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GLYCOGEN METABOLISM IN EMBRYONIC CHICK AND NEONATAL RAT LIVER

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SUMMARY

The enzymes UDPG α-glucan glucosyl transferase, α-glucan phosphorylase, glucose-1-phosphate uridyl transferase and phosphoglucomutase all increase in activity throughout the period of glycogen synthesis in the embryonic chick and foetal rat liver. UDPG α-glucan glucosyl transferase activity appears at the time of initial glycogen synthesis in rat liver. There is a 2-fold increase in hepatic α-glucan phosphorylase activity during the 24 h after birth in the rat, when the liver glycogen store is utilised.

Measurement of phosphoenolpyruvate synthesis from malate shows that this system is present in the embryonic chick liver and post-natal rat liver but is absent in foetal rat liver. Studies on the incorporation of [^{14}C]glucose and [^{14}C]pyruvate into glycogen in liver slices also indicate that rat liver develops glyconeogenetic activity only after birth of the animal. There is good correlation between the results of the studies in slices and the enzyme experiments *in vitro*. In the chick embryo liver, enzymes obligatory for glyconeogenesis are present as early as the 11th day of incubation, and radiotracer studies have confirmed that the appearance of such activities is closely correlated with the development of glyconeogenetic ability.

INTRODUCTION

In many mammals, *e.g.* rat, rabbit, dog and guinea-pig, the foetal liver is devoid of glycogen until the last quarter of pregnancy[1]. A second group of animals including man, rhesus monkey[1] and chick embryo[2] have glycogen present in the liver at an earlier stage of development. In all embryos which have been studied, whether glycogen appears early or late in gestation, large increases in liver glycogen levels occur immediately before birth[1].

As glycogen appears in other body tissues before liver[1], it is suggested that the beginning of liver glycogenesis is due to some changes occurring in the hepatic cells rather than extrinsic factors such as circulating hormones. This is also supported by results from tissue transplants[2] and from tissue-culture experiments[3].

In this paper, the metabolic origin of glycogen and the factors involved in the initiation of glycogen synthesis in embryonic liver have been investigated. In previous

Abbreviation: PEP, phosphoenolpyruvate.

work[4] it has been shown that D-fructose-1,6-diphosphate 1-phosphohydrolase (hexosediphosphatase E.C. 3.1.3.11), an enzyme essential for the synthesis of glycogen from pyruvate by a reversal of anaerobic glycolysis, has a very low activity in the foetal rat liver, but increases strikingly after birth. This result suggests that foetal rat-liver glycogen may be synthesised mainly from hexose units and that the capacity to synthesise glycogen from 3-carbon compounds does not appear until after birth. This suggestion has been further tested in this paper by an examinaton of another enzyme system of the reversed glycolytic path, and also by measuring the incorporation of [^{14}C]pyruvate and [^{14}C]glucose into liver glycogen.

The second problem has been approached by measurements of the activities of enzymes involved in glycogen synthesis. In this way it is hoped to find a correlation between the first appearance of liver glycogen and the appearance of one or more enzymic activities.

Equivalent studies have also been made in both these fields in chick-embryo liver since the embryological environment of this animal is so markedly different from that of mammals.

MATERIALS AND METHODS

Eggs of White Leghorn crossed with Black Australorp fowls were obtained at various stages of incubation from Hampton's Poultry stud, Belmont, Western Australia. Rats of the Wistar Albino strain of the species *Rattus norvegicus* were bred in the Preclinical Animal House of this University.

Glycogen determinations: Liver samples (15–30 mg) were dissolved in 0.5 ml of 37% KOH at 100° immediately after removal from the animals. The glycogen was precipitated with two volumes of 2% Na_2SO_4–ethanol at −30° and the precipitate estimated as total carbohydrate by the anthrone method[5].

α-1,4-Glucan: orthophosphate glucosyl transferase (α-glucan phosphorylase E.C. 2.4.1.1) was assayed in fluoride–citrate homogenates at pH 5.8 (see ref. 6) by orthophosphate production from Glu-1-*P*.

Orthophosphate was measured by the method of TAUSSKY AND SHORR[7].

Hexosediphosphatase was measured as described by POGELL AND MCGILVERY[8] in borate homogenates at pH 9.3 as orthophosphate production from Fru-1,6-P_2.

D-*Glu-6-P: NADP oxidoreductase* (Glu-6-*P* dehydrogenase, E.C. 1.1.1.49) was prepared from dried Baker's yeast by an unpublished method. It was free of glucokinase (E.C. 2.7.1.2), glucosephosphate isomerase (E.C. 5.3.1.9), phosphoglucomutase (E.C. 2.7.5.1) and glucose-6-phosphatase (E.C. 3.1.3.9). It was assayed as described by KORNBERG[9].

UDP-glucose: NAD oxidoreductase (UDPG dehydrogenase E.C. 1.1.1.22) was prepared from fresh or frozen calf liver by the following procedure.

Fresh calf livers are obtained at the abattoirs and packed in ice for transport to the laboratory. They are cut into slices and frozen at −25° for storage. All operations are carried out at 0° unless otherwise stated. Fresh or frozen liver, cut into small pieces, is treated in 100-g batches with 400 ml of 0.15 M KCl in a Waring blendor at full speed for 5 min. The homogenate is filtered through gauze and centrifuged at 4000 × *g* for 40 min. The cloudy supernatant is decanted and brought to 35% saturation (at 0°) by slow addition of solid ammonium sulphate. After equilibration

for 60 min, the solution is centrifuged for 90 min at 4000 × *g*. The precipitate is discarded and the clear red supernatant brought to 55 % saturation by addition of solid ammonium sulphate. The suspension is equilibrated as before and the precipitate collected by centrifugation as before. The precipitate is dissolved in the minimum volume of ice-cold water and the concentration of ammonium sulphate determined conductimetrically. The solution is then adjusted to pH 5.0 with 2 N acetic acid and brought to 50° by immersion with stirring in a hot water bath, such that 50° is reached in 90–120 sec. After 90 sec at 50° the solution is cooled in ice water to 10° and centrifuged at 4000 × *g* for 15 min. To the supernatant, 0.2 volume of 0.1 M phosphate buffer in 0.01 M EDTA at pH 6.8 is added and the pH adjusted to 6.8. Taking into account the previously established concentration of ammonium sulphate the solution is brought to 40 % saturation with solid ammonium sulphate and after equilibration for 30 min, centrifuged at 4000 × *g* for 30 min. The supernatant is collected and the concentration of ammonium sulphate increased to 50 % saturation. After equilibration and centrifugation the precipitate is dissolved in 0.01 M phosphate buffer in 0.001 M EDTA at pH 6.8.

The enzyme solution is titrated with $Ca_3(PO_4)_2$ gel[10].

The exact conditions are established by a pilot run. Usually for a preparation from 500 g liver (volume 70 ml) about 20 ml of gel (26 mg solids/ml) are added. No enzyme is adsorbed, and in some preparations increased activity appears. The gel is discarded and a further 150 ml of gel added to adsorb the enzyme. The gel is centrifuged down and washed twice by suspension and centrifugation in 0.01 M phosphate, 0.001 M EDTA pH 6.8.

The enzyme is eluted with two 100-ml batches of 0.1 M phosphate buffer (pH 7.4) followed by three 100-ml batches of 0.2 M phosphate buffer (pH 7.4). The first eluate which is highly coloured and of low specific activity, is discarded. The other eluates are combined and the enzyme precipitated at 55 % saturation with ammonium sulphate, centrifuged down, dissolved in 0.005 M phosphate buffer (pH 7.4) and dialysed against the same buffer.

The enzyme is assayed according to STROMINGER *et al.*[11]. It is free from Glu-1-*P* uridyl transferase activity. The yield is about 40 % and the overall purification is twenty-five-fold from the original extract. It is stored frozen in small batches.

ATP: pyruvate phosphotransferase (pyruvate kinase, E.C. 2.7.1.40) was prepared from rabbit muscle as described by BUCHER AND PFLEIDERER[12] up to the first crystallization. The "crystals" were dissolved in water and dialysed to remove ammonium sulphate. The enzyme was titrated on to calcium phosphate gel in 0.05 M phosphate buffered at pH 5.5. After washing twice in 0.05 M phosphate buffer (pH 5.5), the enzyme was eluted from the gel with 0.1 M phosphate buffer (pH 7.5). The pyruvate kinase preparation was free of lactate dehydrogenase (E.C. 1.1.1.27) and adenylate-kinase (E.C. 2.7.4.3) and did not lose activity when stored at — 30° in 0.4 M KCl for over 3 months.

UDP-glucose: α-1,4-glucan α-4-glucosyl transferase (UDPG α-glucan glucosyl transferase E.C. 2.4.1.11) was assayed according to LELOIR AND GOLDEMBERG[13] by the rate of uridine diphosphate production from UDPG in the presence of 1 % oyster glycogen at 37°. UDP production was estimated by its phosphorylation by PEP in the presence of pyruvate kinase, and the resulting pyruvate was assayed by the dinitrophenyl-hydrazine reaction[14]. Owing to the rapid appearance of glucose-6-

phosphatase at birth in the rat[15], the concentration of Glu-6-*P* in all assays of rat-liver UDPG α-glucan glucosyl transferase was increased tenfold over that used by LELOIR AND GOLDEMBERG. Under these conditions the Glu-6-*P* concentration was never rate limiting.

UTP: α-D-Glu-1-P uridylyl transferase (Glu-1-*P* uridyl transferase, E.C. 2.7.7.9). Liver samples were homogenised in 20 volumes of 0.03 M Tris containing 0.005 M EDTA at pH 8.5, and centrifuged at 25000 × *g* at 0° for 1 h in the high speed head of an International Refrigerated Centrifuge (PR-2). 43 μl of the supernatant was used for the enzyme assay. The reaction mixture contained 0.5 μmole NAD, excess UDPG dehydrogenase, 200 μmoles Tris buffer (pH 8.5) and 2 μmoles $MgSO_4$. The substrates, 0.15 μmole UTP + 1.5 μmoles Glu-1-*P* together with liver extract were added giving a total volume of 0.84 ml, and the absorbancy at 340 mμ measured over 5 min at 30-sec intervals at 30°. The rate of NAD reduction was the same whether endogenous substrates were removed or not. Absorbancy increments were converted to μmoles UDPG produced/g tissue/h according to STROMINGER *et al.*[16]. Glu-1-*P* uridyl transferase activity was found to be maximal at pH 8.5 in foetal livers.

Phosphoglucomutase: 2% liver homogenates in water were prepared and centrifuged at 25000 × *g* for 1 h at 0°. Phosphoglucomutase activity was assayed in the supernatants by the method of BODANSKY[17]. The assay medium contained 40 μmoles Tris, 20 μmoles histidine, 8 μmoles Glu-1-*P*, 0.5 μmole NADP, 5 μmoles $MgSO_4$ and excess Glu-6-*P* dehydrogenase in a spectrophotometer cuvette at pH 7.4 and 37°. The change in absorbancy at 340 mμ was used to measure the reduction of NADP and thus Glu-6-*P* production. The activity was calculated in mmoles Glu-6-*P* produced/g (wet wt.) tissue/h.

The formation of PEP from malate

The isolation of liver mitochondria was carried out at 2°. 400 mg of liver after being washed in 0.9% KCl was homogenised in 2.0 ml of 0.9% KCl and centrifuged at 2000 × *g* for 30 min. The pellet was suspended in 0.9% KCl and centrifuged for 20 min at 2000 × *g*. This washing was repeated. The upper layers of the twice-washed pellet containing mitochondria and nuclei were suspended in 2.0 ml 0.9% KCl. 0.2 ml of this suspension was added to 0.2 ml of a medium containing 0.002 M ATP, 0.002 M $MgCl_2$, 0.02 M potassium phosphate buffer (pH 7.4) and 0.025 M sodium malate and incubated in open tubes in a metabolic shaker at 20° for 10-, 20- and 30-min periods. The reaction was stopped by placing the tubes in boiling water for 2 min.

After cooling, 1 μmole ADP, 10 μmoles Tris buffer (pH 7.5) 2 μmoles $MgSO_4$ and 1 μmole EDTA were added to each tube together with pyruvate kinase, and after incubation for a further 5 min pyruvate was estimated with dinitrophenylhydrazine[14]. No keto acids could be detected in the absence of pyruvate kinase. Thus the pyruvate estimated must have been formed from PEP.

The results were expressed as μmoles PEP formed/g wet wt. of liver/h at 20°.

Incorporation of [^{14}C]pyruvate or [^{14}C]glucose into glycogen

Slices were cut from foetal, young and embryonic livers by sliding a razor blade along the surface of a perspex slide with a 0.3-mm deep groove which was pressed on to the liver sample. The liver slice adhered to the perspex and was transferred to stop-

pered gassed vessels containing 1 ml of a high potassium medium (110 μmoles K^+, 20 μmoles Mg^{2+}, 10 μmoles Ca^{2+}, 70 μmoles Cl^-, 40 μmoles HCO_3^-, 60 μmoles pyruvate and 30 μmoles glucose at pH 7.45)[18] containing ^{14}C substrate. Slices (100 mg per tube) were incubated with 1 μC [^{14}C]glucose or [^{14}C]pyruvate for 2 h at 37° in an atmosphere of O_2–CO_2 (95:5) with shaking.

2 ml of 60 % KOH were added to each vessel after incubation and after digestion in boiling water for 10 min, glycogen was precipitated by the addition of 5 ml ethanol. The glycogen samples were purified according to COWGILL AND PARDEE[19] and washed with ethanol until no radioactivity could be detected in the ethanol. The glycogen samples were dissolved in water and glycogen determined in aliquots by the anthrone method[5]. Further aliquots (containing less than 0.5 mg glycogen in 200 μl) were added to 5.0 ml Diotol[20] and counted in a liquid scintillation counter with an efficiency of 50 %. Internal glucose standards were counted after the samples, but no quenching corrections were necessary. The results were expressed as counts/min/mg of glycogen.

Chemicals

Fru-1,6-P_2, PEP, UTP, UDPG, ATP, ADP, Glu-6-*P* were obtained from the Sigma Chemical Co., Glu-1-*P* from L. Light & Co. Colnbrook (Great Britain) and NAD, NADP from Calbiochem.

[2-^{14}C]Pyruvate and uniformly labelled [$^{14}C_6$]glucose were obtained from the Radiochemical Centre, Amersham, Bucks (Great Britain).

RESULTS

The points on each figure represent the mean of from 4–10 determinations. The limits are plus and minus one standard error.

Glycogen first appears in the foetal rat liver on the seventeenth day of gestation (Fig. 1) and increases rapidly to a concentration of almost 10 % (wet wt.) by the time of birth on the twenty-second day. The concentration falls immediately after birth, and then rises over the next 3 weeks to the adult value of 30 mg/g. The postnatal

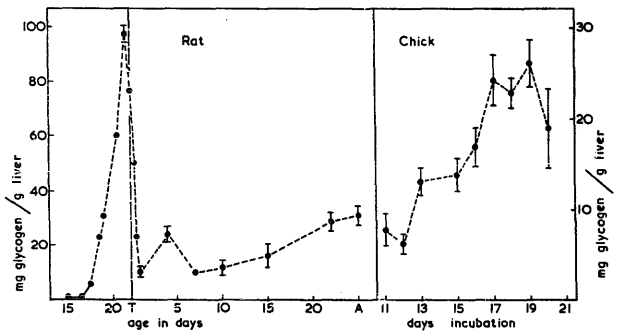

Fig. 1. Neonatal rat and embryonic chick-liver glycogen content. T, term; A, adult. Limits of ± 1 S.E. are shown where they are large enough to be indicated.

glycogen values were extremely variable. The glycogen content of the chick embryo liver increases from 7 mg/g at 10 days of incubation to 26 mg/g at 19 days. There is a fall to the twentieth day.

In the rat-liver UDPG α-glucan glucosyl transferase (Fig. 2) rises from 14 μmoles/g/h on the fifteenth day of gestation to 40 by the seventeenth day with a large increase to 214 μmoles/g/h by the twentieth day of foetal life. It remains at this high level even in the adult. This enzyme increases in activity by a factor of 3 from the tenth to the nineteenth day of incubation in the chick liver.

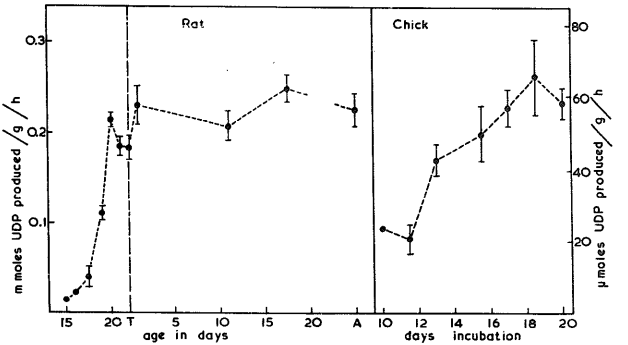

Fig. 2. Neonatal rat and embryonic chick liver UDPG α-glucan glucosyl transferase activity, expressed as UDP produced from UDPG with glycogen primer. Assayed by method of LELOIR AND GOLDEMBERG[13]. T, term; A, adult.

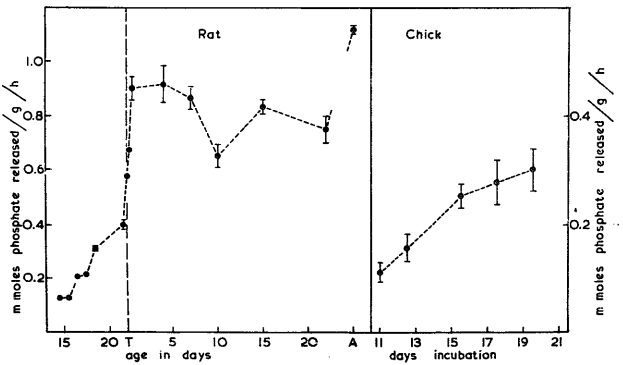

Fig. 3. Neonatal rat and embryonic chick liver α-glucan phosphorylase activity, expressed as inorganic phosphate released from Glu-1-*P* with glycogen primer. Assayed by method of SHULL *et al.*[6]. T, term; A, adult.

In both the foetal rat and chick embryo hepatic α-glucan phosphorylase shows a threefold increase throughout the period studied (Fig. 3). However, rat liver α-glucan phosphorylase shows a striking increase in activity during the day of birth, reaching

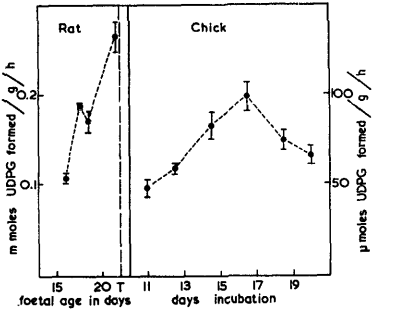

Fig. 4. Foetal rat and embryonic chick-liver Glu-1-*P* uridyl transferase activity, expressed as UDPG formed from UTP and Glu-1-*P*. The assay method is described under methods. T, term.

a maximum activity of nearly 1 mmole/g/h. This high level of activity remains in the adult.

Foetal rat-liver Glu-1-*P* uridyl transferase increases from a value of 0.1 mmole/g/h at sixteen days to 0.26 mmole/g/h at birth (Fig. 4). A peak in activity of this enzyme is found at the sixteenth day of incubation in the chick embryo at which stage it has an activity of 0.1 mmole/g/h.

In both animals phosphoglucomutase activity increases considerably throughout the period studied. It has a high activity at the earliest stage we could measure (Fig. 5).

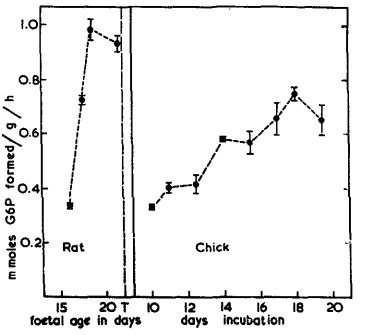

Fig. 5. Foetal rat and embryonic chick-liver phosphoglucomutase activity, expressed as Glu-6-*P* formed from Glu-1-*P* assayed as described by BODANSKY[17]. T, term.

The synthesis of PEP from malate in washed mitochondria could not be demonstrated in 20-day foetal rat liver. This point represents 8 values obtained from 24 animals in 2 litters. The ability to synthesise PEP increases soon after birth, reaching a value of 1 μmole/g/h after 20 days (Fig. 6). This system had a significant activity (1.3 μmoles/g/h) at the eleventh day of incubation in the chick and reached a maximum of 10.8 μmoles/g/h after 17 days. By hatching the activity had halved.

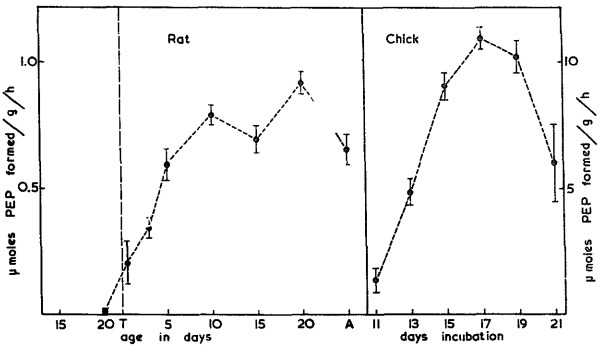

Fig. 6. The rate of PEP synthesis from malate in mitochondria prepared from neonatal rat and embryonic chick liver. Assay is described under METHODS. T, term; A, adult.

TABLE I

SPECIFIC RADIOACTIVITY OF GLYCOGEN ISOLATED FROM LIVER SLICES INCUBATED FOR 2 H AT 37° WITH ^{14}C-LABELLED SUBSTRATES

Age of rats	*20-day foetus*	*22-day foetus*	*1 day*	*5 day*	*10 day*	*15 day*	*20 day*	*Adult*
Counts/min/mg								
($\times 10^{-3}$) from [^{14}C]pyruvate	0.065	0.45	36.7	37.6	76.6	63.2	60.2	24.3
± standard error	0.026	0.025	2.7	5.1	9.9	2.1	6.4	2.5
Counts/min/mg								
($\times 10^{-3}$) from [^{14}C]glucose	7.7	4.8	34.3	14.8	19.5	21.2	55.1	89.0
± standard error	0.45	0.78	3.7	5.1	2.8	1.8	7.0	11.7

Age of chick embryos	*11 day*	*13 day*	*15 day*	*17 day*	*19 day*	*21 day*
Counts/min/mg						
($\times 10^{-3}$) from [^{14}C]pyruvate	14.2	13.65	9.75	11.75	0.38	3.25
± standard error	1.42	2.84	4.0	6.0	0.011	0.93
Counts/min/mg						
($\times 10^{-3}$) from [^{14}C]glucose	21.8	6.6	3.13	4.75	2.44	6.87
± standard error	6.1	1.36	0.60	0.90	0.74	1.87

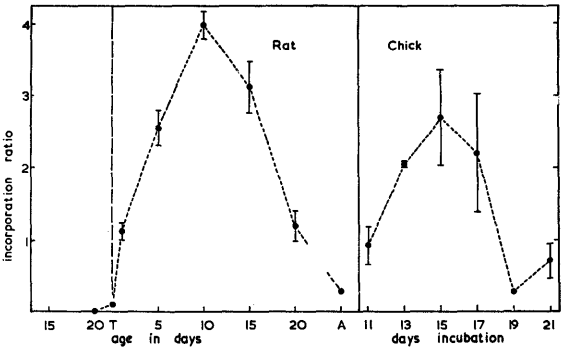

Fig. 7. The ratio between counts/min incorporated from [^{14}C]pyruvate into liver glycogen and counts/min incorporated from [^{14}C]glucose into liver glycogen. Slices from neonatal rat and embryonic chick liver were incubated with labelled substrates as described under METHODS. T, term; A, adult.

Table I shows the specific radioactivity of glycogen isolated from rat-liver slices incubated with either [^{14}C]pyruvate or [^{14}C]glucose. The most significant result is that the synthesis of glycogen from [^{14}C]pyruvate is non-existent in the youngest foetus, and this capacity increases rapidly after birth. These results have been plotted in Fig. 7 as the ratio counts/min/mg glycogen from pyruvate to counts/min/mg glycogen from glucose. We suggest that this gives a more meaningful presentation of results, as first, the fragility of the liver slices decreases with age as connective tissue is formed, and secondly, the enormous variation in liver glycogen content must have a large effect on the rate of isotope incorporation.

Similar results are presented for chick-liver slices incubated with [^{14}C]pyruvate

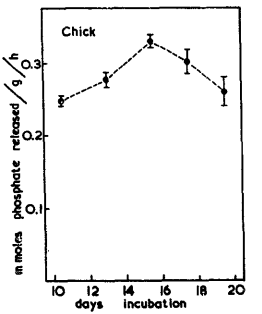

Fig 8. Embryonic chick-liver hexosediphosphatase activity, expressed as inorganic phosphate released from Fru-1,6-P_2. Assayed by method of POGELL AND MCGILVERY[8].

or [^{14}C]glucose in Table I and Fig. 7. The wide scatter of results is probably due to the extreme fragility of these slices.

Hexosediphosphatase (Fig. 8) has a high activity in the chick liver from the tenth to the twentieth day, with a peak in activity of 0.33 mmole/g/h on the sixteenth day.

DISCUSSION

It was not possible to study enzymic changes at the stage when glycogen first appears in the chick-embryo liver (6 days[2]), but the glycogen content of the 10-day liver was still very low. As the hepatic glycogen level increases by a factor of four to five from this stage to that of a twenty-day embryo, it would seem that changes in enzymic activity may be the direct cause of the increase in glycogen deposition.

It has not been possible to relate this glycogen increase with a single enzyme, as all the enzymes studied show considerable increases in activity. RINAUDO[21] has also shown increases in four glycolytic enzymes, phosphopyruvate hydratase (E.C. 4.2.1.11), glyceraldehyde phosphate dehydrogenase (E.C. 1.2.1.12), aldolase (E.C. 4.1.2.7) and glucose phosphate isomerase during this period.

The ^{14}C results indicate an increasing importance of glyconeogenesis up to the fifteenth day, with a fall towards hatching. It should be noted that even at the 11-day stage pyruvate was at least as good a substrate for glycogen synthesis as glucose.

Hexose diphosphatase and the synthesis of PEP from malate, which are essential for the process of glyconeogenesis, show peaks in activity at the sixteenth and seventeenth days, lending support to the contention that during the period of active glycogen formation, 3 carbon compounds are more important substrates than glucose. This is further supported by the results of SOLOMON[22] who showed that lactate dehydrogenase and malate dehydrogenase (E.C. 1.1.1.37), have peaks in activity at the fifteenth and sixteenth days of incubation. Both these enzymes are required for the synthesis of glycogen from lactate, the 3-carbon acid of highest concentration in the blood.

The enzymes of glycogenesis in the rat show increases in activity throughout foetal development, although UDPG α-glucan glucosyl transferase increases by a factor of 15 while phosphoglucomutase, α-glucan phosphorylase and Glu-1-*P* uridyl transferase increase only two- or three-fold. It is tempting to assign the major rate-limiting step to UDPG α-glucan glucosyl transferase, although a knowledge of enzyme-substrate affinities, enzyme turn-over numbers and the steady-state concentration of substrates *in vivo* would be required to define rigorously the rate-limiting step.

The lack of incorporation of [^{14}C]pyruvate into glycogen in the foetal rat-liver slices clearly demonstrates that glycogen cannot be synthesised from 3-carbon acids in these animals. The hexosediphosphatase results previously reported[4] and the rate of conversion of malate to PEP reported here confirm this finding. It is likely that one or both of these enzymes restrict the synthesis of glycogen from pyruvate in the foetal liver as both have extremely low activities.

It is difficult to interpret the finding that glycogen is formed 4 times as readily in the 10-day young rat from pyruvate as from glucose, while in the adult the ratio is reversed. However, the changes observed in this ratio mirror more closely the results on hexosediphosphatase than on PEP synthesis. Hexosediphosphatase activity may thus be the rate-limiting factor for glyconeogenesis in older animals.

The sudden increase of α-glucan phosphorylase activity at the time of birth corresponds with the almost complete degradation of the hepatic glycogen store. It is well known that the activity of glucose-6-phosphatase increases considerably during this period[15] and the degradation of glycogen has been attributed to this enzyme. It is probable that the increase in α-glucan phosphorylase activity also plays a significant part in the degradation of glycogen during this period.

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