Hormonal Regulation of Glycogen Metabolism in Neonatal Rat Liver

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(Received 19 March 1973)

1. The development of active and inactive phosphorylase was determined in rat liver during the perinatal period. No inactive form could be found in tissues from animals less than 19 days gestation or older than the fifth postnatal day. 2. The regulation of phosphorylase in organ cultures of foetal rat liver was examined. None of the agents examined [glucagon, insulin or dibutyryl cyclic AMP (6-*N*,2'-*O*-dibutyryladenosine 3':5'-cyclic monophosphate)] changed the amount of phosphorylase activity. 3. Glycogen concentration in these explants were nevertheless decreased more than twofold by 4h of incubation with glucagon or dibutyryl cyclic AMP. Incubation with insulin for 4h increased the glycogen content twofold. 4. Glycogen synthetase activity was examined in these explants. I-form activity (without glucose 6-phosphate) was found to decrease by a factor of two after 4h of incubation with dibutyryl cyclic AMP, whereas I+D activity (with glucose 6-phosphate) remained nearly constant. Incubation for 4h with insulin increased I-form activity threefold, with only a slight increase in I+D activity. 5. When explants were incubated with insulin followed by addition of dibutyryl cyclic AMP, the effects of insulin on glycogen concentration and glycogen synthetase activity were reversed. 6. These results indicate that the regulation of glycogen synthesis may be the major factor in the hormonal control of glycogen metabolism in neonatal rat liver.

The hormonal regulation of liver glycogenolysis in the adult rat is thought to occur via the cyclic AMP-mediated interconversion of phosphorylase, largely by analogy to the situation in rabbit muscle (Villar-Palazi *et al.*, 1971). Adult liver phosphorylase exists in two forms, active and inactive, the latter lacking two crucial phosphate residues (Wosilait, 1958). Less is known about neonatal liver glycogenolysis. Glycogen begins to appear in foetal rat liver some 3 days before birth and by term may represent 10% by weight (Dawkins, 1963a). At birth there is a dramatic depletion of these stores, which have been linked to the appearance of neonatal hypoglycaemia (Dawkins, 1963b). As the administration of glucagon or cyclic AMP to rat fetuses *in utero* rapidly decreases liver glycogen content (Girard & Bal, 1970; Greengard & Dewey, 1970), it has been concluded that the regulation of foetal liver glycogenolysis is similar to that in the adult. Although neonatal and adult liver glycogen regulation may be qualitatively similar, there are quantitative differences, as seen in hepatic glucose output (glycogenolysis) in the neonatal dog after glucagon infusion (Kornhauser *et al.*, 1970).

For hormonal control of glycogenolysis to be exerted by the generally accepted cyclic AMP mechanism (Robison *et al.*, 1971) phosphorylase and phosphorylase kinase must both be present and available for activation, protein kinase must be available for activation by cyclic AMP and adenylate

cyclase must be present. The presence of cyclic AMP-activatable protein kinase and phosphorylase kinase has been demonstrated in neonatal rat liver by Novak *et al.* (1972) and Hommes & Beere (1971). Phosphorylase activity increases during development in neonatal rat liver (Novak *et al.*, 1972), but the relative proportions of inactive and active phosphorylase activity are not known. Some inactive phosphorylase is presumably present since Cake & Oliver (1969) have shown a 15% increase in phosphorylase activity in neonatal rats injected *in vivo* with cyclic AMP.

In the present study we report the availability of inactive phosphorylase in the neonatal period in the rat. In addition the regulation of glycogenolysis in organ cultures of foetal rat liver was examined as the organ-culture system provides for the prolonged viability of isolated foetal liver removed from the circulating factors present *in utero*.

Experimental

Materials

UDP-[U-¹⁴C]glucose (specific radioactivity 210mCi/mmol) was purchased from International Chemical and Nuclear Corp., Irvine, Calif., U.S.A. Glucagon and insulin (glucagon-free) were obtained from Eli Lilly Co., Indianapolis, Ind., U.S.A.; dibutyryl cyclic AMP was from Calbiochem, La

Jolla, Calif., U.S.A.; and glucose oxidase from Worthington Biochemicals, Freehold, N.J., U.S.A.

The culture medium, Eagle's minimum essential medium in Hank's balanced salt solution (Grand Island Biological Co., New York, N.Y., U.S.A.) with twice the usual concentration of glucose, bicarbonate and L-glutamine and with 100 units of penicillin and 100 μ g of streptomycin added per ml, was purchased from Grand Island Biological Co.

Methods

Assay of phosphorylase. Phosphorylase was assayed by the method of Kakiuchi & Rall (1968) in which the release of P_i from glucose 1-phosphate is measured in the presence of 5'-AMP. Liver was homogenized in ice-cold 75 mM-NaF-5 mM-EDTA (pH 6.7). Samples (0.1 ml) of the whole homogenate were incubated in a system with a final volume of 0.65 ml containing 11.2 mM-glucose 1-phosphate (pH 6.1), 0.1 mM-NaF, 3.4 mM-EDTA, 1.25 mg of glycogen/ml and 2.5 mM-5'-AMP. Incubations were for 30 min at 37°C and were terminated by the addition of 0.65 ml of ice-cold 5% (w/v) trichloroacetic acid. P_i was measured in the supernatant by the method of Fiske & SubbaRow (1925). Appropriate unincubated blanks were run on each sample. Omission of glycogen from the reaction mixture served as an independent blank. Protein concentration in the homogenate was determined by the method of Lowry *et al.* (1951) with dry bovine serum albumin as a standard. Enzyme activity is expressed as μ mol of P_i released/30 min per mg of protein.

To assess the amount of phosphorylase present in the inactive form a portion of the sample homogenate was assayed with and without pretreatment with excess of purified phosphorylase kinase, MgATP and F^- . The treatment was performed by incubating a portion (0.01 ml) of the phosphorylase kinase with 0.1 ml of the sample homogenate in the presence of 20 mM-MgCl₂-3.5 mM-ATP (pH 6.8) in a final volume of 0.15 ml for 5 min at 37°C. The optimum duration of kinase pretreatment was 5 min. The use of 1 μ l of the same phosphorylase kinase preparation produced the same degree of phosphorylase conversion under these conditions. Appropriate blanks were run on all samples. For this study inactive phosphorylase is defined as the difference between these two values.

Phosphorylase kinase was prepared from rabbit muscle by the method of Krebs *et al.* (1964) and was activated by incubation at 27°C for 5 min in the presence of 10 μ M-cyclic AMP, 20 mM-MgCl₂, 6 mM-ATP and 25 mM-glycerol phosphate (pH 6.8) (Krebs *et al.*, 1964).

Assay of glycogen synthetase. Glycogen synthetase activity was assayed as the ability to incorporate UDP-[¹⁴C]glucose into glycogen in both the absence and the presence of 5 mM-glucose 6-phosphate, by the

method of Thomas *et al.* (1968). Briefly, the liver tissue was homogenized in approx. 20 vol. (w/v) of ice-cold 5 mM-EDTA-0.25 M-sucrose (pH 7.5). A sample (0.025 ml) of the whole homogenate was incubated with 0.050 ml of a reaction mixture to yield final concentrations of 5 mM-UDP-glucose (specific radioactivity 120 000 c.p.m./mmol), 34 mM-Tris (pH 7.8), 15 mM-EDTA and 14.4 mg of glycogen/ml for 10 min at 30°C. The reaction was stopped by chilling the sample at -25°C for 5 min and 0.050 ml of the mixture was spotted on filter-paper discs and immediately suspended in ice-cold 66% (v/v) ethanol. The discs were washed three times, once for 30 min and twice for 15 min each, with >30 ml of 66% ethanol/disc. This removed >99% of the unincorporated radioactivity. The discs were then dried and placed in 10 ml of 0.01% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene and counted in a Packard Tri-Carb liquid-scintillation counter. I-form activity is measured in the absence of glucose 6-phosphate and I+D activity in the presence of 5 mM-glucose 6-phosphate.

Protein was determined on a sample of the whole homogenate. Enzyme activity is expressed as nmol incorporated/min per mg of protein.

Assay of glycogen. Liver glycogen concentration was measured as follows. Liver tissue was homogenized in approx. 25 vol. (w/v) of ice-cold water and 0.5 ml of the homogenate was boiled in 1.0 ml of 30% (w/v) KOH for 30 min. The homogenate was cooled, 1 drop of saturated Na₂SO₄ and 1.5 ml of ice-cold 100% ethanol were added and the sample was left at 4°C for at least 1 h. The precipitate was dried, hydrolysed with 1 M-HCl at 100°C for 1 h and neutralized with NaOH. The glucose concentration was determined by the glucose oxidase micro method (Worthington Biochemical Co.). Protein concentration was also measured on the whole homogenate and glycogen concentration is expressed as μ g of glucose/mg of protein.

Animals. Adult female Holtzman Sprague-Dawley strain (Holtzman Rat Co., Madison, Wis., U.S.A.) rats were mated for a 24 h period after which vaginal smears were taken and those animals which had sperm present were considered to be at day 1 of gestation. At the appropriate time of gestation, the mother was killed by a blow to the head and the fetuses were immediately removed and their livers excised. For developmental studies the livers were quick-frozen in liquid N₂.

Postnatal ages were determined from the time of birth. All animals remained with their mother until they were killed. The livers were rapidly excised and frozen in liquid N₂. Samples could be stored at -80°C for months without loss of enzyme activities.

Organ culture. The organ culture system has been completely described including both ultramicroscopic and biochemical criteria for the viability of the ex-

plants (Schwartz, 1972). Each experiment was performed with livers pooled from foetuses of the same litter. The livers were cut into 1 mm cubes and placed on stainless-steel grids, which supported the tissue between the liquid medium and the circulating gas phase, $O_2 + CO_2$ (95:5). All explants were maintained in culture at least 24 h before experiments began.

Each assay for phosphorylase or glycogen synthetase on organ-cultured tissue required 25 explants. Each assay for glycogen required 50 explants.

Statistics. The statistical significance was determined by Student's *t* test.

Results

Development of phosphorylase

Although the development of active phosphorylase has been partially detailed in neonatal rat liver (Novak *et al.*, 1972) no information is available on the total amount of phosphorylase during this period. Unlike muscle phosphorylase, the proportion of inactive and active liver phosphorylase cannot be assessed by assay in the presence and absence of

5'-AMP. In order to determine inactive liver phosphorylase it is necessary to allow the action of phosphorylase kinase (either endogenous or exogenous) by incubation of tissue homogenates with Mg^{2+} and ATP in the presence of F^- ions which inhibit phosphorylase phosphatase action. This method for the determination of total and active phosphorylase activities was validated with samples of various proportions of active and inactive phosphorylase generated in liver slices.

In liver from young adult rats (21 days old) there was no evidence for the presence of inactive phosphorylase in rapidly frozen tissue, because incubation of the homogenate with MgATP and F^- with or without the addition of muscle phosphorylase kinase produced no increase over the initial phosphorylase activity values. Upon incubation of liver slices from such animals, the amount of active phosphorylase activity decreased rapidly, levelling off at 55% of the initial value by 20 min (Fig. 1). Incubation of homogenate with MgATP and F^- for various times produced little or no change in phosphorylase activity (results not presented); however, at any point, incubation of the homogenate with muscle phosphorylase

Fig. 1. *Determination of inactive and active phosphorylase in rat liver*

The liver of a 21-day-old rat was excised, a portion immediately frozen and slices prepared at 4°C from the remainder. Slices were incubated at 37°C in a Ca^{2+} - and Mg^{2+} -free Krebs-Ringer bicarbonate solution (Umbreit *et al.*, 1964) for 0–40 min. Adrenaline was added to some slices at 0 or 20 min to a final concentration of 0.5 mM. At the appropriate times, the slices were frozen in liquid N_2 . Phosphorylase activity was determined as described in the text. □, Initial sample; △, ▲, slices incubated with adrenaline; ○, △, no phosphorylase kinase treatment (i.e. active phosphorylase activity); ●, ▲, phosphorylase kinase pretreatment for 2, 5 and 10 min, respectively (i.e. total phosphorylase activity). The absolute value for the initial sample was $5.66 \mu\text{mol of } P_i/30 \text{ min per mg of protein}$.

kinase restored phosphorylase activity to the initial value within 5 min (Fig. 1). Unless incubation of liver slices removed an inhibitor of phosphorylase kinase, then the pretreatment with muscle phosphorylase kinase under these conditions converts all inactive phosphorylase into the active form. Therefore, these

conditions were used to assess the content of inactive phosphorylase in liver samples from neonatal animals (see the Discussion section). The experiment described in Fig. 1 also shows that the addition of adrenaline can increase appreciably the proportion of active phosphorylase in incubated rat liver slices.

Fig. 2. Development of phosphorylase in perinatal rat liver

Livers of rats of known gestational or postnatal age were excised, frozen and phosphorylase activity was determined. Each point represents one animal, except on day -5.5 (16.5 days of gestation), when two livers were pooled for each point. All points at each day are from one litter of animals. (a) Active phosphorylase, (b) total phosphorylase and (c) inactive phosphorylase. B, indicates day of birth.

The development of phosphorylase in perinatal rat liver is described in Fig. 2. Phosphorylase activity is already present in the liver from 16.5 day foetuses and is almost entirely present as the active form. This total phosphorylase activity increases until about 20 (–2 days) days of gestation. The activity during the remainder of foetal life and during the first six post-natal days fluctuates from 3.5–5 μmol/30 min per mg of protein (Fig. 2). The active phosphorylase activity also increases to 20 days of gestation and thereafter follows a pattern similar to that observed for the total phosphorylase activity. The inactive phosphorylase starts at a value near zero until about the second day before birth, thereafter increasing in activity until birth. After birth the amount of inactive phosphorylase steadily declined, again reaching near zero at the sixth postnatal day.

Novak *et al.* (1972) examined the development of active phosphorylase, assayed both in the absence and presence of 5'-AMP, in the rat. Their results are similar to those in Fig. 1 for active phosphorylase. In addition, in samples assayed in the presence of 5'-AMP, the active phosphorylase activity (Fig. 2a; e.g. at –1.5 day, 3.92±0.22) was about 15% higher than in the absence of 5'-AMP (3.25±0.17), similar to that found by Novak *et al.* (1972).

Development of glycogen synthetase

Glycogen synthetase is present in –5.5-day foetal liver (Table 1). The amount of I+D activity, measured in the presence of glucose 6-phosphate, is highest in the liver of 19.5 day foetus and decreased to a constant value at 21 days of gestation. The I/(I+D) ratio of 0.44 at 19.5-days gestation decreased to 0.26 at 21.5 days of gestation and to 0.16 at birth, then increased slightly during the first week of life (Table 1).

Table 1. Normal development of glycogen synthetase in perinatal rat liver

Livers from rats of known gestational (16.5, 19.5 and 21.5 days respectively) or postnatal age were excised, frozen and glycogen synthetase activity was determined. Each value represents the mean±s.e.m. for six animals.

Days	Glycogen synthetase activity (nmol/min per mg of protein)		
	I-form	I+D	I/(I+D)
–5.5	0.13±0.05	2.88±0.20	0.05±0.02
–2.5	7.10±1.42	16.07±2.68	0.44±0.04
–0.5	3.86±0.45	14.99±1.34	0.26±0.01
0 (i.e., 2–4 h)	2.83±0.72	17.81±2.64	0.16±0.03
+3	3.40±0.30	16.02±1.09	0.22±0.03
+6	3.17±0.49	13.60±1.45	0.23±0.03

Table 2. Effect of dibutyryl cyclic AMP (plus theophylline) on glycogen, phosphorylase and glycogen synthetase in foetal liver explants

Explants were prepared from 19.5-day foetuses (Expt. A) or 21.5-day foetuses (Expt. B). After 24h preincubation, dibutyryl cyclic AMP and theophylline were added to some cultures to yield concentrations of 0.1 and 0.5 mM respectively. After 4h explants were removed, pooled and immediately frozen for analysis. Each value represents the mean ±s.e.m. (number of determinations). *P* values were calculated by Student's *t* test; n.s. >0.05.

	Glycogen conc. (μg of glucose/mg of protein)	Phosphorylase activity (μmol of P _i /30 min per mg of protein)		Glycogen synthetase activity (nmol/min per mg of protein)		
		Active	Total	I-form	I+D	I/(I+D)
Expt. A						
Control	10.7±0.4 (3)	1.82±0.13 (8)	3.13±0.26 (8)	1.17±0.04 (3)	9.73±0.98 (3)	0.12±0.01 (3)
Dibutyryl cyclic AMP (+theophylline)	4.0±0.4 (3)	1.72±0.17 (8)	3.01±0.32 (7)	0.51±0.13 (3)	7.85±0.52 (3)	0.06±0.01 (3)
<i>P</i>	<0.005	n.s.	n.s.	<0.005	n.s.	<0.005
Expt. B.						
Control	15.9±4.9 (3)	2.20±0.18 (4)	3.67±0.23 (4)	2.02±0.21 (6)	16.94±2.15 (6)	0.12±0.01 (6)
Dibutyryl cyclic AMP (+ theophylline)	5.7±0.6 (3)	2.29±0.14 (4)	3.31±0.24 (3)	0.89±0.13 (6)	14.57±1.68 (6)	0.07±0.01 (6)
<i>P</i>	<0.05	n.s.	n.s.	<0.005	n.s.	<0.005

Studies in organ culture

Phosphorylase activity in organ culture. When organ explants are prepared from livers from 19.5-day foetuses, the phosphorylase activities maintain their relative activities throughout the 48 h incubation period (Fig. 3). There is a small but consistent decrease in both active form and total activity during incubation; the amount of inactive enzyme remains constant at approximately the freshly excised values. Since the time of conception for all foetal samples was ± 12 h, the enzyme activities for any individual litter may vary and thus may seem to relate to a slightly older or younger specimen.

Fig. 3. Changes in phosphorylase activity during organ culture of foetal rat liver explants

Explants were prepared from liver of 19.5-day foetus at time zero. At the appropriate times explants were removed, frozen and phosphorylase activity determined. $\square$, total phosphorylase; $\circ$, active phosphorylase; $\triangle$, inactive phosphorylase. Note that the time of conception was within ± 12 h, the initial values for all foetal studies may appear to be from animals slightly older or younger.

The incubation of foetal liver explants with glucagon or dibutyryl cyclic AMP for up to 24h had no significant effect upon the phosphorylase activity, either active or total. A concentration of $6.7 \mu\text{g}$ of glucagon/ml, which elicited maximal effects in organ culture in previous studies with tyrosine aminotransferase (EC 2.6.1.5) (Räihä *et al.*, 1971) and arginine synthetase system (Schwartz, 1972), elicited no changes in phosphorylase activity in explants incubated for 24h. Dibutyryl cyclic AMP, which mimics the effects of glucagon in foetal liver explants (Schwartz, 1972), produced no change in phosphorylase activity at concentrations of $0.1 \mu\text{M}$ – 1 mM in the absence or the presence of 0.5 mM -theophylline during incubations for 4h of explants of livers from 19.5-day or 21.5-day foetuses (Table 2, Expts. A and B). In other experiments, similar results were obtained with incubation for 10min through 24h.

Glycogen in organ culture. The glycogen concentration was examined in these explants. The tissue concentration rapidly decreased during the preincubation period, but reached a constant value by the end of the first 24h. As seen in Table 3 incubation with glucagon, or dibutyryl cyclic AMP plus theophylline, caused a decrease in glycogen concentration to less than 50% of the control values. The change in concentration occurred within 4h. The glycogen concentration remained decreased in the presence of dibutyryl cyclic AMP for 24h, whereas 24h after the addition of glucagon, the glycogen concentration had returned to control values. There was a consistent decrease in glycogen concentration in all experiments where explants were exposed to glucagon or dibutyryl cyclic AMP, including tissues from 19.5-day foetuses (Table 2, Expt. A) and 21.5-day foetuses (Table 2, Expt. B).

Glycogen synthetase in organ culture. Since in foetal liver explants in organ culture phosphorylase activities were unaffected by glucagon or dibutyryl cyclic AMP

Table 3. Effect of glucagon and dibutyryl cyclic AMP on glycogen concentrations in foetal liver explants

Explants were prepared from 21.5-day foetuses. After 24h preincubation, dibutyryl cyclic AMP or glucagon was added to yield final concentrations of 0.1 mM and $100 \mu\text{g/ml}$ respectively. Theophylline at 0.5 mM was present in the cultures receiving dibutyryl cyclic AMP. At the appropriate time, explants were removed and frozen for analysis. Each value represents the mean $\pm$ S.E.M. (number of determinations). The glycogen concentration of the liver explants at 0h preincubation was 143 ± 10 (3).

Agent	Time present (h)	Glycogen concn. (μg of glucose/mg of protein)
Control	0	29 ± 5 (3)
Dibutyryl cyclic AMP	1	20 ± 3 (3)
Dibutyryl cyclic AMP	4	13 ± 2 (3)
Glucagon	4	13 ± 3 (3)
Dibutyryl cyclic AMP	24	17 ± 4 (3)
Glucagon	24	31 ± 3 (3)
Control	24	28 ± 4 (3)

Table 4. Effect of insulin and dibutyryl cyclic AMP on glycogen, phosphorylase and glycogen synthetase in foetal liver explants

Explants were prepared from 19.5-day foetuses. After 24h preincubation, insulin (final concentration = 0.1 unit/ml) was added to some cultures. After 4h some explants were removed and frozen for analysis, whereas dibutyryl cyclic AMP and theophylline (final concentrations 0.1 and 0.5 mM respectively) were added for 4h to other cultures. Each value represents the mean $\pm$ s.e.m. (number of determinations). The glycogen synthetase activity in fresh tissue was 7.97 ± 1.06 (3) for the I-form and 19.88 ± 1.88 (3) for the I+D activity.

	Glycogen concn. (μ g of glucose/mg of protein)	Phosphorylase activity (μ mol of P_i /30 min per mg of protein)		Glycogen synthetase activity (nmol/min per mg of protein)		
		Active	Total	I-form	I+D	I/(I+D)
Control (4h)	6.99 $\pm$ 0.77 (3)	1.63 $\pm$ 0.12 (4)	2.10 $\pm$ 0.26 (4)	1.08 $\pm$ 0.14 (6)	11.87 $\pm$ 0.83 (6)	0.09 $\pm$ 0.01 (6)
Insulin (4h)	13.34 $\pm$ 1.33 (3)	1.53 $\pm$ 0.10 (4)	2.28 $\pm$ 0.19 (4)	3.04 $\pm$ 0.28 (6)	14.70 $\pm$ 0.60 (6)	0.21 $\pm$ 0.02 (6)
Insulin (4h) +dibutyryl cyclic AMP (4h)	—	—	—	1.44 $\pm$ 0.18 (6)	10.99 $\pm$ 0.82 (6)	0.13 $\pm$ 0.01 (6)
Control (8h)	—	—	—	1.03 $\pm$ 0.28 (3)	12.16 $\pm$ 1.41 (3)	0.08 $\pm$ 0.02 (3)

incubation, but glycogen concentrations decreased, glycogen synthetase activity in explants during incubation was examined. Within the 24h preincubation period there was a decrease in both the I-form activity and the I+D activity (Table 4); however, the I-form activity decreased more markedly, thus decreasing the I/(I+D) ratio, which is used as an expression of the activity capable of synthesizing glycogen (Craig *et al.*, 1969). The activities became constant within the first 24h of incubation and remained there during experiments.

Incubation of liver explants from 19.5-day foetuses with 0.1 mM-dibutyryl cyclic AMP (in the presence of 0.5 mM-theophylline) for 4h decreased the I-form activity of glycogen synthetase to less than 50% of the control value (Table 2, Expt. A). There was also a small decrease in I+D activity, but the I/(I+D) ratio decreased by a factor of two. This same pattern of response was evident in explants prepared from livers of 21.5-day foetuses (Table 2, Expt. B).

Effects of insulin on glycogen metabolism. Since insulin has been shown to exhibit effects on glycogen metabolism opposite to those of glucagon and cyclic AMP (Levine, 1965), we examined the effect of insulin (the insulin used in all experiments was glucagon-free) on phosphorylase, glycogen synthetase and glycogen concentration in the foetal liver explants. No effect on active or total phosphorylase activity could be seen with 4h incubation of insulin at concentrations of 10^{-4} – 10^{-1} units/ml (Table 4); however, the glycogen concentration in the explants doubled (Table 4). The glycogen synthetase I+D activity increased slightly with a threefold increase in I-form activity yielding a more than twofold increase in the I/(I+D) ratio (Table 4).

The addition of dibutyryl cyclic AMP for 4h to explants already incubated with insulin for 4h reversed the effects of insulin on glycogen synthetase (Table 4). The I-form activity decreased to slightly greater than the control values and the I/(I+D) ratio decreased from 0.21 with insulin to 0.13, as compared with the control values of 0.08–0.09 (Table 4).

Discussion

In both the 6-day and 21-day rat liver, negligible inactive phosphorylase was demonstrable. The question arises as to whether this reflects the situation *in vivo* or is it an artifact arising during death and sample acquisition. Indeed, special experimental conditions (e.g., removal of serial biopsies under careful hexobarbital anaesthesia) were required before Sutherland & Cori (1951) were able to demonstrate any change in phosphorylase activity *in vivo* after adrenaline injection to rabbits. Thus it is difficult to assess with any degree of accuracy the availability of inactive phosphorylase under various states *in vivo*.

It is possible that the relatively small proportion of

inactive phosphorylase found in foetal rat liver *in situ* or in culture could result from the inability of muscle phosphorylase kinase to act upon a foetal form of the enzyme. However, Sato *et al.* (1972) recently reported that the vast majority of phosphorylase present in late foetal rat liver demonstrates the properties of the adult enzyme.

Our results with phosphorylase in organ cultures of foetal rat liver demonstrate a striking difference in biochemical homeostasis between previously reported liver systems *in vitro* and the organ-culture system, since the relative proportions of active and inactive phosphorylase are maintained for an extended period, i.e. at least 48 h. Studies with liver slices (Rall *et al.*, 1956; Fig. 1) demonstrate a rapid process of enzyme inactivation, but this is not evident in the culture system.

Whereas the organ-culture system maintains tissue in an environment devoid of the humoral influences present *in vivo*, the presence or absence of certain regulatory phenomena in this system may be dependent upon specific factors. The presence of inactive phosphorylase in organ culture would allow for increased accumulation of the active form of phosphorylase in the presence of suitable stimuli. The absence of any effect on these activities in late foetal liver by dibutyl cyclic AMP may be the result of a limiting amount of phosphorylase kinase, reported to be present at low activity at this stage of development (Novak *et al.*, 1972).

Since changes in phosphorylase activity may not be important in the regulation of glycogenolysis in foetal rat liver, it is of interest that in adult rat liver only small changes in phosphorylase activity can be elicited by glycogenolytic hormones (Fig. 1; Levine, 1965). However, there are species (e.g., dog) in which large changes in phosphorylase activity occur in the adult (Rall *et al.*, 1956).

However, changes in glycogen metabolism during perinatal development are consistent with changes in the glycogen synthetase activity. Glycogen in the foetus accumulates in parallel with the increase in glycogen synthetase I-form activity (Table 1), and postnatally both decline concomitantly. The large changes in glycogen synthetase activity during the period immediately before and after birth (Table 1) and the relatively minor fluctuations in active phosphorylase activity during this period (Fig. 2) might suggest that the control of glycogen metabolism *in vivo* may largely result from changes in the rate of synthesis with a nearly constant rate of breakdown.

The results from organ culture studies also suggest that the regulation of glycogen synthetase may be of key importance in the metabolism of foetal liver glycogen. The ability to assess the capacity of the tissue to respond to stimuli in a quantitatively comparable manner is of importance in this regard. For example, the changes in glycogen synthetase (I-form)

activity observed in Table 2 (Expt. A) could provide for a decrease of 158 nmol of glucose/mg of protein during the 4 h period of glycogen synthesis. The decrease in tissue glycogen concentration was 37 nmol of glucose/mg of protein during this time. Thus, since one would expect that the change in enzyme activity was not instantaneous, the decrease in synthetase activity could account for the decreased glycogen concentration without requiring that phosphorylase activity be changed. Calculation of values from Table 2 (Expt. B) yields similar relationships. Therefore liver organ cultures can provide a system for the continuous observation of quantitative changes similar to those observed in the perfused liver, but for much longer periods of time.

The changes in glycogen synthetase activity that occur upon incubation of foetal liver explants may be due to the lack of insulin in the culture system, i.e. the synthetase I-form activity is not maintained (Table 4). Also this could explain the decrease in glycogen concentration during the preincubation (Table 3), as a new steady-state balance is achieved. The increased glycogen accumulation after incubation of the explants with insulin is consistent with an increased glycogen synthetase I-form activity and no change in the phosphorylase activity (Table 4).

Hence changes in glycogen metabolism in neonatal rat liver may reflect changes in glycogen synthesis and its regulation by insulin and cyclic AMP. Perhaps the dramatic decrease in plasma insulin concentrations in the newborn rat (Blazquez *et al.*, 1970) may be important in an understanding of the regulation of glycogen metabolism in the neonatal rat liver.

The authors acknowledge the fine technical assistance of Miss Deborah Little and thank Dr. Irving Rothchild for placing the facilities of his department at our disposal. This study was supported by the Cleveland Diabetes Fund, U.S. Public Health Service (Grant no. GM 0061-10 to the Pharmacology Department, Case Western Reserve University, and Grant no. NS 5716 to T. W. R.). The glucagon-free insulin was a gift of Eli Lilly Co.

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