Carbohydrate Metabolism in Liver from Foetal and Neonatal Sheep

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(Received 6 July 1964)

1. During development of the sheep, the activities of UDP-glucose- α -glucan glucosyltransferase and UDP-glucose pyrophosphorylase and the glycogen content are highest in the liver of lambs 2 weeks old and considerably lower in liver from adult sheep. 2. The activity of hexokinase and the rate of incorporation of [^{14}C]-glucose into glycogen are much lower in liver from postnatal sheep than in rat liver. 3. The activities of hexose diphosphatase and glucose 6-phosphatase and the rates of incorporation of [^{14}C]pyruvate and [^{14}C]propionate into glycogen increase from low levels in the liver of foetal sheep to maxima a few weeks after birth. The activities in the liver of adult sheep are slightly lower. 4. The incorporation rate of [^{14}C]pyruvate into glucose has been measured in liver slices from rats, sheep and chick embryos at several ages of these animals. This pathway is active in liver from foetal sheep, embryonic chicks and postnatal rats or sheep, but is absent from the liver from foetal rats. 5. Fructose metabolism, as measured by the rates of incorporation of [^{14}C]fructose into glycogen and glucose in liver slices and by assays of liver ketohexokinase, is barely detectable in the liver of foetal sheep and appears soon after birth. 6. During development of the sheep, the incorporation rate of [^{14}C]galactose into glycogen in liver slices is highest in foetal sheep and decreases with increasing age of the animal. 7. These findings are discussed with reference to the changing pattern of carbohydrate metabolism during neonatal development of liver in the sheep.

The activities of enzymes involved in the conversion of glucose into glycogen in liver are known to change during foetal and postnatal development of the mammal. Ballard & Oliver (1963), Dawkins (1963) and Burch *et al.* (1963), working with rat liver, and Kornfeld & Brown (1963), using guinea-pig liver, found that several enzymes concerned with glycogen synthesis increased in activity towards the end of gestation. In addition, Ballard & Oliver (1963) showed that glycogenesis was absent from liver from foetal rats and that this pathway appeared at birth.

Studies of this nature have not been undertaken with sheep liver. Filsell, Jarrett, Atkinson, Caiger & Morton (1963) have measured hexokinase activity in livers from growing lambs, and Dawkins (1961) has found that glucose 6-phosphatase activity increases at the birth of the lamb. Changes in glucose utilization during development of the lamb have been determined by McCandless & Dye (1950), Jarrett & Potter (1952) and Jarrett, Jones & Potter (1964). From these results it is clear that the metabolism of the young lamb resembles that of monogastric animals more closely than that of adult sheep.

The discovery of two glucose-phosphorylating enzymes in extracts of liver from adult rats and guinea pigs (Walker, 1962; Viñuela, Salas & Sols, 1963; Walker, 1963*a*; Sharma, Manjeshwar & Weinhouse, 1963; Ballard & Oliver, 1964*a*) indicates that in the liver of these animals there is a concentration-dependent system for the control of glucose phosphorylation. In the liver of the foetuses of these animals no such system is present; glucokinase (EC 2.7.1.2), the enzyme with a high Michaelis constant for glucose, is absent. We have shown (Ballard & Oliver, 1964*b*) that glucokinase is not present in the liver of foetal or adult sheep and that this absence is reflected in the rate of incorporation of glucose into glycogen in liver slices.

In most mammals, fructose is present in the diet although usually at a concentration considerably lower than glucose. In the foetus, the blood of sheep, together with that of other ungulates and also the Cetaceae, has a low concentration of glucose and is rich in fructose (Huggett, 1961). Surprisingly, Andrews, Britton, Huggett & Nixon (1960) found that liver from foetal sheep was unable to metabolize fructose.

In the present work, the utilization of glucose,

fructose, galactose, pyruvate and propionate for the synthesis of liver glycogen has been investigated during foetal development in the sheep. In addition, a relatively simple method is described for the measurement of the incorporation of pyruvate, propionate or fructose into glucose in liver slices. This method has been used to measure the rate of incorporation of pyruvate into glucose in liver slices from sheep, rat and chick embryos at various ages, and the incorporation of fructose into glucose in sheep-liver and lamb-liver slices.

MATERIALS AND METHODS

Chemicals. ATP, ADP, UTP, UDP, glucose 6-phosphate, glucose 1-phosphate, and UDP-glucose were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. [^{14}C]Glucose, [^{14}C]fructose, [^{14}C]galactose, sodium [^{14}C]pyruvate and sodium [^{14}C]propionate were obtained from The Radiochemical Centre, Amersham, Bucks.

Animals. Rats of the Wistar albino strain of *Rattus norvegicus* were bred in the preclinical animal house of this University. Adult sheep were Merino wethers (aged 1–2 years) that were pasture-fed at the Institute of Agriculture of this University. Foetal sheep were Merinos of either sex obtained from the abattoirs. Lambs were Merinos of either sex kept with their mothers until killed. Both ewes and lambs had access to pasture and were supplemented with a diet containing two parts (by wt.) of oat chaff, one part of lucerne chaff and one part of sheep nuts (21% of protein). All sheep were killed by cutting the neck blood vessels. The livers were removed and placed on frozen blocks of a medium consisting of KCl (70 mm), MgCl_2 (20 mm), KHCO_3 (40 mm) and CaCl_2 (10 mm), adjusted to pH 7.45 with 2N-HCl, for transport to the laboratory.

Assay of hexokinase (EC 2.7.1.1). Liver extracts were prepared and hexokinase was assayed in a manner similar to that described by Ballard & Oliver (1964a) for glucokinase, except that the change in extinction at $340\text{ m}\mu$ in a reaction mixture with ATP omitted was subtracted from the extinction change in the complete system. This was necessary because, at the glucose concentration used (0.1 M), the presence of a glucose dehydrogenase (EC 1.1.1.47) in sheep-liver extracts interfered with the hexokinase assay. As sheep liver does not contain a glucokinase with a high K_m for glucose (Ballard & Oliver, 1964b), the sheep-liver hexokinase may be assayed at a glucose concentration of 1 mm. At this concentration the assay is not complicated by an apparent activity due to the glucose dehydrogenase as this latter enzyme has K_m approx. 150 mm for glucose (Strecker & Korkes, 1952). The hexokinase activities in sheep liver at glucose concentrations of 0.1 M or 1 mm are always similar as long as the blank due to glucose-dehydrogenase activity has been subtracted.

Assay of UDP-glucose- α -glucan glucosyltransferase (EC 2.4.1.11). This enzyme was assayed according to the method of Leloir & Goldemberg (1960), in the presence of 1% of oyster glycogen at 37° . UDP production was measured by phosphorylation with phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40), and the pyruvate formed was measured by using the dinitrophenylhydrazine reaction (Cabib & Leloir, 1958). Owing to the high activity of glucose 6-phosphatase (EC 3.1.3.9) in liver from neonatal sheep, the

concentration of glucose 6-phosphate in the reaction mixture was increased tenfold over that used by Leloir & Goldemberg (1960). Under these conditions the glucose 6-phosphate concentration gave maximum stimulation of activity.

Pyruvate kinase. This enzyme was prepared by the method of Tietz & Ochoa (1962).

Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) This enzyme was prepared as described by Ballard & Oliver (1964a).

Phosphoglucumutase (EC 2.7.5.1). This enzyme was prepared from dried baker's yeast by the method of McCoy & Najjar (1959).

Assay of UDP-glucose pyrophosphorylase (EC 2.7.7.9). Liver samples were homogenized in 20 vol. (w/v) of 30 mM-tris containing EDTA (5 mM) (adjusted to pH 8.5 with 2N-HCl) and centrifuged at $25\,000g$ at 0° for 1 hr. in an International refrigerated centrifuge model PR 2 with rotor 296. The supernatant was diluted 1:5 (v/v) with homogenization medium. The assay cuvettes contained tris (0.2 M) (adjusted to pH 7.45 with 2N-HCl), UDP-glucose (0.4 mM), MgCl_2 (2 mM), NADP (0.5 mM), excess of glucose 6-phosphate dehydrogenase and excess of phosphoglucumutase. To each cuvette was added $25\text{ }\mu\text{l}$. of the diluted liver extract, giving a total volume of 1.125 ml. The cuvettes were maintained at 37° for 10 min. to incubate out any endogenous substrates, and the reaction was started by the addition of $50\text{ }\mu\text{l}$. of 50 mM- $\text{K}_4\text{P}_2\text{O}_7$, which was prepared immediately before use. The change in extinction was measured at $340\text{ m}\mu$ at 37° for 10 min. at 30 sec. intervals. The rate of glucose 1-phosphate production was calculated from the rate of NADP reduction, and expressed as m-moles of glucose 1-phosphate produced/g. wet wt. of liver/hr.

Assay of phosphoglucumutase. Liver homogenates (5%, w/v) in 50 mM-histidine (adjusted to pH 7.0 with N-HCl) were prepared and centrifuged at $25\,000g$ for 1 hr. at 0° . Phosphoglucumutase activity was measured in the supernatant by the method of Bodansky (1961). The assay cuvettes contained tris (40 mM), histidine (20 mM), NADP (0.5 mM), MgCl_2 (5 mM) (adjusted to pH 7.4 with N-HCl) and $25\text{ }\mu\text{l}$. of supernatant. Excess of glucose 6-phosphate dehydrogenase was added and endogenous substrates were incubated out. The change in extinction at $340\text{ m}\mu$ and 37° was followed for 10 min. at 30 sec. intervals after the addition of glucose 1-phosphate (to 8 mM). The activity was calculated in m-moles of glucose 6-phosphate produced/g. wet wt. of liver/hr.

Assay of glucose 6-phosphatase. Approx. 100 mg. of liver was homogenized in 1 ml. of 0.1 M-citric acid (adjusted to pH 6.5 with N-KOH). A 0.1 ml. portion of homogenate was incubated with 0.1 ml. of 0.025 M-potassium glucose 6-phosphate (adjusted to pH 6.5 with N-KOH) for 0, 10 and 20 min. at 37° (Shull, Ashmore, & Mayer, 1956). The reaction was stopped by the addition of 2 ml. of 5% (w/v) trichloroacetic acid and the precipitated protein removed by centrifuging. Inorganic phosphate was measured in 1 ml. samples of the supernatant by the method of Taussky & Shorr (1953). Glucose 6-phosphatase activity was expressed as m-moles of inorganic phosphate released/g. wet wt. of liver/hr.

Assay of hexose diphosphatase (EC 3.1.3.11). Hexose-diphosphatase activity was measured, as described by Pogell & McGilvery (1954), as inorganic phosphate production from fructose 1,6-diphosphate.

Assay of α -glucan phosphorylase (EC 2.4.1.1). α -Glucan phosphorylase was assayed, as described by Shull *et al.* (1956), as inorganic phosphate production from glucose 1-phosphate.

Assay of ketohexokinase (EC 2.7.1.3). Ketohexokinase was assayed as described by Ballard & Oliver (1964a).

Isotope experiments. (1) Incorporation of [^{14}C]glucose, [^{14}C]galactose, [^{14}C]fructose, [^{14}C]pyruvate and [^{14}C]propionate into glycogen in liver slices. The methods used were as described by Ballard & Oliver (1964a), except that the volume of the incubation medium was 2 ml. The substrate concentrations used were 30 mM for glucose, galactose and fructose, and 60 mM for pyruvate and propionate. When pyruvate or propionate were substrates the concentration of KCl was decreased from 70 mM to 10 mM. The results are expressed as μmoles of substrate incorporated into the glycogen present in 1 g. of liver in the 2 hr. incubation. For an accurate estimate of the glycogen content of the liver slices, the purification procedure must result in complete glycogen recovery. This does not occur when the glycogen content of the incubated slices is below 10 mg./g. This value was exceeded in all experiments.

The 'incorporation rate' of labelled substrate into liver glycogen is based on measurements after incubation for 2 hr. Table 1 summarizes experiments with liver slices from foetal or newborn and adult sheep incubated with ^{14}C -labelled substrates for various times from 0 to 3 hr. In all experiments it was found that the incorporation of ^{14}C continues at a roughly linear rate throughout the incubation.

(2) Incorporation of [^{14}C]pyruvate and [^{14}C]propionate

into glucose in liver slices. Slices were cut and incubated as described by Ballard & Oliver (1964a). After incubation, the incorporation was stopped by the addition of 1 ml. of 10% (w/v) perchloric acid. The contents of each tube were poured into a Perspex homogenizer designed to (a) hold up to 4 ml. of fluid, and (b) fit into the cups of an Ecco bench centrifuge. The contents were homogenized and centrifuged for 5 min. at 2000g. The supernatant was decanted into a centrifuge tube with a capacity of 15 ml. The precipitate was homogenized in 1.0 ml. of 3.3% (w/v) perchloric acid and centrifuged as before. The supernatant was added to the previous one. This washing was repeated once. To each centrifuge tube containing approx. 5 ml. of extract was added 1 drop of bromocresol purple indicator. The contents of each tube were neutralized (pH 6-8) by adding 2N-KOH dropwise with stirring. Then 7.5 ml. of ethanol was added to each tube, which was then placed in boiling water until the ethanolic solution came to the boil. The tubes were then cooled to -15° and centrifuged for 10 min. in the cold room. The precipitate contains the glycogen extracted from the tissue slices (this extraction is not quantitative) and most of the potassium perchlorate, as it is only slightly soluble at 2° . The supernatants were poured into 50 ml. test tubes and diluted to 25 ml. with water. To each tube was added 1 g. of finely ground (smaller than 250 mesh) Bio-Deminrolit, a mixed-bed ion-exchange resin. The tubes were shaken vigorously and, after standing for 30 min., filtered through Whatman no. 5 filter paper. To each filtrate 0.3 g. of Bio-Deminrolit was added. The tubes were shaken, stood and the contents filtered as before. In all cases at least 99.9% of labelled propionate or pyruvate was removed by

Table 1. *Effect of incubation time on the incorporation of ^{14}C from labelled substrates into glycogen or glucose in liver slices*

The methods used are described under 'Isotope experiments' in the Materials and Methods section and in Ballard & Oliver (1964a). In those cases where sufficient liver could not be obtained from a single animal, livers from rat litter mates or from chick embryos of similar ages were pooled.

Labelled product	Labelled substrate	Source of liver	Incubation time (hr.)...	Substrate incorporated ($\mu\text{moles/g. of liver}$)			
				0	1	2	3
Glycogen	Glucose	Lamb (5 days)	0	0	0.50	0.89	1.41
		Adult sheep	0	0	0.41	0.46	0.67
	Fructose	Lamb (2 days)	0	0	0.61	0.75	1.03
		Adult sheep	0	0	6.80	11.5	21.1
	Galactose	Foetal sheep (4 months)	0	0	14.4	36.3	60.7
		Adult sheep	0	0	1.31	1.85	4.17
	Pyruvate	Lamb (5 days)	0	0	0.81	2.47	3.92
		Adult sheep	0	0	0.83	4.60	7.13
	Propionate	Lamb (5 days)	0	0	3.21	3.63	5.98
		Adult sheep	0	0	2.83	5.10	8.05
Glucose	Propionate	Lamb (5 days)	0	0	4.3	7.9	13.4
		Adult sheep	0	0	8.2	25.4	39.4
	Fructose	Foetal sheep (4 months)	0	0	< 5	< 5	< 5
		Adult sheep	0	0	48	80	111
	Pyruvate	Lamb (5 days)	0	0	23.4	57.9	76.6
		Adult sheep	0	0	8.4	18.8	30.5
	Pyruvate	Foetal rat (22 days)	0	0	2.04	2.32	4.56
		Young rat (10 days)	0	0	80	125	191
		Adult rat	0	0	14.5	26.1	38.1
	Pyruvate	Chick embryo (15 days)	0	0	65	176	216

Table 2. Removal of ^{14}C -labelled pyruvate or propionate by treatment with Bio-Deminrolit

Liver slices (100 mg.) from an adult rat were added to 2.0 ml. of the incubation medium at 0° (Ballard & Oliver, 1964a) containing KHCO_3 (40 mm), KCl (10 mm), CaCl_2 (10 mm), MgCl_2 (20 mm) and either potassium pyruvate (60 mm) or potassium propionate (60 mm), which had been adjusted to pH 7.45 with 0.01 N-HCl. The additions are indicated below. Then 1 ml. of 10% (w/v) perchloric acid was immediately added and the contents of each tube were treated as described in 'Isotope experiments (2)' in the Materials and Methods section. Radioactivity was determined in samples of the deionized solution by counting in an Ekco liquid-scintillation detector. Each result is the mean of two similar experiments.

Medium	$10^{-6} \times [^{14}\text{C}]$ - Pyruvate added (counts/min.)	$10^{-6} \times [^{14}\text{C}]$ - Propionate added (counts/min.)	$10^{-4} \times [^{14}\text{C}]$ - Glucose added (counts/min.)	$10^{-4} \times$ Radio- activity recovered (counts/min.)	Pyruvate remaining (%)	Propionate remaining (%)	Glucose remaining (%)
Pyruvate	2.01	—	0	0.02	0.01	—	—
	2.01	—	1.85	1.86	—	—	101
	0	—	1.85	1.82	—	—	98
Propionate	—	2.06	0	0.01	—	0.005	—
	—	2.06	1.85	1.84	—	—	99
	—	0	1.85	1.74	—	—	94

this double treatment (Table 2). A 5 ml. portion of the second filtrate was pipetted into liquid-scintillation pots and evaporated to dryness. Then 0.2 ml. of water followed by 5.0 ml. of Diotol (Herberg, 1960) was added to the pots, and, after a thorough mixing, the radioactivity was determined in an Ekco liquid-scintillation detector. Although at least 99.9% of pyruvate or propionate was removed, the recovery of glucose was quantitative (Table 2). In experiments on several ages of each animal species 5 ml. portions of the second filtrate were freeze-dried and dissolved in 0.3 ml. of water. A 100 μl . sample of this concentrated solution was chromatographed on Whatman no. 1 paper with either phenol-water (4:1, w/v) or ethyl acetate-pyridine-water (12:5:4, by vol.), (Smith, 1960, p. 248). The dried strips were scanned for radioactivity with a Nuclear-Chicago 4 π Actigraph. In all cases a single radioactive spot was found. When the chromatogram strips were stained for hexose with the benzidine reagent (Smith, 1960, p. 250), a single spot was found at the same position as the radioactivity and corresponding in R_F to a glucose standard.

The results are expressed as μmoles of substrate converted into glucose/g. wet wt. of liver. The incorporation rate of ^{14}C into liver glucose is based on measurements after 2 hr. incubation. Liver slices from lambs 5 days old or from adult sheep were incubated with ^{14}C -labelled pyruvate or propionate for various times from 0 to 3 hr. (Table 1). In all experiments the incorporation of ^{14}C continued at a roughly linear rate throughout the incubation period.

(3) Incorporation of [^{14}C]fructose into glucose in liver slices. Slices were cut and incubated as described by Ballard & Oliver (1964a). After 2 hr. the incorporation was stopped, glucose was extracted and the solution deionized with Bio-Deminrolit as described in the preceding section. The deionized solution (approx. 10 ml.) was freeze-dried, and the solid dissolved in 0.5 ml. of water. A 100 μl . portion of this solution was placed in a 1.7 cm.-long band on Whatman no. 1 chromatography paper and the chromatogram developed by descending chromatography for 15 hr. at room temperature (20–25°). The solvent used was phenol-water (4:1, w/v). The paper was dried and cut into strips 3.5 cm. wide. By staining with benzidine (Smith, 1960), it was shown that the two hexose spots were well separated. The strips were scanned for radioactivity with a Nuclear-

Chicago 4 π Actigraph gas-glow detector. From the relative areas of the peaks, the percentage conversion of fructose into glucose was calculated, and thus the rate of incorporation in $\mu\text{moles/g. wet wt. of liver/2 hr.}$

To test whether fructose conversion into glucose was proceeding at a linear rate throughout the 2 hr. incubation period, liver slices from foetal and adult sheep were incubated for various times from 0 to 3 hr. Table 1 shows that the incorporation was roughly linear in liver slices from adult sheep whereas incorporation was barely detectable in liver slices from foetal sheep.

RESULTS

Glycogen synthesis from glucose. The concentrations of glycogen in livers from postnatal sheep are shown in Fig. 1(a). The glycogen content of the liver samples from foetal sheep were generally high, but are not included in the Figure as the fetuses were obtained from ewes slaughtered at the abattoirs and no information could be obtained on the time that the ewes had been kept without food before being killed. The glycogen content of liver from postnatal sheep increases from 11 mg./g. 1 day after birth to a maximum of 45 mg./g. in the liver of 4-week-old lambs and then decreases to about 20 mg./g. in lambs older than 7 weeks.

UDP-glucose- α -glucan glucosyltransferase activity (Fig. 1b) increases from 181 $\mu\text{moles/g./hr.}$ in livers from foetal sheep (gestational age 4 months) to 210 $\mu\text{moles/g./hr.}$ in liver from newborn lambs. There is a fall in activity throughout development to that (55 $\mu\text{moles/g./hr.}$) found in the adult.

The activity of UDP-glucose pyrophosphorylase (Fig. 1c) in the 4-month sheep foetus is 2.67 m-moles/g./hr. The activity is slightly higher in the newborn and then falls to the value (0.85 m-mole/g./hr.) found in the adult.

Phosphoglucumutase activity (Fig. 2a) increases from 0.89 m-mole/g./hr. in liver from foetal sheep

Fig. 1. (a) Glycogen content of liver from neonatal sheep. (b) UDP-glucose- α -glucan glucosyltransferase activity determined by UDP production from UDP-glucose and primer glycogen at 37° in 3000g supernatants of sheep liver. (c) UDP-glucose-pyrophosphorylase activity determined by glucose 1-phosphate formation from UDP-glucose and pyrophosphate at 37° in 25000g supernatants of sheep liver. Additional experimental details are given in the Materials and Methods section. F, 4-month sheep foetus; T, term; A, adult. Each point represents the mean $\pm$ S.E.M. of at least four duplicate determinations.

to 1.27 m-moles/g./hr. in liver from adult sheep. The activity of α -glucan phosphorylase in sheep liver (Fig. 2b) increases from 0.77 m-mole/g./hr. in the 4-month foetus to 2.4 m-moles/g./hr. in the newborn lamb. The activity of α -glucan phosphorylase is variable from birth to the value (1.85 m-moles/g./hr.) found in the adult.

The activity of hexokinase and the rate of incorporation of [14 C]glucose into glycogen are shown in Fig. 3. Liver hexokinase has an activity of 13.9 μ moles/g./hr. in the foetus (4 months), 21.5 μ moles/g./hr. in newborn lambs and 7.5 μ moles/g./hr. in adult sheep. The incorporation rate of glucose into sheep liver glycogen falls from 3.34 μ moles/g./2 hr. in 3-month foetal sheep to 1.09 μ moles/g./2 hr. at birth and to a minimum of 0.31 μ moles/g./2 hr. in lambs 6 weeks old. There is a rise to an incorporation rate of 0.67 μ moles/g./2 hr. in liver from adult sheep.

Glucose and glycogen formation from pyruvate and

Fig. 2. (a) Phosphoglucumutase activity determined as glucose 6-phosphate formation from glucose 1-phosphate in 25000g supernatants of sheep liver at 37°. (b) α -Glucan-phosphorylase activity assayed as inorganic phosphate formation from glucose 1-phosphate and primer glycogen at 37° in homogenates of sheep liver. Additional experimental details are given in the Materials and Methods section. F, 4-month sheep foetus; T, term; A, adult. Each point represents the mean $\pm$ S.E.M. of four to six determinations.

Fig. 3. (a) Hexokinase activity of liver from neonatal sheep. Supernatants obtained after centrifuging liver extracts at 25000g were used for the assay after endogenous glucose had been removed by dialysis. The hexokinase was estimated spectrophotometrically at 340 m μ and 37° by glucose 6-phosphate production from ATP and glucose with NADP and glucose 6-phosphate dehydrogenase. (b) Incorporation of [14 C]glucose into glycogen in sheep-liver slices incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. F, 4-month sheep foetus; T, term; A, adult. Each point represents the mean $\pm$ S.E.M. of four to eight duplicate determinations.

Table 3. *Incorporation rate of [2-¹⁴C]pyruvate or [2-¹⁴C]propionate into glycogen or glucose in sheep-liver slices*

Slices from liver were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section and in Ballard & Oliver (1964a). Each determination is the mean of duplicates. All results are expressed as means $\pm$ s.e.m., with the numbers of determinations given in parentheses.

Labelled product	Labelled substrate	Age of	Substrate incorporated (μ moles/g. of liver/2 hr.)					
			0	2	4	7	10	Adult
Glycogen	Pyruvate	Foetus (4 months)	1.88 $\pm$ 0.38 (13)	2.33 $\pm$ 0.93 (7)	4.82 $\pm$ 1.51 (7)	4.67 $\pm$ 1.28 (12)	4.73 $\pm$ 0.95 (6)	3.87 $\pm$ 1.05 (8)
	Propionate		0.61 $\pm$ 0.15 (10)	4.42 $\pm$ 1.45 (7)	7.31 $\pm$ 2.15 (10)	5.38 $\pm$ 1.27 (6)	4.90 $\pm$ 0.73 (6)	5.30 $\pm$ 1.28 (8)
Glucose	Pyruvate	Age of sheep (weeks) ...	44.9 $\pm$ 12.9 (4)	78.4 $\pm$ 9.1 (4)	56.2 $\pm$ 3.0 (4)	—	17.9 $\pm$ 4.6 (5)	16.6 $\pm$ 6.3 (7)
	Propionate		17.7 $\pm$ 6.0 (4)	38.6 $\pm$ 6.4 (4)	35.7 $\pm$ 6.2 (4)	—	23.3 $\pm$ 2.9 (7)	22.3 $\pm$ 2.7 (7)

propionate. Glyconeogenesis was measured by the incorporation rate of [2-¹⁴C]pyruvate or [2-¹⁴C]propionate into glycogen in liver slices. The incorporation rates of both substrates increase from low activities in liver from foetal sheep to maxima in liver from lambs 4 weeks after birth. The rates of incorporation fall slightly to values found in the adult (Table 3). These results were extremely variable among animals of the same age, as shown by the large standard errors.

As the concentration of glycogen in liver from adult sheep is lower than in liver from adult rats, and the incorporation rate of pyruvate into glycogen in liver from adult sheep (3.87 μ moles/g./2 hr.) is much lower than the corresponding incorporation rate in liver from adult rats (24.7 μ moles/g./2 hr.; calculated from Ballard & Oliver, 1963), it would not be surprising if the incorporation rate of pyruvate into glycogen in sheep-liver slices is relatively insignificant compared with the incorporation rate into glucose. The incorporation rates of [2-¹⁴C]pyruvate and [2-¹⁴C]propionate into glucose in sheep-liver slices are also given in Table 3. Incorporation rates of [2-¹⁴C]pyruvate into glucose in liver slices from rats and chick embryos are included in Table 4. The incorporation rate of pyruvate into glucose in sheep-liver slices increases from 13.4 μ moles/g./2 hr. in liver from 4-month foetuses to a maximum of 78.4 μ moles/g./2 hr. in liver from lambs 2 months after birth. There is a fall to the incorporation rate of 16.6 μ moles/g./2 hr. in liver from adult sheep. The rate of incorporation of propionate into glucose in sheep-liver slices increases from 5.6 μ moles/g./2 hr. in foetal sheep (4 months) to a maximum of 38.6 μ moles/g./2 hr. in lambs 2 weeks after birth, and then falls to the values (22.3 μ moles/g./2 hr.) found in the adult.

In liver from chick embryos, the rate of incorporation of pyruvate into glucose increases to a maximum of 193 μ moles/g./2 hr. on the seventeenth day of incubation. The incorporation rate is lower after this age. The incorporation rate of pyruvate into glucose in liver slices from foetal rats (19 days) is barely detectable, and increases 200-fold to a maximum activity of 120.9 μ moles/g./2 hr. by 18 days after birth. The incorporation rate in liver from adult rats is 26.8 μ moles/g./2 hr. (Table 4).

Glucose 6-phosphatase and hexose diphosphatase, which are obligatory enzymes for the conversion of propionate or pyruvate into glucose, have low activities in liver from foetal sheep (Fig. 4). Glucose 6-phosphatase increases in activity from 70 μ moles/g./hr. in liver from foetuses of 3 months gestation to 183 μ moles/g./hr. by 4 months. At birth the activity is 545 μ moles/g./hr., and the activity is maximal between 4 and 7 weeks after birth. There is a slight fall to the activity (525 μ moles/g./hr.) in liver from adult sheep (Fig. 4a). Hexose-diphosphatase ac-

Table 4. Incorporation of [2-¹⁴C]pyruvate into glucose in liver slices from rats and chick embryos

Slices from liver were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section and in Ballard & Oliver (1964a). In those cases where sufficient liver could not be obtained from a single animal, livers from rat litter mates or from chick embryos of similar ages were pooled. Each determination is the mean of duplicates. All results are expressed as means $\pm$ S.E.M. of four determinations.

		Pyruvate incorporated (μ moles/g. of liver/2hr.)						
Animal	Age of rats (days)...	Foetus (19 days)	Foetus (22 days)	2	10	18	26	Adult
Rat		0.63 $\pm$ 0.11	2.47 $\pm$ 0.39	74.7 $\pm$ 12.3	107.8 $\pm$ 10.5	120.9 $\pm$ 14.9	41.2 $\pm$ 8.3	26.8 $\pm$ 3.9
	Age of chick embryos (days of incubation) ...	11	13	15	17	19	21	
Chick embryo		147 $\pm$ 7.5	149 $\pm$ 9.3	167.5 $\pm$ 3.3	193 $\pm$ 34.9	159 $\pm$ 30.5	126 $\pm$ 10.6

Fig. 4. (a). Glucose 6-phosphatase activity determined by inorganic phosphate production from glucose 6-phosphate at 37° in homogenates of sheep liver. (b) Hexose-diphosphatase activity determined by inorganic phosphate production from fructose 1,6-diphosphate at 37° in homogenates of sheep liver. T, term; A, adult. Each point represents the mean $\pm$ S.E.M. of at least four determinations.

Fig. 5. Fructose metabolism in liver from neonatal sheep. (a) Ketohexokinase activity estimated by fructose disappearance at 37° in 25000g supernatants of sheep liver. (b) Incorporation of [¹⁴C]fructose into glycogen in liver slices. (c) Incorporation of [¹⁴C]fructose into glucose in liver slices. Slices were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. F, 4-month sheep foetus; T, term; A, adult. Each point represents the mean $\pm$ S.E.M. of at least four duplicate determinations.

tivity, 67 μ moles/g./hr. in liver from foetal sheep (3 months), increases to 365 μ moles/g./hr. at birth and to a maximum of 732 μ moles/g./hr. 4 weeks after birth. The activity in liver from adult sheep is 580 μ moles/g./hr.

Glucose and glycogen formation from fructose and glycogen formation from galactose. In sheep liver ketohexokinase has an activity of 1.4 μ moles/g./hr. in the late foetus. This is unchanged at birth and then increases to a maximum activity of 24.6 μ moles/g./hr. 4 weeks after birth. The activity in liver from the adult is 13.7 μ moles/g./hr. (Fig. 5a). The incorporation of fructose into glycogen in sheep-liver slices is just detectable in foetal and postnatal animals up to 2 days after birth. There is a rise to 4.0 μ moles/g./2hr. at 4 days after birth and

then a further increase to a maximum incorporation rate of 14.6 μ moles/g./2hr. in livers from lambs 2 weeks old. From this age through to the adult there is little change in activity of the rate of fructose incorporation into glycogen (Fig. 5b). The rate of

Fig. 6. Incorporation of [^{14}C]galactose into glycogen in sheep-liver slices. Slices were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. F, 4-month sheep foetus; T, term; A, adult.

fructose conversion into glucose in liver slices from sheep is shown in Fig. 5(c). In liver from foetal sheep, although some conversion of fructose into glucose is indicated by the activity found ($3.6 \mu\text{moles/g./2 hr.}$), this approximates to the sensitivity limit of the method. The incorporation rate of fructose into glucose is $35 \mu\text{moles/g./2 hr.}$ in 2-day-old lambs and $70 \mu\text{moles/g./2 hr.}$ in lambs 2 weeks old. This rate of conversion is maintained throughout later development in the liver.

In slices from sheep liver (Fig. 6), the incorporation rate of galactose into liver glycogen is at a maximum in the foetus ($29.8 \mu\text{moles/g./2 hr.}$). There is a fall in incorporation by birth to $21.5 \mu\text{moles/g./2 hr.}$, and a further fall to the activity in the adult of $2.0 \mu\text{moles/g./2 hr.}$

DISCUSSION

Glycogen synthesis from glucose. In liver from foetal sheep glycogen appears at an early stage of gestation, whereas in livers from foetal rats, guinea pigs or rabbits glycogen cannot be detected until a few days before term (Shelley, 1961). Although we have not included findings for liver glycogen from foetal sheep in Fig. 1(a), livers from animals in the third month of gestation (wt. 80–130 g.) contained glycogen up to 20 mg./g. As glycogen is present in sheep liver early in gestation, it would be expected that the enzymes that are known to catalyse reactions in the synthesis of glycogen in adult liver would be present. UDP-glucose- α -glucan glucosyltransferase, UDP-glucose pyrophosphorylase and phosphoglucomutase have high activities in liver from foetal sheep at 4 months. Similar high activities have been found in livers from foetal rats and guinea pigs late in gestation (Ballard & Oliver, 1963; Kornfeld & Brown, 1963).

In rat liver the rate of [^{14}C]glucose incorporation into glycogen in liver slices changed considerably with the age of the animal, and these changes roughly paralleled the activity of liver glucokinase plus hexokinase (Ballard & Oliver, 1964a). In liver slices from foetal rats (19 days), glucose was incorporated into glycogen at a rate that was about one-sixth of the activity in liver from adult rat (Ballard & Oliver, 1964a). In liver slices from foetal sheep (3 months) the incorporation rate of [^{14}C]glucose into glycogen was five times the incorporation rate in liver from adult sheep. As the incorporation rate in liver from adult rats is almost 100 times the incorporation rate in liver from adult sheep, it is clear that with respect to glucose conversion into glycogen the metabolism in liver from adult sheep differs very considerably from that in liver from adult rats. Sheep liver does not contain a glucokinase (Ballard & Oliver, 1964b), and the activity of hexokinase ($7.5 \mu\text{moles/g./hr.}$) in liver from adult sheep (Fig. 3a) is much lower than the activity of glucokinase plus hexokinase ($165 \mu\text{moles/g./hr.}$) in liver from adult rats (Ballard & Oliver, 1964a). Although this difference in activity is probably the main reason for the difference in the rate of glucose conversion into glycogen between livers from adult rats and adult sheep, the physiological significance of glycogen and glycogen metabolism is probably less in the liver of adult sheep than in young lambs or in rats. As shown in Fig. 1, the glycogen content and the activities of UDP-glucose- α -glucan glucosyltransferase and UDP-glucose pyrophosphorylase are less in liver from adult sheep than in liver from young lambs. In livers of adult rats, the glycogen content and the activity of UDP-glucose- α -glucan glucosyltransferase are as high as in liver from young rats (Ballard & Oliver, 1963).

The liver of monogastric animals removes glucose from the blood for storage as glycogen during feeding and will release glucose into the blood when the animal is starved (Soskin, Essex, Herrick & Mann, 1938; Shoemaker, Yanof, Turk & Wilson, 1963). In sheep glucose is not obtained in quantity from the gut, as it is fermented by the rumen micro-organisms. It is unlikely that net uptake of glucose into the liver of adult sheep ever occurs under physiological conditions. The liver glycogen would be used as a store for glucose formed by gluconeogenesis rather than glucose obtained in the diet. The results described in the present paper support this hypothesis by showing that the sheep liver is well equipped enzymically for the release of glucose into the blood and for glucose synthesis from pyruvate and propionate, but has only a low activity of hexokinase. Schambye (1951a,b) and Annison, Hill & Lewis (1957) showed that the concentrations of glucose in the portal vein and the carotid artery were similar in both fed and starved sheep, but in the

marsupial *Setonix brachyurus*, which has a similar nutrition to ruminants, glucose is passed from the liver into the blood in fed and starved animals (Barker, 1961). Barker (1961) found that the difference in glucose concentration between the portal vein and the carotid artery was considerably higher in fed *Setonix* than in starved animals. This is the opposite to the situation in monogastric animals (Shoemaker *et al.* 1963).

Glyconeogenesis and gluconeogenesis. Ballard & Oliver (1963) could not detect any significant activity of the glyconeogenic pathway in liver from foetal rats, but found that this pathway appeared at or soon after birth. In the present work we have shown that gluconeogenesis from pyruvate is also absent in liver from foetal rats and that this synthesis occurs in newborn rats.

Liver from foetal sheep (4 months) can convert pyruvate into glucose at 20 times the rate of the liver of the foetal rat (19 days), and the increase in activity of this pathway at the birth of the lamb is very much less than in the rat. In addition, liver from foetal sheep has considerable glyconeogenic activity and there is little increase in activity of this pathway at birth. Glucose 6-phosphatase (Weber & Cantero, 1955) and hexose diphosphatase (Ballard & Oliver, 1962) have very low activities in liver from foetal rats, whereas the activities of these enzymes are relatively high in the liver of foetal sheep (4 months). As both enzymes are obligatory for the conversion of pyruvate into glucose in liver, the differences in the activities of these enzymes in liver from foetal sheep and foetal rats may account for the differences in the rate of incorporation of pyruvate into glucose that was found in the slice experiments. The rates of conversion of propionate into glycogen or glucose in sheep livers at various ages are similar to the rates of conversion of pyruvate into glycogen or glucose respectively. The rate of incorporation of [2-¹⁴C]propionate into glucose in liver slices from adult sheep is similar to the results of Leng & Annison (1963). In the present work it is further shown that [2-¹⁴C]propionate is incorporated into glycogen in liver slices from adult sheep. Leng & Annison (1963) were unable to demonstrate appearance of radioactivity in glycogen with similar incubation methods, although the method of counting 'labelled' glycogen was not stated.

Ballard & Oliver (1963) demonstrated that glyconeogenesis was active in liver from chick embryos. The results presented in Table 4 indicate that gluconeogenesis in liver slices from chick embryos is also active in these animals. Because the chick embryo has only 300–500 mg. of carbohydrate (Needham, 1963) it is likely that gluconeogenesis in the liver of these embryos has an important function in the maintenance of the blood glucose concentration.

Fructose metabolism. Walker (1963b) has measured the activity of liver ketohexokinase at various ages in rats, guinea pigs and rabbits. He was unable to measure any activity in livers from foetuses of these animals, but found that the enzyme appeared at birth and increased to maximum activity within a few days. The low activities of ketohexokinase that we found in livers from foetal sheep (Fig. 5a), and the increase in activity soon after birth, indicate that the pattern in sheep liver is similar to that in the rat, guinea pig and rabbit. The incorporation experiments confirm that fructose is not converted into glucose or glycogen in sheep-liver slices. These results support the finding by Andrews *et al.* (1960) that fructose was not removed from the perfusion medium in liver-perfusion experiments with foetal sheep.

Galactose metabolism. The incorporation rates of [¹⁴C]galactose into glycogen in sheep-liver slices indicate that this sugar can be converted into glycogen at a greater rate in liver from foetal or newborn sheep than in liver from adult sheep. In the rat, Alvarez & Sas (1961) found that β -galactosidase (EC 3.2.1.23) was present at a high activity in the intestine during suckling and had a much lower activity both before and after this period. β -Galactosidase hydrolyses the lactose obtained from milk to galactose and glucose. Segal, Roth & Bertoli (1963) measured galactose uptake and the rate of ¹⁴CO₂ production from [1-¹⁴C]galactose in livers from foetal, newborn, 20-day-old and adult rats. The galactose uptake and oxidation to carbon dioxide were greatest in livers from foetal or newborn animals and were least in livers from adult rats. From these results it is likely that the pattern of galactose metabolism with age is similar in sheep and rat liver.

The authors are indebted to the National Health and Medical Research Council of Australia and to the Medical Research Grants Committee of the University of Western Australia for financial assistance. The co-operation of the staff of the Institute of Agriculture of the University of Western Australia in the care of sheep and lambs is gratefully acknowledged.

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