Pathways of Fructose Conversion into Glucose in Foetal Rat Liver

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1. Glucose, formed from [1-¹⁴C]fructose or [6-¹⁴C]fructose in rat-liver slices, has been isolated as gluconate and degraded to give the radioactivity in C-1, C-2-5 and C-6. 2. By using this method it has been shown that, in liver from foetal rats younger than 20 days, glucose is formed from fructose without splitting of the molecule by the aldolase reaction. The rate of glucose formation from fructose in liver from these foetuses is approximately half of the rate in adult liver. 3. The direct conversion of fructose into glucose in foetal rat liver is not via sorbitol as in seminal vesicles, as this pathway cannot be detected. 4. When liver slices are incubated with [U-¹⁴C]fructose of high specific activity, the labelled intermediates are similar whether from liver from 18-day foetal, newborn or adult rats. 5. These findings are discussed with reference to the changing pathways of fructose metabolism during perinatal development of the liver in the rat.

The investigations of Hers and others have shown that the pathways of fructose metabolism differ in mammalian tissues. In liver (Hers & Kusaka, 1953; Hers, 1955; Leuthardt, Testa & Wolf, 1953), fructose is phosphorylated by ketohexokinase (EC 2.7.1.3) to fructose 1-phosphate, which is split by aldolase (EC 4.1.2.7) to C₃ units before conversion into glucose. In muscle (Hers, 1955), fructose is phosphorylated by hexokinase (EC 2.7.1.1) to fructose 6-phosphate, which is converted into glucose 6-phosphate without splitting. A third pathway exists in seminal vesicles (Hers, 1956) and in ocular lens (van Heyningen, 1959), in which fructose is reduced to sorbitol by L-iditol dehydrogenase (EC 1.1.1.14) and sorbitol is oxidized to glucose by aldose reductase (EC 1.1.1.21). This pathway normally acts in the direction of fructose rather than glucose formation.

Experiments by Muntz & Vanko (1962) and by DiPietro (1964) indicate that, in liver, pathways other than the one described by Hers & Kusaka (1953) may contribute in some degree to the metabolism of fructose. Preliminary experiments have shown that the rate of conversion of fructose into glucose in liver slices from foetal rats is about half of that in slices from adult animals. It seemed likely that the pathway of conversion of fructose into glucose in foetal liver was different from that in adult liver as the activities of ketohexokinase (Walker, 1963a; Ballard & Oliver, 1964), hexose diphosphatase (EC 3.1.3.11; Ballard & Oliver, 1962) and glucose 6-phosphatase (EC 3.1.3.9; Weber & Cantero, 1955; Dawkins, 1961), which catalyse reactions in the conversion of fructose into glucose

in adult liver, were found to be low or negligible in foetal rat liver.

The following combined enzyme and tracer study was undertaken to evaluate these pathways of glucose formation from fructose in foetal rat liver.

MATERIALS AND METHODS

Chemicals. Glucose oxidase, NAD, NADP, NADH₂, NADPH₂ and sugar phosphates for chromatography were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Amberlite CG-400 (100-200 mesh) and Amberlite MB-3 were purchased from Mallinckrodt Chemical Works, New York, N.Y., U.S.A. [1-¹⁴C]Fructose was obtained from Nuclear Research Chemicals Inc., Orlando, Fla., U.S.A. This labelled sugar was found to contain 4% of a contaminant having *R_f* similar to that of glucose on paper chromatography with phenol-water (4:1, w/v) (Smith, 1960). It was purified by paper chromatography with this solvent. The radioactive spots corresponding to fructose were eluted from the paper. [6-¹⁴C]Fructose was obtained chromatographically pure from Chemtrac, Cambridge, Mass., U.S.A.

[1-¹⁴C]Glucose, [2-¹⁴C]glucose, [6-¹⁴C]glucose, [1-¹⁴C]-sorbitol and [U-¹⁴C]fructose (high specific activity, 86.2 μC/mg.) were purchased from The Radiochemical Centre, Amersham, Bucks.

Animals. Rats of the Wistar albino strain of the species *Rattus norvegicus* were obtained from Carworth Farms Co., New City, N.Y., U.S.A. All adult rats used were males.

Liver-slice experiments. Slices were cut and incubated as described by Ballard & Oliver (1964), except that the volume of the incubation medium was 4 ml. and the concentration of fructose was 15 mM. After incubation for 2 hr. at 37° the incorporation was stopped by the addition of 1 ml. of 20% (w/v) HClO₄.

Preparation of gluconate. The contents of each tube from the liver-slice incubations were homogenized and centrifuged

for 5 min. at 2000g. Each supernatant was decanted into a centrifuge tube with a capacity of 20 ml. The precipitate was homogenized in 0.5 ml. of 3.3% (w/v) HClO_4 and centrifuged as before. The supernatant was added to the previous one. This washing was repeated once. To each centrifuge tube was added 1.0 ml. of carrier glucose (100 μ -moles), and a portion (200 μ l.) was removed for glucose estimation (Hill, Kessler, Hill & Chauncey, 1961). The extracts were neutralized (pH 6–8 with bromocresol purple) by adding 2N- KHCO_3 dropwise with stirring. Then 10 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 in an International refrigerated centrifuge (model PR-2) at 2000g for 20 min. at 0° .

Each supernatant was passed through a 5g. column of Amberlite MB-3 previously treated with CO_2 to convert the OH^- into the HCO_3^- form. If this CO_2 treatment was omitted, the Amberlite MB-3 column converted approx. 20% of the sugars into acids. Each column was washed with water after the solutions had been passed through to give a volume of 50 ml. The eluates were freeze-dried and dissolved in 1 ml. of water. A sample was chromatographed on Whatman no. 1 paper with phenol–water as solvent, and scanned for radioactivity with a Nuclear–Chicago Corp. 4 π Actigraph detector. Two peaks, corresponding in R_F to glucose and fructose, were present. The remainder of each solution was diluted to 5 ml. with water, and 1 mg. (20 units) of glucose oxidase was added. The solutions were kept overnight at room temperature. Each solution containing the gluconolactone thus formed was made slightly alkaline (phenolphthalein indicator) with 0.1N-NaOH, passed through a 2g. column of Amberlite CG-400 (Cl-form) and washed with 25 ml. of water. The gluconate was eluted in a volume of 25 ml. with 0.1M-NaCl.

Degradation of ^{14}C -labelled gluconate. This method was adapted from that of B. Friedmann & S. Weinhouse (personal communication) to liquid-scintillation counting. A 1 ml. sample of the gluconate solution was counted in a Packard scintillation detector (model 314 EX) with 20 ml. of Ditol (Herberg, 1960). To the remainder of the solution was added 4 ml. of M-sodium acetate (adjusted to pH 5.8 with N-acetic acid) and 3 ml. of freshly prepared 7% (w/v) HIO_4 solution. The CO_2 produced by the oxidation (Salomon & Johnson, 1959) of C-1 of gluconate was collected in 5 ml. of 0.5N-NaOH in a bead tower, with the aid of a stream of CO_2 -free air. After 2 hr., 4 ml. of N-HCl was added to the incubation mixture and CO_2 collected for a further 15 min. The bead column was washed with water to give a volume of 24 ml. A 1 ml. sample of this solution was counted with 20 ml. of Ditol. The HIO_4 remaining in the reaction mixture was destroyed and I_2 evolved by the addition of 3 ml. of 1.2M-NaAsO₂. When all the I_2 had been liberated, 2 ml. of M-sodium acetate was added and the pH of the solution adjusted to 5 with N-NaOH (pH indicator paper). Then 1 ml. of a dimedone solution (containing 80 mg. of 5,5-dimethylcyclohexane-1,3-dione dissolved in ethanol) was added and the dimedone complex with the formaldehyde, formed from C-6 of the gluconate, precipitated on a steam bath (Dunn, Friedmann, Maass, Reichard & Weinhouse, 1957). After the solution had cooled the precipitate was collected in weighed sintered-glass funnels. The volume of the filtrate (containing formic acid from C-2–5 of gluconate) was measured and 1 ml. counted with 20 ml.

of Ditol. The precipitate was washed with water, the filters were dried under a lamp (less than 70°) and the filters were reweighed to give the weight of the dimedone complex. From this weight the recovery of glucose in the purification and degradation procedure was calculated. The precipitate was dissolved in 5 ml. of toluene and 1 ml. of this solution was counted in the scintillation detector with 1 ml. of 0.1N-NaOH and 19 ml. of a solution similar to Ditol so that the final concentrations of toluene, dioxan, methanol, naphthalene and 2,5-diphenyloxazole were equivalent to those found in the Ditol solution. If the samples were counted in this manner, the efficiency of detection was unchanged in all the samples counted. The methods were tested with [^{14}C]glucose, [^{14}C]glucose and [^{14}C]glucose (Table 1).

The partial randomization of label from C-1 (or C-6) of fructose to C-6 (or C-1 respectively) of glucose was expressed as the percentage relocation, which is twice the ratio of the counts in the previously unlabelled position (C-1 or C-6) to the total counts recovered. Complete randomization of label between C-1 and C-6, with no label in C-2–5, would give 100% relocation.

Assay of L-iditol dehydrogenase. Liver extracts were prepared by homogenizing 1g. of liver in 7 ml. of a solution containing KCl (0.15M), MgCl_2 (5mM) and β -mercaptoethanol (10mM), adjusted to pH 7.4 with 0.01N-NaOH. The homogenates were centrifuged at 105000g in a Spinco model L ultracentrifuge for 45 min. The supernatants were dialysed overnight against 200 vol. of the homogenizing solution.

The enzyme was assayed spectrophotometrically either by decrease in extinction at 340 m μ with NADH_2 and fructose as substrates, or by increase in extinction with NAD and sorbitol as substrates at 37° , in a Zeiss PMQ2 spectrophotometer. The final concentrations of reactants (total vol. 2 ml.) in the cuvettes for fructose reduction were tris (40 mM, adjusted to pH 7.4 with 2N-HCl), NADH_2 (0.12 mM) and MgCl_2 (5 mM). A 20 μ l. portion of dialysed liver extract was added and the change in extinction followed for 5 min. In all cases this blank activity was negligible. The reaction was started by the addition of fructose to give a final concentration of 20 mM. The enzyme activity was calculated from the extinction change for the first 3 min.

The reverse reaction was measured in the same manner except that NAD at a final concentration of 0.5 mM replaced NADH_2 , sorbitol (20 mM) replaced fructose and the tris buffer was at pH 9.0. These methods are similar to those described by Touster & Montesi (1962).

Assay of aldose reductase. This enzyme was assayed as described by Hers (1960).

Table 1. Recovery and distribution of ^{14}C in gluconate formed from ^{14}C -labelled glucose

The gluconate formed by oxidation of variously labelled glucose was degraded by the HIO_4 oxidation technique given in the Materials and Methods section.

Position of label in glucose	Percentage of radioactivity			Percentage recovery
	C-1	C-2–5	C-6	
C-1	89.1	3.0	0.7	92.8
C-2	0.1	95.6	1.4	97.1
C-6	1.0	0.2	93.0	94.2

Chromatography of labelled cell extracts. Approx. 25mg. portions of liver slices from 18-day foetal, newborn and adult rats were incubated in 0.5 ml. of Krebs-Ringer phosphate solution (Umbreit, Burris & Stauffer, 1964) containing $10\mu\text{C}$ of [^{14}C]fructose ($20\mu\text{g.}$) at 37° for 30 min. The gas phase was air and the tubes were shaken at 100 oscillations/min. At the end of the incubation, the tissues were washed and extracted as described by Smith (1960). The ethanolic extracts were concentrated under vacuum to a volume of 0.2 ml., and 0.1 ml. samples were chromatographed on oxalic acid-washed Whatman no. 4 paper (Smith, 1960) with phenol-water as solvent. The developed chromatogram was scanned for radioactivity and the radioactive areas were eluted with water. The eluates were concentrated to 0.2 ml. The identity of the radioactive products was determined by co-chromatography with known derivatives with suitable solvent systems.

RESULTS

The rates of incorporation of [^{14}C]fructose into glucose in liver slices from rats of various ages are

Fig. 1. Incorporation of [^{14}C]fructose into glucose in rat-liver slices. Slices were incubated for 2 hr. at 37° as described under 'Liver-slice experiments' in the Materials and Methods section. T, Term; A, adult. Each point represents the mean $\pm$ s.e.m. of from four to 12 duplicate determinations.

given in Fig. 1. Although the incorporation was greater in post-natal livers, the incorporation by foetal liver was also considerable. The distribution of ^{14}C among the glucose carbon atoms is shown in Table 2. In adult rats, the percentage relocation was between 58 and 91%. The amount of relocation was unchanged in liver slices from 22-day foetal rats. However, the relocation in the liver from 20-day foetal rats was lower, and in that from 17-day foetal rats was only 3%.

Evaluation of the sorbitol pathway from fructose to glucose. As no randomization of label between C-1 and C-6 of glucose was found in the experiments with liver from 17-day foetal rats, the possibility was considered that glucose could be formed from fructose via sorbitol, without phosphorylation. [^{14}C]Sorbitol (15mm) was incubated with liver slices and glucose was isolated and degraded in a manner identical with that described for labelled fructose. The average rate of sorbitol incorporation in liver slices from adult rats was $8.5\mu\text{moles/g.}$ of liver/2hr. (four animals), and the average percentage relocation of label to C-6 of glucose was 44%. In foetal liver glucose labelling from sorbitol could not be detected (less than $2\mu\text{moles/g.}$ of liver/2hr.).

The activity of L-iditol dehydrogenase in liver extracts from foetal and adult rats is shown in Table 3. The enzyme was measured with either fructose or sorbitol as substrate. The activity in adult liver was approx. $6-7\mu\text{moles/g.}$ of liver/min., whereas in the 18-day foetus it was about 10% of this value. The ratio of the rates of fructose reduction to sorbitol oxidation was relatively constant in the animals of different ages, suggesting that the same enzyme was being measured in rats of all ages.

Attempts were made to measure the activity of aldose reductase in liver extracts. No activity could be detected in foetal or adult liver with either NADPH₂ plus sorbitol or glucose plus NADP as substrates.

Chromatography in the phenol-water solvent of

Table 2. Distribution of ^{14}C in glucose formed from [^{14}C]fructose or [^{14}C]fructose by liver slices from rats of various ages

The experimental techniques used are described in the Materials and Methods section. Each incubation vessel contained $60\mu\text{moles}$ and $1\mu\text{C}$ of fructose in a volume of 4 ml. All results are expressed as means $\pm$ s.e.m.

Animals	Position of label in fructose	No. of animals	Percentage of radioactivity			Total percentage recovery	Percentage relocation
			C-1	C-2-5	C-6		
Adults	C-1	8	57.2 ± 1.5	6.6 ± 2.6	31.6 ± 1.2	95.2 ± 1.3	66.4 ± 2.8
	C-6	8	32.0 ± 0.6	11.5 ± 1.4	47.9 ± 1.2	91.4 ± 1.1	70.0 ± 1.5
Foetuses, gestational age 22 days	C-1	5	59.9 ± 5.7	3.1 ± 1.3	31.4 ± 4.5	94.4 ± 2.4	66.7 ± 9.4
Foetuses, gestational age 20 days	C-1	5	75.2 ± 5.4	4.9 ± 1.1	11.9 ± 3.0	92.0 ± 2.5	26.3 ± 7.0
Foetuses, gestational age 17 days	C-1	7	91.2 ± 2.7	1.6 ± 0.4	1.3 ± 0.3	94.1 ± 1.3	2.8 ± 0.6
	C-6	8	1.5 ± 0.3	1.7 ± 0.2	91.6 ± 0.9	94.8 ± 1.3	3.2 ± 0.6

cell extracts labelled by incubation of liver slices with [$U\text{-}^{14}\text{C}$]fructose showed radioactive peaks with R_f values similar to that of sorbitol. However, sorbitol and fructose have similar R_f values in that solvent. The spots were eluted and chromatographed with butan-2-one-acetic acid-water saturated with boric acid (9:1:1, by vol.) (Rees & Reynolds, 1958), in which fructose and sorbitol are separated. It was found that the liver extracts from 18-day foetal rats did not contain labelled sorbitol, although some could be detected in liver extracts from adult rats.

Table 3. Activity of L-iditol dehydrogenase measured as D-fructose reduction and sorbitol oxidation in liver extracts from foetal and adult rats

The details of the assay are given in the Materials and Methods section. The activity is expressed in either μ moles of NADH_2 oxidized or NAD reduced/g. of liver/min. at 37°.

Animals	Activity of fructose reduction	Activity of sorbitol oxidation	Fructose reduction Sorbitol oxidation
Adult	7.2	6.25	1.15
Adult	7.1	5.75	1.28
Foetus, gestational age 22 days	1.95	1.93	1.01
Foetus, gestational age 20 days	1.0	0.77	1.30
Foetus, gestational age 18 days	0.77	0.58	1.32

Labelling of intermediates of fructose metabolism. When cell extracts labelled by the incubation of liver slices with [$U\text{-}^{14}\text{C}$]fructose were chromatographed with phenol-water, six peaks of radioactivity were found. These corresponded to hexose diphosphates, hexose monophosphates, glycerol 1-phosphate, glucose, fructose-sorbitol and lactate. Each area of radioactivity on the chromatogram, after elution and concentration, was co-chromatographed with known markers. The amount of radioactivity in each area on the chromatogram was estimated roughly by scanning the chromatograms with a Nuclear-Chicago Corp. 4 π Actigraph. The results of these experiments are shown in Table 4. Radioactivity could not be detected in glucose 1-phosphate, glyceraldehyde, glyceraldehyde 3-phosphate, ribose phosphates, dihydroxyacetone phosphate or sugars other than glucose and fructose. These results indicate that the same compounds were labelled in liver slices from rats of different ages, although less glucose and more glucose 6-phosphate were labelled in the foetal liver, and sorbitol was not labelled. It is likely that a low activity of glucose 6-phosphatase causes the increased labelling of glucose 6-phosphate.

DISCUSSION

Hers (1955) has shown that, in liver, glycogen formed from fructose labelled at C-1 shows considerable relocation to C-6. That this randomization was not complete has been taken as evidence for a slow equilibration between glyceraldehyde 3-

Table 4. Estimates of the relative radioactivity in compounds in extracts of liver slices incubated with [$U\text{-}^{14}\text{C}$]fructose of high specific radioactivity

Experimental methods are given in the Materials and Methods section. The scale of radioactivity is from absent (—) through trace ($\pm$) to highly radioactive ($+++$). The chromatographic solvents, in addition to phenol-water (4:1, w/v) (Smith, 1960), which was used to separate the groups of compounds, are: 1, butan-1-ol-propionic acid-water (10:5:7, by vol.) (Smith, 1960); 2, ethyl acetate-acetic acid-water (3:3:1, by vol.) (Mortimer, 1952); 3, methanol-aq. 28% NH_3 -water (6:1:3, by vol.) (Bandurski & Axelrod, 1951); 4, methanol-formic acid-water (16:3:1, by vol.) (Bandurski & Axelrod, 1951); 5, butan-2-one-acetic acid-water saturated with H_3BO_3 (9:1:1, by vol.) (Rees & Reynolds, 1958).

Radioactive substance	Solvents used	Liver slices, from ...		
		18-Day foetus	Newborn	Adult
Fructose 1,6-diphosphate	1	+	$\pm$	$\pm$
Glucose 6-phosphate	1, 2, 3, 4	$+++$	+	+
Fructose 1-phosphate		+	+	+
Fructose 6-phosphate		+	$\pm$	$\pm$
Glycerol 1-phosphate	1, 2, 3, 4	+	+	$++$
Glucose	1, 5	$\pm$	+	+
Fructose	1, 5	$+++$	$+++$	$+++$
Sorbitol		—	+	+
Lactate	1	+	+	+

phosphate and dihydroxyacetone phosphate. Rose, Kellermeyer, Stjernholm & Wood (1962), using ^{14}C -labelled and also ^3H -labelled glycerol, found that the equilibration reaction catalysed by triose phosphate isomerase (EC 5.3.1.1) may not be sufficiently active to produce complete randomization between C-1 and C-6 of glucose. In the experiments of Rose *et al.* (1962), the glucose for analysis was formed by hydrolysis of liver glycogen labelled *in vivo*.

In the present paper a method is described whereby the labelling pattern of glucose, rather than glycogen, may be measured. By using this technique, it was shown that in liver slices from adult rats incubated with $[1-^{14}\text{C}]$ fructose an excess of label occurs at C-1 relative to C-6. These results agree closely with the pattern of labelling found when glycogen was the radioactive product (Hers, 1955; Landau & Merlevede, 1963).

It has been reported that the activity of ketohexokinase is very low (Ballard & Oliver, 1964) or even absent (Walker, 1963a) in foetal rat liver, and that the rate of incorporation of fructose into glycogen is low in liver slices from foetal rats (Ballard & Oliver, 1964). Several other enzymes on the metabolic pathway used in adult liver are stated to have extremely low activities in foetal liver, e.g. glucose 6-phosphatase (Weber & Cantero, 1955), hexose diphosphatase (Ballard & Oliver, 1962) and the rate of fructose 1-phosphate cleavage by aldolase (Rutter, Blostein, Woodfin & Weber, 1963). The present results indicate a high rate of conversion of fructose into glucose in the foetal rat liver. The labelling pattern of glucose formed from $[1-^{14}\text{C}]$ fructose in liver slices from 22-day fetuses is similar to that found in adult liver. Although the enzyme activities are low, they must be sufficient to catalyse the conversion of fructose into glucose at the rates observed in the present paper.

In the foetus younger than 20 days, glucose isolated from liver slices incubated with either $[1-^{14}\text{C}]$ fructose or $[6-^{14}\text{C}]$ fructose did not show any relocation of label. It is likely that this glucose has been formed by phosphorylation of fructose at C-6 by hexokinase as this enzyme phosphorylates fructose and is present in foetal rat liver (Walker, 1963b; Ballard & Oliver, 1964).

Studies on hexokinase in liver (Walker, 1963b) have shown that the affinity for glucose is much greater than the affinity for fructose. DiPietro (1963) found that fructose phosphorylation by hexokinase from adipose tissue was inhibited by glucose. If this inhibition occurred in foetal rat liver, where glucose is present at concentrations greater than fructose, any phosphorylation of fructose by hexokinase would be unlikely. However, many experiments with foetal or adult liver, in

which glucose was added to the slice incubation medium together with $[1-^{14}\text{C}]$ fructose, have shown no inhibition of fructose conversion into glucose. In addition, no change in the labelling pattern of the glucose formed from fructose has been found.

The pathway of conversion of fructose into glucose via sorbitol can be excluded in foetal liver as the activity of sorbitol dehydrogenase was very low; the activity of aldose reductase was undetectable; fructose of high specific radioactivity did not form labelled sorbitol in liver slices; and sorbitol was not converted into glucose at a measurable rate in liver slices. This pathway may contribute to glucose formation in adult liver if an aldose reductase is present in a particulate fraction of the liver homogenate. If so, this would account for the findings of Muntz & Vanko (1962) that glucose formed in liver from intraportally injected $[^{14}\text{C}]$ -fructose has a specific activity higher than could be explained by its labelling by pathways through phosphorylated hexose.

It has been shown (Ballard & Oliver, 1965) that the rates of incorporation of labelled fructose into glucose and glycogen in slices of foetal sheep liver are very low, although the foetal blood contains a high fructose concentration (Huggett, 1961). The blood of the foetal rat contains little if any fructose, but the liver of this animal can convert into glucose any small amounts of fructose that are present.

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