2. No marked dependence on structure was observed.

3. Glucosides and ethereal sulphates of *m*-aminophenol, 1-naphthol and 4-methylumbelliferone were detected in excreta of houseflies dosed with these phenols.

4. Glucuronic acid conjugates were not detected by the colorimetric and fluorimetric procedures used.

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The Effect of Concentration on Glucose Phosphorylation and Incorporation into Glycogen in the Livers of Foetal and Adult Rats and Sheep

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Adult rat and guinea-pig livers have been shown to contain two types of hexokinase, one specific for glucose and mannose, glucokinase (EC 2.7.1.2), and a second non-specific enzyme, hexokinase (EC 2.7.1.1), both of which phosphorylate glucose in the 6-position (Walker, 1962; Viñuela, Salas & Sols, 1963; Ballard & Oliver, 1964). The glucokinase present in adult rat liver, but absent in foetal rat liver, has a Michaelis constant for glucose of 10–40 mM (DiPietro, Sharma & Weinhouse, 1962; Walker, 1962; Ballard & Oliver, 1964). This property would permit enzymic control over the rate of glucose phosphorylation in response to changes in the concentration of blood glucose over

the normal range 3–10 mM (Cole & Harned, 1938). This form of glucokinase would thus enable the rat liver to regulate the uptake of glucose from the blood and thus the amount of glycogen stored in the liver.

In ruminants, e.g. sheep, large quantities of volatile fatty acids, principally acetic acid, propionic acid and butyric acid, are produced by fermentation of carbohydrate in the rumen and provide much of the energy for the animal (Elsden & Phillipson, 1948). Glucose is not absorbed even if present in the diet, because it is rapidly metabolized by the rumen micro-organisms (Weller & Gray, 1954). Glucose required for the metabolism of

extrahepatic tissues must be obtained by gluconeogenesis, probably in the liver. In the sheep it might be expected that systems for the control of glucose uptake from the blood by the liver would be decreased in activity or absent. The metabolism of young or foetal sheep would be expected to resemble that of the young of monogastric animals, as lambs do not become effective ruminants until 1–2 months after birth.

In the present work a kinetic comparison has been made of the phosphorylation of glucose by liver extracts, and the conversion of glucose into glycogen in liver slices. Foetal and adult sheep and rats have been compared. The concentration-dependence of the incorporation of glucose into liver-slice glycogen shows characteristics reflecting the Michaelis kinetics of liver hexokinase activity in all the animals studied, even though the precise spectra of hexokinase types differ.

METHODS

Chemicals. ATP, NADP and glucose 6-phosphate were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. [^{14}C]Glucose was obtained from The Radiochemical Centre, Amersham, Bucks. Calcium gluconate was obtained from Drug Houses of Australia Ltd. Calcium gluconate was dissolved in the calculated amount of 2N-HCl and the Ca^{2+} ions were removed by treatment with Dowex 50 cation-exchange resin (H^+ form). The solutions were neutralized with 2N-KOH.

Animals. Foetal and adult rats of the Wistar albino strain of the species *Rattus norvegicus* were bred in the Preclinical Animal House of this University. Adult male rats were 4–6 months old.

Sheep were Merino wethers (1–2 years old) pasture-fed at the Institute of Agriculture of this University. The animals were killed by cutting the neck blood vessels. Liver samples were obtained after bleeding had nearly stopped, usually within 5 min.

Foetal sheep were Merinos of either sex obtained from pregnant ewes immediately after death at the abattoirs. Livers removed from the foetuses were 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 0.01N-HCl) for transport to the laboratory.

Liver extracts. Liver extracts for the determination of hexokinase, glucokinase and glucose-dehydrogenase (EC 1.1.1.47) activities were prepared and dialysed as described by Ballard & Oliver (1964). Gluconokinase (EC 2.7.1.12) activity was determined in extracts prepared as follows: 2 g. of liver was homogenized in 6 ml. of 0.1M- KH_2PO_4 –1 mM-EDTA (adjusted to pH 7.4 with 2N-KOH) and centrifuged at 25000g for 1 hr. in rotor no. 295 of the International refrigerated centrifuge (model PR2). The supernatant solution was dialysed overnight on a rocking machine against 100 vol. of the phosphate–EDTA buffer.

Hexokinase activity. This was assayed in the presence of different glucose concentrations by coupling the production of glucose 6-phosphate from ATP and glucose to the reduction of NADP in the presence of added glucose 6-phosphate dehydrogenase (EC 1.1.1.49) (Ballard & Oliver,

1964). The cuvettes contained tris buffer (44 mM) (adjusted to pH 7.5 with $\text{m-H}_3\text{PO}_4$), NADP (0.75 mM), MgSO_4 (7.5 mM), ATP (3 mM) (adjusted to pH 7.5 with 0.01N-KOH) and excess of glucose 6-phosphate dehydrogenase. The reaction was started by adding liver extract and the rate of reaction at 37° determined by measuring the extinction at 340 m μ at 60 sec. intervals in a Beckman model DU spectrophotometer. Blank estimations in the absence of glucose were carried out in similar reaction mixtures.

The glucose 6-phosphate dehydrogenase was prepared as described by Ballard & Oliver (1964), and was free of glucokinase, phosphoglucumutase (EC 2.7.5.1), phosphogluconate dehydrogenase (EC 1.1.1.43), glucose 6-phosphatase (EC 3.1.3.9) and glucose dehydrogenase.

Glucose-dehydrogenase activity. This was determined by adding a similar volume of extract to a cuvette containing the same solution but with ATP omitted.

Gluconokinase activity. This was determined at 37° by the method of Leder (1957). Reaction mixtures contained maleic acid (40 mM) (adjusted to pH 6.2 with 2N-KOH), ATP (10 mM), MgCl_2 (6 mM) and potassium gluconate (2.5 mM) in a volume of 0.5 ml. Reactions were started by addition of 0.1 ml. of liver extract and stopped after various incubation times by heating for 2 min. in a boiling-water bath. Blank determinations were similarly treated but gluconate was omitted. After cooling the tubes in ice and centrifuging, 6-phosphogluconate was determined in 0.2 ml. samples of the supernatant solution by reaction with NADP in the presence of 6-phosphogluconate dehydrogenase. The enzyme was prepared from dried baker's yeast by the method of Horecker & Smyrniotis (1955). Spectrophotometer cuvettes contained glycylglycine (80 mM) (adjusted to pH 7.4 with 2N-NaOH), NADP (0.5 mM) and MgCl_2 (6 mM) in a volume of 1.0 ml., and 0.2 ml. of the solution to be analysed. The initial extinction at 340 m μ was determined and 25 μl . of 6-phosphogluconate dehydrogenase solution then added. The extinction at 340 m μ was measured at 1 min. intervals until no further change occurred.

The amount of 6-phosphogluconate present was calculated from the change in extinction, corrected for the blank (in the absence of gluconate), and the molar extinction coefficient of reduced NADP (6.22 $\text{cm}^2/\mu\text{mole}$; Horecker & Kornberg, 1948).

The gluconate was tested for glucose by measurement of NADP reduction in the presence of ATP, purified yeast hexokinase and 6-phosphogluconate dehydrogenase, which contains substantial activity of glucose 6-phosphate dehydrogenase. No glucose could be detected even at high concentration (50 mM) of gluconate.

Isotope experiments. The methods of preparing and incubating the liver slices and of isolating and counting the glycogen of the slices were identical with those described by Ballard & Oliver (1964), except that the concentration of glucose was varied in the different experiments. The specific radioactivity of the glucose substrate is given for each glucose concentration in Tables 1 and 3.

Foetal rat-liver slices were obtained from one litter of animals and pooled.

RESULTS

The results on glucokinase (Fig. 1) show that an enzyme with a high K_m for glucose is present in adult rat liver but none can be detected in foetal

rat liver. The single hexokinase of foetal rat liver is about five times as active as the corresponding enzyme in the adult rat tissue.

The amount of [^{14}C]glucose incorporated into glycogen in rat-liver slices is shown in Table 1. The glucose concentrations were in the range 1–200 mM. As the specific radioactivity of the glucose substrate was lowered for experiments at the higher concentrations, the results are expressed as μmoles of glycogen formed from glucose/g. of liver in the 2 hr. incubation period. The incorporation in foetal rat liver is about 15 times that in adult rat liver at a glucose concentration of 1 mM, whereas at high

concentrations (50–200 mM) the incorporation in adult rat liver is about twice that of foetal rat-liver slices.

The K_m values of hexokinase in foetal and adult sheep liver, determined by the method of Lineweaver & Burk (1934), are 0.06–0.1 mM and 0.05 mM respectively for glucose. There is no indication of a hexokinase of higher K_m in extracts of either foetal or adult sheep liver.

In sheep liver the direct oxidation of glucose by glucose dehydrogenase interferes with the standard assay of glucokinase. The activity of glucose dehydrogenase has been measured in the absence of ATP and has been subtracted from the rate of NADP reduction in the presence of ATP. The activities of glucose dehydrogenase in the livers of adult and foetal rats and sheep are shown at several glucose concentrations in Table 2. Adult sheep liver contains the highest glucose-dehydrogenase activity. This activity at 200 mM-glucose is about four times the glucokinase activity, whereas in adult rat liver it is less than one-tenth of the glucokinase activity.

The incorporation of [^{14}C]glucose into glycogen in sheep-liver slices is shown in Table 3. In foetal sheep-liver slices the incorporation rises rapidly over the concentration range 1–5 mM-glucose and is fairly constant at higher concentrations. In adult sheep-liver slices the incorporation at 1–5 mM-glucose is 5–10 % of that in foetal slices, and it rises to about 15 % at 20 mM-glucose. At higher glucose concentrations, and especially at 200 mM, the incorporation in adult sheep-liver slices has increased enormously.

The activity of gluconokinase in the livers of foetal and adult rats and sheep is shown in Table 4. No activity could be detected in foetal rat liver but the enzyme occurs in foetal and adult sheep liver and adult rat liver.

DISCUSSION

The hexokinase assays indicate the absence of a glucokinase with high K_m from both adult and foetal sheep liver. Nevertheless, in the slice experiments with adult sheep liver, there is a striking increase in glucose incorporation into glycogen at high glucose concentration, which is much greater than that found in any of the other tissues studied. Glucose dehydrogenase has a very high K_m for glucose (150 mM) (Strecker & Korkes, 1952) and is of high activity in adult sheep liver. Gluconokinase, which catalyses the phosphorylation of gluconate to 6-phosphogluconate, is about one-tenth as active in adult sheep liver (Table 4) and has a low K_m for gluconate (Leder, 1957). Since glucose dehydrogenase reduces both NADP and NAD at similar rates (Strecker, 1955), the operation of an

Fig. 1. Effect of glucose concentration on hexokinase activity. The activities are expressed as percentages of the activity at a glucose concentration of 0.2M. The assay method is described in the text. (a) Foetal (21 days) rat-liver hexokinase. The livers from litter mates were pooled for the preparation of the liver extract. The activity at a glucose concentration of 0.2M was 61 μmoles of NADP reduced/g. wet wt./hr. (b) Adult rat-liver hexokinase. The activity at a glucose concentration of 0.2M was 219 μmoles of NADP reduced/g./hr. (c) Foetal (4-months) sheep-liver hexokinase. The activity at a glucose concentration of 0.2M was 14.4 μmoles of NADP reduced/g./hr. (d) Adult sheep-liver hexokinase. The activity at a glucose concentration of 0.2M was 16.0 μmoles of NADP reduced/g./hr.

Table 1. *Incorporation of [¹⁴C]glucose into glycogen in rat-liver slices*

Incorporation is measured in a high-K⁺ ion medium by determining the incorporation of [¹⁴C]glucose in 2 hr. at 37°. Experimental details are given in the text and by Ballard & Oliver (1964). Each determination is the mean of duplicates.

Concn. of glucose (mM)	Sp. radio-activity of glucose (μmc/mg.)	Glycogen formed (μmoles/g. wet wt. of liver/2 hr. incubation at 37°)			
		Foetal (21 days) litter 1	Foetal (21 days) litter 2	Adult rat 1	Adult rat 2
1	280	0.76	1.08	0.06	0.06
2	280	1.48	1.09	0.09	0.10
5	280	1.52	3.35	0.52	0.38
10	280	1.96	4.05	1.29	1.56
20	140	3.74	6.03	5.90	5.38
50	56	4.22	9.58	8.70	23.9
100	28	6.36	11.68	12.44	24.7
200	28	7.36	12.32	14.02	31.9

Table 2. *Glucose-dehydrogenase activity in the livers of foetal and adult rats and sheep*

Glucose-dehydrogenase activity was determined in liver extracts. The assay medium is described in the text.

Concn. of glucose ...	Activity (μmoles of NADP reduced/g. wet wt. of liver/hr. at 37°)			
	1 mM	10 mM	100 mM	200 mM
Foetal rat liver	<0.5	1.0	7.2	12
Adult rat liver	<0.5	<0.5	6.8	16
Foetal sheep liver	1.0	2.4	11.2	14.7
Adult sheep liver	2.5	9.2	51	67

Table 3. *Incorporation of [¹⁴C]glucose into glycogen in sheep-liver slices*

Incorporation was measured in a medium consisting of KCl (70 mM), MgCl₂ (20 mM), KHCO₃ (40 mM) and CaCl₂ (10 mM), adjusted to pH 7.45 with 0.01 N-HCl, by determining the incorporation of [¹⁴C]glucose in 2 hr. at 37°. Experimental details are given in the text and by Ballard & Oliver (1964). Each determination is the mean of duplicates.

Concn. of glucose (mM)	Sp. radio-activity of glucose (μmc/mg.)	Glycogen formed (μmoles/g. wet wt. of liver/2 hr. incubation at 37°)			
		Foetal (4 months) sheep	Foetal (5 months) sheep	Adult (1 year old) sheep 1	Adult (1 year old) sheep 2
1	280	0.48	0.56	0.038	0.023
2	280	0.78	0.72	0.061	0.035
5	280	1.05	1.19	0.066	0.081
10	280	1.05	1.33	0.14	0.12
20	140	1.05	1.91	0.22	0.18
50	56	1.08	2.52	0.68	0.39
100	28	0.81	3.16	0.90	1.34
200	28	0.57	3.40	2.88	3.36

Table 4. *Gluconokinase activity in the livers of foetal and adult rats and sheep*

Gluconokinase activity was determined in extracts as described in the text.

Animal	6-Phosphogluconate produced (μmoles/g. wet wt. of liver/hr. at 37°)
Foetal (21 days) rat	<0.1
Adult rat	9.6
Foetal (4 months) sheep	1.6
Adult sheep	6.0

internal oxidation-reduction cycle in the sheep-liver slices could account for the reduction of 6-phosphogluconate to glucose 6-phosphate, and hence incorporation of labelled glucose into liver glycogen at high glucose concentrations. Although the energetics of the last reaction are unfavourable *in vitro*, this enzyme is very active in sheep liver (Filsell, Jarrett, Atkinson, Caiger & Morton, 1963), and the generation of NADPH₂ by glucose dehydrogenase and the removal of glucose 6-phosphate by glycolysis and also by incorporation via

glucose 1-phosphate into glycogen may drive the reaction. The 6-phosphogluconate could also be converted into glucose 6-phosphate via the pentose phosphate pathway. Similar explanations may hold for the other tissues, where the increase in the incorporation of glucose into glycogen at high glucose concentration is less than that found in adult sheep liver.

A further effect of high glucose concentration may be inhibition of glucose 6-phosphatase; this would also result in greater incorporation into glycogen (Ashmore & Weber, 1959).

At a lower glucose concentration (20 mM) the rate of incorporation into glycogen is about $5 \mu\text{moles/g. of liver/2 hr.}$ incubation in liver slices from both adult and foetal rats. In foetal sheep the value is about $1 \mu\text{mole/g. of liver/2 hr.}$, whereas in adult sheep liver a lower rate of incorporation of about $0.2 \mu\text{mole/g. of liver/2 hr.}$ is found.

The activities of hexokinase in the four tissues studied at a glucose concentration of 20 mM are $150 \mu\text{moles/g./hr.}$ in adult rat liver (derived from Fig. 1), $60 \mu\text{moles/g./hr.}$ in foetal rat liver and about $15 \mu\text{moles/g./hr.}$ in both sheep livers. Therefore the results of both the glucokinase assays and of the liver-slice experiments show that sheep liver, and in particular adult sheep liver, has a poor ability to utilize glucose, and that adult rat liver has the greatest ability.

The concentration of glucose (20 mM; 360 mg./100 ml.) is probably greater than the maximum reached in any of the four tissues. The concentration of glucose in liver cells is approximately equal to that in the portal vein. In foetal animals the portal blood glucose is maintained at a relatively constant concentration by the placenta and the maternal glucose-regulation systems. The blood glucose is also constant in adult sheep, as glucose is not normally available from the intestine, where it is converted into volatile fatty acids by the rumen micro-organisms. Glucose in the blood is produced by gluconeogenesis from propionate and amino acids in the liver (Lindsay, 1959). In the adult rat, however, the blood glucose concentration varies greatly with the dietary state (between 3 and 10 mM). The concentration of glucose available for phosphorylation by hexokinase in the liver will also vary within this range (Cahill, Ashmore, Renold & Hastings, 1959). The hexokinase assays indicate that only in adult rat liver does the glucose phosphorylation rate vary with glucose concentration over the physiological concentration range.

In the livers of foetal sheep, adult sheep and foetal rats hexokinase activity is constant at glucose concentrations in the range 1–20 mM, whereas hexokinase activity in adult rat liver increases fivefold over this range. Similarly, in the two liver-slice experiments, the rate of incorpora-

tion of glucose into glycogen within the glucose concentration limits of 1–20 mM increases 2.2- and 3.5-fold in foetal sheep liver, 5.8- and 7.8-fold in adult sheep liver, and 5.0- and 5.6-fold in foetal rat liver, but in adult rat liver the corresponding increases are 90- and 99-fold.

The experiments indicate that only in adult rat liver is there a powerful concentration-dependent system for the control of glucose uptake.

SUMMARY

1. The glucokinase with Michaelis constant for glucose 10–40 mM, which is present in adult rat liver, is absent in the livers of foetal rats, foetal sheep and adult sheep.

2. At glucose concentrations up to 20 mM, the rate of glucose incorporation into glycogen in adult rat-liver slices increases greatly with glucose concentration.

3. Much smaller increases are apparent in liver slices from foetal rats, foetal sheep or adult sheep under similar conditions.

4. At glucose concentrations above 50 mM a large increase in glucose incorporation into glycogen occurs in liver slices from adult sheep.

5. The activities of glucose dehydrogenase and gluconokinase in adult sheep liver are sufficient to account for a pathway of the conversion of glucose via gluconate into glycogen at high glucose concentrations.

6. These results are discussed in terms of the glucose uptake and metabolism in the livers of foetal and adult rats and sheep.

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γ -Methyleneglutamic Acid and Related Compounds from Plants

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Many amino and imino acids, additional to those normally present in protein molecules, are now known as plant constituents (for reviews see Fowden, 1958, 1962). Several of these newly recognized compounds are dicarboxylic acids possessing a common branched C_6 carbon skeleton.

γ -Methyleneglutamic acid and γ -methyleneglutamine, first isolated from peanut plants, form the most rigorously characterized members of the group (Done & Fowden, 1952; Wailes, Whiting & Fowden, 1954). These two substances have been isolated subsequently from tulips (Zacharius, Pollard & Steward, 1954) and *Amorpha fruticosa* (Tschiersch, 1962) and identified as constituents of several other species on paper-chromatographic evidence (see Fowden, 1958). Chemically synthesized γ -methylglutamic acid has been resolved via the action of brucine or of cinchonine on its *N*-formyl derivative, and the configurations of the *L*- and *D*-isomers were established by degradation to the corresponding isomers of aspartic acid (Nakayama & Kaneko, 1957). However, the stereochemistry of the natural form has remained in doubt because its optical rotation in mineral acid has never been recorded: γ -methylene-*L*-glutamic acid has $[\alpha]_D^{20}$ 0° in water (Nakayama & Kaneko, 1957). The configuration of natural γ -methyleneglutamine is likewise uncertain.

In several species, substances identified as γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid coexist with the γ -methylene compounds (Fowden, 1958). γ -Methylglutamic acid was isolated from *Phyllitis scolopendrium* (Virtanen & Berg, 1955): the minute amount obtained permitted only the determination of C and H content, and identification of the isolate was dependent entirely on chromatographic comparison with synthetic material (Done & Fowden, 1952).

γ -Hydroxy- γ -methylglutamic acid was obtained from *Adiantum pedatum*, the maidenhair fern (Steward, Wetmore & Pollard, 1955; Grobbelaar, Pollard & Steward, 1955), in slightly larger amount (37 mg.); analysis of crystalline material indicated that it was a hemihydrate, but no further evidence was given to confirm this view.

The present paper describes the isolation of larger quantities of all four compounds from either tulips or *Phyllitis*. In addition, some properties of a further unidentified dicarboxylic amino acid from tulips are given. The materials isolated have been used to establish with certainty the identities of γ -methylglutamic acid and γ -hydroxy- γ -methylglutamic acid and to study the stereochemistry of the natural isomers. The possibility that the compounds cyclize under conditions which yield pyrrolid-2-one-5-carboxylic acid from glutamic acid and glutamine has been tested. An attempted demonstration of the presence in peanut seedlings of an enzyme similar to glutamine synthetase but utilizing γ -methyleneglutamine and γ -methylene-glutamic acid as substrates was unconvincing.

Extractions

Extraction from tulips. An extract of 55 kg. of mature tulip leaves was fractionated by ion-exchange chromatographic techniques to yield γ -methyleneglutamine (16.7 g.) and γ -methylene-glutamic acid (1.1 g.). Certain mixed acid fractions eluted from Dowex 1 resin were separated further by countercurrent and paper-electrophoretic methods to give a small quantity of crude γ -methylglutamic acid and the unidentified substance (72 mg.).

Extraction from Phyllitis. The amino acid fraction of an extract of 18 kg. of fern fronds was separated by anion-exchange resin chromatography to give γ -methylglutamic acid (9.3 g.) and γ -