The Post-Natal Development of Hepatic Fructokinase

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The presence of fructose in the foetal blood and fluids of some species has been indicated for a long time (for review, see Needham, 1931) and was confirmed by Bacon & Bell (1948). Attempts to decide which of two postulated mechanisms is involved in the formation of foetal fructose have been inconclusive (Neil, Walker & Warren, 1961). Relative impermeability of the placenta to fructose is observed in both fructogenic and non-fructogenic species and may only be indirectly related to the presence of foetal fructose by assisting in its accumulation once formed (Walker, 1960).

The accumulation of fructose in the conceptus will also depend on its utilization by the foetal tissues. Absence of liver enzymes involved in the metabolism of fructose is likely to be of major importance. Parks, Ben-Gershom & Lardy (1957) reviewed methods for the assay of specific fructokinase (adenosine triphosphate-d-fructose 1-phosphotransferase, EC 2.7.1.3) and described an improved procedure giving higher activities and zero-order kinetics. Using earlier methods, Kuypers (1955) reported that liver fructokinase is absent in the foetal rat and that it develops after birth, and Hers (1957) briefly noted the absence of the enzyme in the foetal sheep. Andrews, Britton, Huggett & Nixon (1960) demonstrated by a perfusion technique that the foetal-sheep liver is unable to utilize fructose. Andrews, Britton & Nixon (1961) showed that conceptual age and possibly premature delivery are factors determining the commencement of the hepatic metabolism of fructose in sheep as indicated by the perfusion technique, and suggested that this was due to the post-natal appearance and development of hepatic fructokinase. The indirect evidence for this is therefore good. As part of a wider programme of work on carbohydrate metabolism in the developing foetal and new-born mammal (Walker, 1962; Lea & Walker, 1962), direct evidence for the post-natal development of hepatic fructokinase in several species has been obtained and is reported in the present paper. Evidence is also presented indicating that the absence of the enzyme in the new-born rabbit (a non-fructogenic species) lowers the fructose tolerance of the animal compared with the adult.

MATERIALS AND METHODS

Animals. The male and female rats were an ordinary laboratory albino strain, and the rabbits were of no special variety. The guinea pigs were of the Pirbright albino strain (the original stock having been obtained from the Animal Virus Diseases Research Institute, Pirbright, Surrey) and were bred by an intensive polygamous group method (Paterson, 1957). They were maintained on a diet consisting of equal parts (w/w) of Spiller's Intensive Poultry Pellets with added vitamins and Diet R.G.P. (C. Hill Ltd., Poole, Dorset) plus a liberal supply of fresh greensuff daily and hay and water ad lib.

Gestational age was assessed on the assumption of post-partum mating (rats), observed mating (rabbits) and a combination of post-partum mating and the tables (Draper, 1920) of crown-rump length and weight against age (guinea pigs). Gravid guinea pigs were used normally during their second or third pregnancy; the mean gestation period was 68 days.

Chemicals and enzyme. Fructose (‘glucose-free’) was obtained from British Drug Houses Ltd. ATP (disodium salt) and creatine phosphate (sodium salt) were obtained from Sigma Chemical Co. (through G.T. Gurr Ltd.,
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London, S.W. 6). All other chemicals were of the best quality obtained from British Drug Houses Ltd.; the tris was recrystallized twice from methanol.

Creatine phosphokinase was prepared by 'procedure B' of Kuby, Noda & Lardy (1954) to the end of 'stage IV'. This preparation was then freeze-dried. Its activity was checked by the method of Chappell & Perry (1954). For use, 30 mg. of the freeze-dried preparation was dissolved in 10 ml. of tris-maleate buffer (see below).

Liver extracts. Young and adult animals were killed by dislocation of the cervical vertebrae; foetal animals were removed from the dead gravid females as quickly as possible and killed by the same method. The livers were then perfused immediately and briefly in situ (by injection into the hepatic veins and cutting the hepatic artery and portal vein) with 10–20 ml. of ice-cold homogenizing medium [potassium chloride (0 15M); EDTA (sodium salt) (5 mM); magnesium chloride (5 mM); pH 7.0] to remove most of the blood; this procedure proved to be technically difficult in the foetal and new-born rats and rabbits and was omitted. The livers were then removed, chilled in the same medium, weighed and homogenized in 2 vol. of medium in a Potter–Elvehjem-type homogenizer (glass tube with Teflon pestle) for 1 min. at 2000 rev./min. More-dilute homogenates were sometimes necessary on liver samples from the smaller animals owing to the limited quantity of liver tissue available. The homogenate was first centrifuged at 1000g for 10 min.; the supernatant thus obtained was then centrifuged at 10 000g for 30 min. (Spinco preparative ultracentrifuge, model L; rotor no 40) and the remainder of the procedure for obtaining the liver supernatant fraction described by DiPietro & Weinhouse (1960) performed, all operations being made at 0C.

Fructokinase activity. This was assayed by measuring the fructose uptake in incubation mixtures (total vol. 0.5 ml.) containing (final concentrations): fructose (400 µg./ml.); tris–maleate buffer (0.1 M), adjusted to pH 7.3 with potassium hydroxide; magnesium chloride (10 mM); ATP (5 mM); creatine phosphate (5 mM); potassium chloride (1M); creatine phosphokinase (400 µg./ml.); liver supernatant preparation (usually 0.15 ml. alone or 0.075 ml. plus 0.075 ml. of homogenizing medium/0.5 ml. of incubation mixture). Assays were performed at 30°C for both 10 and 20 min. when the homogenate concentration was 33–3.5% (w/v) or for both 15 and 30 min. with more-dilute enzyme preparations. All the results reported were obtained by taking the average of four assays with two enzyme concentrations and two incubation periods. The reaction was stopped by the addition of 1 ml. of 5% (w/v) zinc sulphate and 1 ml. of 3N barium hydroxide (Somogyi, 1945). The precipitate was removed by centrifuging and fructose was determined in the protein-free filtrates by the method of Roe, Epstein & Goldstein (1949). Activities were expressed both per g. wet wt. of liver and on a nitrogen basis. Nitrogen was determined on the whole homogenate by a micro-Kjeldahl procedure.

Fructose tolerance. This was determined on adult rabbits, previously starved for 16 hr., by the injection of 1 g. of fructose/kg. body wt. as a 20% (w/v) solution at 57°C into a marginal ear vein over a period of 2 min. Blood samples were taken from the other ear both before and after the injection at specified times. Fructose was determined, in protein-free filtrates obtained after treatment with zinc sulphate and barium hydroxide, as described above; glucose was determined, in filtrates obtained after treatment with perchloric acid, by the glucose-oxidase method with commercial glucose-determination kits (C. F. Boehringer und Soehne G.m.b.H.; obtained through Courtin and Warner Ltd., Lewes, Sussex) used as directed by the manufacturers.

Suitable blood vessels for injection are not available in the new-born rabbits and serial blood sampling is almost impossible. For the assessment of fructose tolerance in the new-born animals, therefore, six litter mates were used for one test. The young rabbits were removed from their mother 4 hr. before the start of the test. Some food was still remaining in the stomach at the time of death. At an appropriate time each animal was anaesthetized with ether and injected with 1 g. of fructose/kg. body wt. as above but directly into the heart. The animal was allowed to recover from the anaesthetic and kept warm. At a specific time from the end of the injection the animal was killed, and blood was collected directly into a heparin-treated syringe after the jugular vein had been cut. One animal of each group was injected with an equivalent volume of 0.9% sodium chloride as a control; one animal was killed immediately after the infusion of fructose, and two animals at 30 min. and another two at either 60 or 90 min. after the administration of the sugar.

RESULTS

Determination of fructokinase. Because of the variety of methods available for the determination of fructokinase, and a number of uncertainties about such factors as the concentration of K+ ions, a series of experiments was performed to confirm the advantages of the method of Parks et al. (1957) and to evaluate other possible factors influencing activity. The following points were verified: (a) The addition of an oxidizable substrate to a basic incubation medium including ATP and Mg2+ ions increased the activity with whole homogenates (Vestling, Mylroie, Irish & Grant, 1950) but not in tissue preparations that had been centrifuged to remove the mitochondria. (b) The inclusion of creatine phosphate and creatine phosphokinase in the incubation medium to rephosphorylate the ADP formed from ATP (Parks et al. 1957) gave much increased activities, compared with all other methods. (c) A high concentration (1 M) of K+ ions is necessary for maximum activities with crude tissue preparations (Hers, 1952b).

Further, because it was hoped that the same tissue preparations might be used for the assay of other enzymic activities such as the phosphorylation of glucose (D. G. Walker, unpublished work), it was established that: (a) almost all the fructokinase activity was present in the supernatant fraction of the homogenate prepared by centrifuging at 100 000g for 30 min., (b) fluoride could be omitted from the incubation medium of Parks et al. (1957) when the supernatant fraction was used, (c) the high concentration of K+ ions is still
necessary with the supernatant fraction; (d) the inclusion of magnesium chloride (5 mM) and EDTA (5 mM) in the potassium chloride homogenizing medium had no effect on the fructokinase activity compared with that in supernatants prepared in 0-15 M potassium chloride alone, and (e) fructose uptakes showing proportionality to enzyme concentration and time could be recorded when over 75% of the fructose had been utilized, thus confirming the high affinity of the enzyme for fructose ($K_m$ less than 0.5 mM according to Hers, 1952a). Throughout the above tests the specificity of the enzyme being assayed was checked by showing that the presence of glucose in the incubation medium in concentrations higher than that of fructose, e.g. 10 mM, had no effect on the result obtained.

Post-natal development of fructokinase. A complete absence of any hepatic fructokinase activity in the foetal liver of guinea pigs, rats and rabbits was found. Increased enzyme concentrations and longer incubation times than in the standard assay procedure (see the Materials and Methods section) gave no indication of any uptake of fructose. The possibilities that the foetal-liver homogenates either contained a fructokinase inhibitor or lacked an activator that was present in adult tissue were eliminated by the fact that assay mixtures containing preparations of both adult and foetal tissue gave the same activity as with the adult sample alone. This type of experiment was performed with foetal-liver preparations from guinea pigs of four different gestational ages ranging from 45 to 67 days; rats and rabbits gave analogous results.

The time-course of the development of fructokinase activity in the guinea pig is shown in Fig. 1, where the fructokinase activities are expressed both in terms of wet wt. of liver and per body wt. The latter should give a better indication of the physiological significance of the activities recorded. Activities were also determined in a few new-born rats and rabbits and these are given in Table 1. All these results on the three species indicate that hepatic fructokinase activity appears after birth and reaches adult levels in 7–10 days. The rather wide variations in activities recorded with the young guinea pigs are at least in part due to a wide variation in the number, birth weights and growth rates of siblings in a given litter.

Fructose tolerance in the new-born animal. Fructose tolerance in rabbits of different ages was determined by the methods described. The various modifications of technique were enforced by the technical difficulties involved in experiments on very young small animals, and in all tests on such animals the sugar concentrations quoted represent determinations on different animals (litter mates) and not on the same animal.

Four typical experiments are shown in Figs. 2 and 3. Fig. 2 shows a normal fructose-tolerance curve on an adult rabbit together with results on a group of 11-day-old litter mates. The rates of decrease of the concentration of fructose shown on the semi-logarithmic plots are much greater than those indicated in Fig. 3, which represent results obtained on two groups of very young (less than 1 day old) litter mates. Many factors such as diffusion of the sugar throughout the extracellular fluids of the whole animal and the excretion of fructose by the kidney may influence these results, but the role of the liver is likely to be a major one and these results indicate a clear distinction between animals possessing a normal or a negligible hepatic fructokinase activity. In the fructose-tolerance test on the adult animal (Fig. 2) the blood glucose concentration increased from a value of 118 to 228 mg./100 ml. 2 hr. after the injection of fructose, and then returned to the
Table 1. Development of hepatic fructokinase activity in new-born rabbits and rats

Each result is the mean of two determinations on litter mates or pooled samples from several litter mates except where otherwise stated. Experimental details are given in the text. N.C., Not calculated.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Age</th>
<th>Fructokinase activity (μmoles of fructose utilized/min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Per g. wet wt. of tissue</td>
</tr>
<tr>
<td>Rabbit foetus</td>
<td>29 days (gestational age)</td>
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</tr>
<tr>
<td>Rabbit foetus</td>
<td>31 days (gestational age)</td>
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<td>Rabbit</td>
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<tr>
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</tr>
<tr>
<td>Rabbit</td>
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<td>0-83</td>
</tr>
<tr>
<td>Rabbit</td>
<td>12 days</td>
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<td>Rabbit</td>
<td>33 days</td>
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</tr>
<tr>
<td>Rabbit (gravid females)</td>
<td>Adult</td>
<td>1-15</td>
</tr>
<tr>
<td>Rabbit (gravid females)</td>
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<td>Adult</td>
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</tbody>
</table>

* Mean ± S.D. of five animals.

Fig. 2. Semi-logarithmic representation of typical fructose-tolerance tests on rabbits having normal hepatic fructokinase activities. Experimental details are given in the text. Fructose (1 g./kg. body wt.) was injected at zero time. O, Adult male animal; ▲, 11-day-old litter mates.

Fig. 3. Semi-logarithmic representation of typical fructose-tolerance tests on rabbits having negligible hepatic fructokinase activities. Experimental details are given in the text. Fructose (1 g./kg. body wt.) was injected at zero time. O, 12-hr.-old litter mates; ▲, 20-hr.-old litter mates.
initial level in less than 4 hr. after injection. Small increases of glucose concentration of up to 50 mg./100 ml. were noted in tests on the new-born animals but the glucose concentrations were very variable and inconclusive, owing probably to variable initial glucose concentrations in the non-starved animals.

DISCUSSION

Studies on the problem of the formation and function of fructose in the foetal blood and fluids of the so-called fructogenic species (Huggett, 1961) are subject to two practical limitations. These are the unavailability of a small fructogenic species of animal that could be maintained in a normal laboratory animal house and the high cost of procuring larger animals (and their young) for tests of a limited nature. However, when considered in conjunction with the results of Kuyper (1955), Hers (1957), Andrews et al. (1960) and Andrews et al. (1961), the present investigations now leave little doubt that hepatic fructokinase is absent from the foetal liver and appears soon after birth in many species, and that adult levels of activity are reached 7–10 days after parturition. They also show that the absence of hepatic fructokinase results in greatly diminished fructose tolerance.

The possible effect of ether anaesthetic must be considered. Full surgical anaesthesia was maintained for less than 5 min. in these experiments. Peters & Van Slyke (1946) reported that ether anaesthesia elevates the blood-sugar (glucose) concentration and decreases the utilization of carbohydrate. Miller, Craig, Drucker & Woodward (1956) confirmed this for glucose and fructose tolerance but showed that the effect of ether anaesthesia on fructose tolerance was negligible.

The post-natal appearance of hepatic fructokinase occurs in both fructogenic and non-fructogenic species. Like the observations on the permeability of the placenta to fructose, therefore, this is only indirectly concerned with the problem of the presence or absence of fructose in foetuses of the various species and is not the reason for the difference. It explains the disappearance of fructose from the blood of the new-born lamb (for review see Huggett, 1961) and the results of the perfusion type of experiments.

The factors controlling the appearance of the enzyme remain unknown. It must be presumed that various changes, perhaps hormonal, that occur after the end of intra-uterine life result in producing the necessary environment for the synthesis of fructokinase. Because comparatively high concentrations of fructose have been present throughout gestation in the fructogenic species, it cannot be a sudden appearance of the substrate fructose which induces the formation of fructokinase. Low concentrations of fructose are also present in the non-fructogenic species. Kuyper (1954, 1955) showed that the feeding of a diet with a high fructose content to rats resulted in an increased hepatic fructokinase activity and that starvation resulted in lower activity. However, the mechanisms for the regulation of the amount of an enzyme in an adult animal may be different from those concerned with the initial induction of enzyme biosynthesis during development.

SUMMARY

1. Hepatic specific-fructokinase activities have been determined on foetal, new-born and adult rats, rabbits and guinea pigs.
2. The enzyme is absent from the foetal livers of these species and appears after birth, reaching adult levels of activity 7–10 days later.
3. New-born rabbits show greatly diminished fructose tolerance before the hepatic fructokinase develops.
4. The general nature of the post-natal development of the enzyme is discussed with special reference to the presence of fructose in the foetal blood and fluids of certain species.

I thank Mr R. B. L. Stagg, who performed some preliminary enzyme assays and made the first preparation of creatine phosphokinase, Mrs S. Rao for valuable technical assistance, and the Wellcome Trust and the Medical Research Council for generous grants towards the research expenses.

REFERENCES

Carbohydrates in Protein

7. THE NATURE OF THE CARBOHYDRATE IN OVOMUCOID*

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The study of covalently linked carbohydrate in various glycoproteins has shown that the oligosaccharide portion of the protein may be present either as a single moiety or in the form of several smaller units. An example of the former is ovalbumin, which has been extensively studied in recent years by a number of groups of investigators (Jevons, 1958; Johansen, Marshall & Neuberger, 1960, 1961; Nuenke & Cunninghan, 1961; Kaverzneva & Bogdanov, 1961; Lee & Montgomery, 1962). This glycoprotein contains its carbohydrate as a single unit which is linked to an aspartic acid residue in the peptide chain. In the submaxillary-gland mucoprotein, however, the carbohydrate appears to consist of a large number of disaccharide units each individually linked to the protein (Gottschalk & Graham, 1959; Graham & Gottschalk, 1960).

In the present paper we report experiments on the nature of the carbohydrate in ovomucoid, the latter being the antitryptic factor found in hen’s egg white (Lineweaver & Murray, 1947). This glycoprotein has a molecular weight of 28 800 (Lineweaver & Murray, 1947; Fredericq & Deutsch, 1949) and contains about 25% of carbohydrate, which has been identified as consisting of glucosamine, mannose and galactose (Sørensen, 1934a, b; Stacey & Woolley, 1940, 1942; Dixon, 1955; Gottschalk & Ada, 1956; Bragg & Hough, 1961).

In the present work ovomucoid was digested with the proteolytic enzyme from Streptomyces griseus (Pronase) and a fraction consisting largely of carbohydrate was isolated. This fraction was studied by procedures that, it was hoped, would indicate whether the carbohydrate existed as a single prosthetic group or was composed of several smaller units. A preliminary description of the procedure for preparing the carbohydrate-rich fraction has been published (Marks, Marshall, Neuberger & Papkoff, 1962).

EXPERIMENTAL

Preparation of ovomucoid. Egg white was diluted with an equal volume of water, solid ammonium sulphate was added to give 50% saturation, and the pH was adjusted to 7-0. After removal of the resultant precipitate by centrifuging, the pH was adjusted to 4-6, which led to the precipitation of the ovalbumin fraction (Warner, 1954). The mixture was centrifuged, and solid ammonium sulphate was added to the supernatant fluid to give 90% saturation. The fraction that was precipitated consisted largely of conalbumin and ovomucoid. Final purification was achieved by chromatography on carboxymethylcellulose and diethylaminoethylcellulose as described by Rhodes, Azari & Feeney (1958) and Rhodes, Bennett & Feeney (1960). The purified ovomucoid fraction was concentrated by precipitation with ammonium sulphate, extensively dialysed against water and freeze-dried.

When examined by free-boundary electrophoresis in a Perkin-Elmer electrophoresis apparatus the preparation migrated essentially as a single component in sodium veronal buffer, pH 8-5 and I 0-1. Antitryptic activity was assessed by the ability to inhibit crystalline trypsin, casein being used as the substrate. Within the limits of experimental error, the ovomucoid preparation completely inhibited the activity of an equal weight of trypsin.