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STUDIES OF THE MECHANISM OF FRUCTOSE PRODUCTION BY HUMAN PLACENTA

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The amount of fructose in the foetal blood is large in ungulates (Bacon & Bell, 1948; Huggett, Warren & Warren, 1951) but small in most other mammals, including monkey and man (Orr, 1924; Hagerman & Villee, 1952; Chinard, Danesino, Hartmann, Huggett, Paul & Reynolds, 1956; Goodwin, 1956). The amniotic and allantoic fluids of ungulates are also characterized by a high fructose content (Barklay, Haas, Huggett, King & Rowley, 1949). The presence of this fructose in the foetal blood poses the question of whether it arises in the foetus or placenta or is transferred across the placenta from the maternal blood. The concentration of fructose is higher in foetal than in maternal blood both in sheep (Cole & Hitchcock, 1946) and in man (Hagerman & Villee, 1952), and thus it could not be passing by simple diffusion from maternal to foetal blood. Holmberg, Kaplan, Karvonen, Lind & Malm (1956) showed that fructose crosses the human placenta much more slowly than glucose, xylose or galactose. In sheep the placenta is clearly the site of fructose formation (Huggett *et al.* 1951) and in man and the rhesus monkey the placenta *in situ* does not produce fructose at a rate sufficient to alter the foetal blood fructose concentration following artificially induced hyperglycaemia in the mother (Chinard *et al.* 1956).

The production of fructose from sorbitol was first demonstrated by Embden & Griesbach (1914) in the perfused liver of dogs. The enzyme catalysing this reaction was partially purified from rat liver and was shown to be a diphosphopyridine nucleotide-linked dehydrogenase (Blakley, 1951). Hers (1957) demonstrated the presence of two enzymes in foetal sheep liver, aldose reductase and ketose reductase, which catalyse the following reactions:

$$\text{Glucose} + \text{TPNH} + \text{H}^+ \rightleftharpoons \text{Sorbitol} + \text{TPN},$$

$$\text{Sorbitol} + \text{DPN} \rightleftharpoons \text{Fructose} + \text{DPNH} + \text{H}^+.$$

Both these reactions, although reversible, would be expected to proceed to the right *in vivo* because in tissues triphosphopyridine nucleotide (TPN) is predominantly in the reduced form and diphosphopyridine nucleotide (DPN) is mostly in the oxidized form (Glock & Mclean, 1955). Together, they provide a mechanism for the production of fructose from glucose without the formation of phosphorylated intermediates. Hers (1957) also demonstrated the presence of aldose reductase in the placenta of foetal sheep and postulated that the fructose of the foetal blood is produced primarily in the foetal liver, although a small amount is derived from the placenta. Huggett *et al.* (1951) had shown earlier that fructose is produced by the perfused sheep placenta and Hagerman & Villee (1952) found that it was produced by slices of human placenta incubated *in vitro*. The present studies were undertaken to determine whether fructose production from glucose in the human placenta occurs via the reduction of glucose to sorbitol and its subsequent oxidation to fructose, or via the intermediate formation of glucose 6-phosphate and fructose 6-phosphate.

MATERIALS AND METHODS

Full-term placentas and a few obtained by Caesarean section were used within 10 min of delivery. Small pieces were cut out and washed with a copious amount of ice-cold 0·9% NaCl solution. The tissue was freed from connective tissue by blunt dissection and then homogenized in two volumes of 0·155 M-KCl. The homogenate was centrifuged at 2000 *g* for 10 min to remove nuclei and cellular debris. In some experiments the resulting 'whole homogenate' was used directly and in others it was separated into fractions by ultracentrifugation. In the latter case the 'whole homogenate' was centrifuged at 10,000 *g* for 20 min to sediment the mitochondria. These were washed twice by resuspension in KCl solution and centrifugation. The supernatant fluid from the initial centrifugation at 10,000 *g* was transferred to another tube and centrifuged at 57,000 *g* for 1 hr to sediment the microsomal fraction. The supernatant fluid from this centrifugation was the non-particulate fraction used in some of the experiments.

The placental preparations were ordinarily incubated in a phosphate buffer which contained (μmole/ml. buffer): phosphate 10, Cl^- 20, Mg^{2+} 10, and K^+ 18·3, adjusted to pH 7·4 with KOH solution. Each flask contained 1 ml. of buffer, 1 ml. of placental preparation and 1 ml. of distilled water containing substrate and co-factors as indicated for each experiment. When fructose production from fructose 6-phosphate or glucose 6-phosphate was measured, 2 ml. of buffer, 1 ml. of placental preparation, and 1 ml. of water was used. The flasks were incubated for 2 hr (or 15 min when phosphate production was measured) with shaking in a water-bath at 37° C.

After incubation, samples of the medium were analysed for total reducing sugar by the method of Nelson (1944), fructose by the method of Roe according to the directions of Higashi & Peters (1950), sorbitol by the method of West & Rapoport (1949), glucose by a glucose oxidase reagent (Eli Lilly and Co.), and inorganic phosphate by the method of Lowry & Lopez (1946). Nitrogen analyses were made on separate samples of the placental preparation by a micro-Kjeldahl method.

For chromatography, samples of the incubation medium were precipitated with zinc sulphate–barium hydroxide, the supernatant fluid after centrifugation was concentrated at 50° C and salts were removed by passing the solution over an ion-exchange resin (Deeminac, Crystal Research Laboratories Inc., Hartford, Conn.). The resulting solution was taken to dryness in vacuo, dissolved in water and placed on Whatman No. 1 paper for ascending chromatography with butanol:acetic acid:water (4:1:1) or phenol:water (4:1) (Block, Durrum & Zweig, 1958). The

chromatograms were then dried and sprayed with an orcinol or naphthoresorcinol reagent for the detection of fructose or with methyl red in boric acid for the detection of sorbitol. The former spray reagent was developed at 110° C and the latter at 37° C. Appropriate standards were always placed on the same chromatogram. S.D. were calculated by the usual formula and the comparison of averages was done by means of Student's test.

RESULTS

Whole 'placental homogenates' were incubated with or without sorbitol and DPN and analysed for fructose and for total reducing sugars. Neither sorbitol nor DPN alone increased the amount of fructose produced, but the completed system produced significantly more ($P < 0{\cdot}05$) fructose than the incomplete system (Table 1).

TABLE 1. Production of fructose and total reducing sugar by homogenized human full-term placenta

Additions	Fructose production	Total reducing sugar production
None	$8{\cdot}0 \pm 0{\cdot}3$	$123 \pm 6{\cdot}4$
Sorbitol	$6{\cdot}1 \pm 0{\cdot}3$	126 ± 6
DPN	$9{\cdot}3 \pm 1{\cdot}6$	122 ± 2
Sorbitol and DPN	$16{\cdot}6 \pm 3{\cdot}1$	177 ± 2

The values represent the μmoles of sugar produced per 2 hr and per g protein $\pm$ the S.D. 0·68 μmoles of DPN and 15 μmoles of sorbitol were added to the flasks as indicated. Each value is the mean of 14 determinations.

In one experiment (incubation and analyses in triplicate) the 'whole homogenate' was incubated in a medium containing DPN and sorbitol. The amount of fructose produced, estimated by the resorcinol method, was 18·2 μmole/g protein in 2 hr. In these same experiments the difference between total reducing sugar and true glucose at the end of the incubation was 87 μmole/g protein in 2 hr. These results support the idea that other ketoses are produced when sorbitol and DPN are present. However, since these ketoses could interfere in the resorcinol method, the sugar produced was identified chromatographically. It was possible to demonstrate the presence of fructose spots which did not differ in their rate of migration from suitable standards.

Washed mitochondria had no ketose reductase activity, i.e. there was no increase in the amount of resorcinol-positive material produced by mitochondria when sorbitol and DPN were added to the incubation medium. However, the non-particulate fraction, when incubated with the co-factor alone, produced $6{\cdot}5 \pm 1{\cdot}0$ μmole fructose/g protein in 2 hr and the same fraction when incubated with both DPN and sorbitol produced $12{\cdot}0 \pm 2{\cdot}3$ μmole/g protein in 2 hr. This difference is significant ($P < 0{\cdot}05$). These experiments provide evidence that human placenta contains an enzyme which catalyses the transformation of sorbitol to fructose. The enzyme is located in the non-particulate

fraction of the placenta homogenate, and there is no evidence that this enzyme differs from the ones described by Blakley (1951) and by Shaw (1956).

TABLE 2. Production of fructose, total reducing sugar and glucose by whole 'homogenates' of human full-term placenta

Experiment	Substrate	Fructose	Total reducing sugar	Glucose
1	None	3·3 ± 2·5	48·5 ± 3·7	—
	Fructose 6-phosphate	31·7 ± 5·3	114·5 ± 19	—
2	Glucose 6-phosphate	63 ± 23	269 ± 88	149 ± 82
	Fructose 6-phosphate	73 ± 21	240 ± 77	114 ± 14

The values are the mean of duplicates ± the S.D. and are given in μmole/g protein per 2 hr. 8 μmoles of fructose 6-phosphate or glucose 6-phosphate were added as indicated.

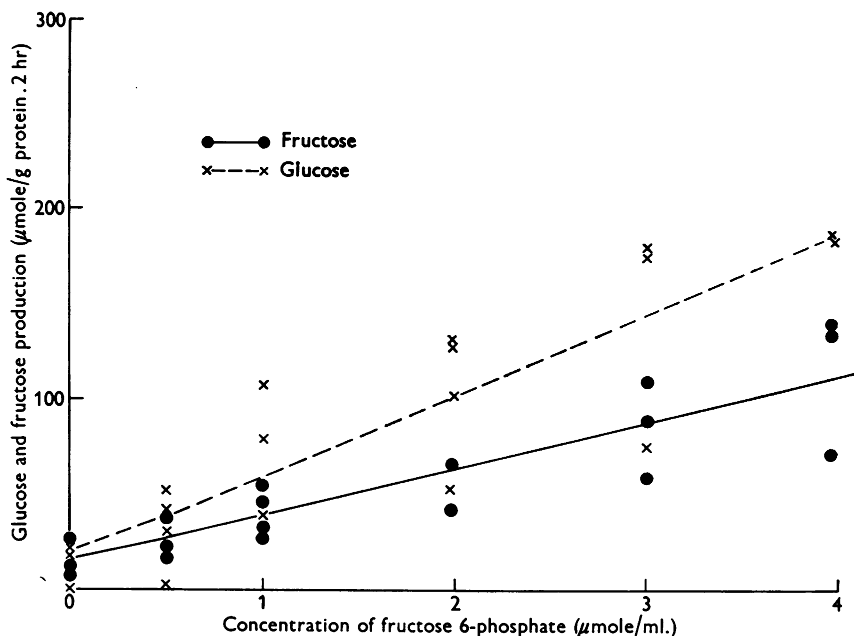

Fig. 1. Fructose and glucose production of homogenates of human full-term placenta as a function of fructose 6-phosphate concentration.

Tissues obtained from one human foetus (36 weeks gestation) were examined for ketose reductase activity by incubating tissue 'homogenates' with sorbitol and DPN. Kidney, liver and lung each produced fructose with or without sorbitol and the co-factor, but kidney made more fructose when they were present than when the substrate and co-factor were omitted.

The amount of sorbitol present in unincubated human placenta was measured. The average of three determinations gave 23 μmole/g protein. This method is not specific, so that an attempt was made to identify sorbitol in placental homogenates by paper chromatography. No material which

migrated at the same rate as authentic sorbitol was detected. Moreover, it was not possible to demonstrate a transformation of glucose to sorbitol after incubating placental homogenates with glucose and TPNH, the specific substrate and co-enzyme for aldose reductase.

Consequently, the effect of the addition of fructose 6-phosphate to a 'whole homogenate' was examined. The results of these experiments are shown in Table 2. Glucose 6-phosphate is as effective as fructose 6-phosphate. Furthermore, the amount of fructose and glucose produced depends upon the concentration of fructose 6-phosphate in the incubation medium (Fig. 1). The production of glucose is somewhat greater than that of fructose.

TABLE 3. Production of fructose, glucose and phosphate by 'whole homogenates' of human full-term placenta incubated with fructose 6-phosphate or glucose 6-phosphate at different pH's

Expt.	Production of	Acetate buffer (pH 5·0)	Citrate buffer (pH 6·4)	Borate buffer (pH 9·0)
1	Fructose from fructose 6-phosphate	0	0	138·4 ± 6·5
	Glucose from fructose 6-phosphate	58·5 ± 14	0	66 ± 10
2	Phosphate from fructose 6-phosphate	0	0	219 ± 54
3	Phosphate from glucose 6-phosphate	0	0	455 ± 59

In Expt. 1 the values are the mean of four determinations ± the S.D. In Expts. 2 and 3 the values are the mean of duplicate determinations. All the results are in μmole/g protein per 2 hr incubation for glucose and fructose production and per 15 min incubation for phosphate production. 8 μmoles of fructose 6-phosphate or glucose 6-phosphate were added to each flask as indicated.

In an attempt to differentiate between specific and non-specific esterases, 'whole homogenates' were incubated at three different pH's (6·4, 5·0 and 9·0), with fructose 6-phosphate as the substrate. The results (Table 3) show that the placenta contains an active alkaline phosphatase which splits both glucose 6-phosphate and fructose 6-phosphate.

DISCUSSION

These experiments show clearly that 'homogenized' human full-term placenta produces both fructose and glucose when incubated without substrate, and that the amount produced is increased by the addition of fructose 6-phosphate, glucose 6-phosphate, or sorbitol and DPN to the incubation medium. They thus confirm previous experiments (Hagerman & Villee, 1952) in which it was shown that slices of human placenta, where the cell membranes are presumably intact, produce fructose when incubated *in vitro*. This activity of the placenta provides an adequate explanation for the presence of fructose in human foetal blood in amounts greater than that in maternal blood.

More detailed consideration of the mechanism of this placental secretion of fructose is now possible. Three enzymic systems might be postulated: first, by a combination of the enzymes aldose reductase and ketose reductase, glucose might be converted into fructose without the intervention of phosphorylated sugars as intermediates. It is clear from the experimental results that the placenta possesses an enzyme system capable of the second reaction in this series, the conversion of sorbitol to fructose. However, it was not possible to demonstrate either the presence of sorbitol in the placenta or an enzyme system capable of converting glucose to sorbitol. This possibility is therefore excluded. Secondly, the placenta might contain a specific fructose 6-phosphatase. No evidence whatsoever for the existence of this enzyme in human full-term placenta was obtained. Thirdly, fructose production in both the slices and homogenates might be the result of the action of a non-specific phosphatase. Such enzymes have been repeatedly demonstrated to be present in placenta, mostly by histochemical techniques. For example, McKay, Hertig, Adams & Richardson (1958) describe the full-term placenta as containing large amounts of alkaline phosphatase concentrated in the syncytial layer of the villi. In our experiments, both glucose and fructose are readily produced when either fructose 6-phosphate or glucose 6-phosphate is incubated with placenta at an alkaline pH. It is reasonable to conclude that this sugar production is due to the same non-specific enzyme that can be demonstrated histochemically, an enzyme whose true function in the cell is unknown. The fact that both substrates produce glucose and fructose implies that the placenta contains an active phosphohexose isomerase. The amount of fructose produced is a function of the concentration of fructose 6-phosphate. That the production of glucose consistently exceeded the production of fructose can probably be attributed to the equilibrium position of phosphohexose isomerase, which favours glucose 6-phosphate formation.

SUMMARY

1. 'Whole homogenates' of human full-term placenta produce fructose when incubated *in vitro* with fructose 6-phosphate, glucose 6-phosphate, or sorbitol and diphosphopyridine nucleotide.

2. Human placenta contains a ketose reductase but does not contain an aldose reductase or significant amounts of sorbitol, so that the production of fructose observed in slice preparations cannot be explained on the basis of a combination of the action of these two enzymes.

3. No evidence could be obtained for the presence in placenta of a specific fructose 6-phosphatase.

4. It is concluded that the production of fructose by human term placenta is the result of the action of a non-specific alkaline phosphatase.

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