose on postnatal induction of phosphopyruvate carboxylase activity in premature rats*

Littermate foetal rats were delivered by uterine section and maintained in a humidicrib at 37°. They were divided into five groups of two animals and treated as shown at the times stated. Animals were killed 5 hr. after delivery for assay of the enzyme in the pooled livers by the method of Chang & Lane (1966). Enzyme activities are expressed as μmoles of oxaloacetate formed/hr./mg. of protein.

Treatment	Time of treatment (hr. after delivery)	Time of assay (hr. after delivery)	Phospho-pyruvate carboxylase activity
None	—	5	0·426
Glucose (25 mg.)	0	5	0·213
Glucagon (25 μg.)	0	5	0·791
Glucagon (25 μg.)+ glucose (25 mg.)	0 1	5	0·782
Glucose (25 mg.)+ glucagon (25 μg.)	0 1	5	0·701

concentration. Glycerol, lactate and pyruvate also cause repression (Table 3). The effects of lactate do not appear to be mediated through the concentration of NADH because ethanol has no effect on enzyme development. In the absence of a reliable assay for plasma glucagon concentrations it appears unwarranted to assume that control of the concentration of the circulating hormone is mediated solely by glucose concentrations, and high blood concentrations of the other carbohydrates mentioned above may have inhibitory effects on the process. In this respect ribose and deoxyglucose have no effect on enzyme formation and may be without activity on the mechanism of glucagon release.

The administration of adrenalin and noradrenalin to foetal rats *in utero* is followed by rapid development of phosphopyruvate carboxylase activity and these hormones, in addition to glucagon, promote the production of 3′,5′-(cyclic)-AMP in liver (Sutherland & Robinson, 1966). It is thus suggested that 3′,5′-(cyclic)-AMP may act in this system as a

critical allosteric-effector molecule, bringing about de-repression of the synthetic system for phosphopyruvate carboxylase when a suitable concentration is reached. The data previously presented on the rapid lag-free development of the enzyme in premature rat liver (Yeung & Oliver, 1967*b*) indicate that the inductive or synthetic system must be exquisitely sensitive to concentration changes of some such critical effector molecule.

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