tion of the structure shown for chorismic acid was obtained by examination of a deuterium oxide solution of barium chorismate by nuclear-magnetic-resonance spectroscopy (Gibson & Jackman, 1963). Chorismic acid can be formulated as the 3-enol-pyruvic ether of *trans*-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid.

SUMMARY

1. Chorismic acid, a new intermediate in aromatic biosynthesis, is the 3-enolpyruvic ether of *trans*-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid.

2. Although chorismic acid itself is a fairly labile compound the barium salt is readily isolated and has been examined.

3. The ultraviolet- and infrared-absorption spectra of chorismate are given together with details of chromatography.

4. Chorismate is readily converted, by warming, into a mixture of prephenate and 4-hydroxybenzoate. Under acid conditions the prephenic acid is further converted into phenylpyruvic acid.

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Ketohexokinase, Isoenzymes of Glucokinase and Glycogen Synthesis from Hexoses in Neonatal Rat Liver

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The activities of many enzymes in the liver are known to change during foetal and post-natal development of the mammal, and such changes reflect important alterations in the physiological functions of the liver. The urea cycle (Kennan & Cohen, 1959), the glucuronide-conjugating system (Brown & Zeulzer, 1958) and the activities of several respiratory enzymes (Dawkins, 1959) have been measured during liver development. In previous reports (Ballard & Oliver, 1962, 1963) evidence was presented for the view that mechanisms of glyconeogenesis develop in the rat liver only after birth of the animal. These results showed that the foetal liver is solely dependent upon blood hexose for the synthesis of its glycogen store.

In the present work, the utilization of glucose, fructose and galactose for the synthesis of liver glycogen has been investigated during neonatal development in the rat. To this end, the incorporation of ¹⁴C-labelled hexoses into hepatic glycogen and the activities of the liver enzymes, ATP-D-fructose 1-phosphotransferase (ketohexokinase, EC 2.7.1.3) and ATP-D-glucose 6-phosphotransferase (glucokinase, EC 2.7.1.2) have been measured. The results obtained in the glucokinase assays suggested the existence of different forms of the enzyme. The Michaelis kinetics and the variation of liver-glucokinase activity with pH indicate the existence of two forms of the enzyme, one found in both the foetal and the adult liver and a second

form which develops with age and predominates in the adult liver. Walker (1962) has recently reported the existence of glucokinase isoenzymes in guinea-pig liver.

MATERIALS AND METHODS

Chemicals. ATP, NADP, glucose 6-phosphate and glucose 1-phosphate were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Uniformly labelled [^{14}C]glucose, uniformly labelled [^{14}C]fructose and [$1\text{-}^{14}\text{C}$]galactose were obtained from The Radiochemical Centre, Amersham, Bucks. Diethylaminoethyl(DEAE)-cellulose (0.43 m-equiv./g.) was obtained from the California Corp. for Biochemical Research, Los Angeles, U.S.A.

Animals. Rats of the Wistar albino strain of *Rattus norvegicus* were bred in the preclinical animal house of this University. Adult rats used were between 4 and 6 months old. Equal numbers of male and female animals were used at random in each series of experiments.

Preparation of glucose 6-phosphate dehydrogenase (EC 1.1.1.49) from dried baker's yeast. Compressed baker's yeast was crumbled by hand and dried at room temperature. The dried yeast was stored in a tightly stoppered bottle at 0–4°.

(1) Autolysis. A portion (200 g.) of dried yeast was suspended in 1 l. of 0.1 M- NaHCO_3 and stirred for 4 hr. at 37°. After chilling it was left at 0–4° for as long as 2 days without loss of activity. The suspension was centrifuged at 4000g for 30 min. at 0°, and the brownish supernatant was decanted and adjusted to pH 8.0 with 2N-NaOH.

(2) Heat-treatment. The fluid was brought to 45° by stirring in a water bath at 55° and then maintained at 45° for 110 min. This treatment destroyed the glucokinase activity but 35–45% of the glucose 6-phosphate dehydrogenase originally present remained. The resulting suspension was cooled in ice, the pH adjusted to 5.7 with 2N-acetic acid and the suspension centrifuged at 4000g for 20 min.

(3) Treatment with protamine sulphate. The supernatant was decanted, the volume measured and 20 ml. of 2% protamine sulphate solution added per 100 ml. of supernatant. Centrifuging at 4000g for 10 min. removed the precipitate.

(4) Fractionation with $(\text{NH}_4)_2\text{SO}_4$. The supernatant solution was made to pH 5.4 with 2N-acetic acid and fractionated by slow addition of solid $(\text{NH}_4)_2\text{SO}_4$ at 0°. A precipitate which appeared at 55% saturation (0°) was centrifuged off at 4000g for 30 min. and the supernatant solution adjusted to 65% saturation with $(\text{NH}_4)_2\text{SO}_4$ at 0°. After standing for 30 min. at 0° the precipitate was harvested by centrifuging at 4000g for 30 min.

(5) Treatment at pH 4.4. The precipitate was dissolved in 120 ml. of 0.1 M-sodium acetate adjusted to pH 4.4 with 0.1 N-acetic acid. The pH of the solution was measured and adjusted to exactly 4.4 with 2N-acetic acid. After standing for 30 min., a flocculent precipitate was removed by centrifuging at 4000g for 10 min. The supernatant, which now was free of glucose phosphate-isomerase (EC 5.3.1.9) activity, was adjusted to pH 5.0 with 2N-NaOH.

(6) Adsorption to $\text{Ca}_3(\text{PO}_4)_2$ gel. A portion (0.1 vol., v/v) of $\text{Ca}_3(\text{PO}_4)_2$ gel (37 mg. dry wt./ml.) (Keilin & Hartree, 1938) was added to the solution and the suspension centri-

fuged. The supernatant was decanted and 0.2 vol. (based on original volume of the enzyme solution) of the gel was added. The suspension was then centrifuged. The gel precipitate was washed twice by suspension and centrifuging with 100 ml. of 0.05 M-sodium acetate adjusted to pH 5.0 with 2N-acetic acid and the enzyme was eluted with four 80 ml. batches of 0.1 M-phosphate buffer, pH 7.4 (0.1 M- NaH_2PO_4 adjusted to pH 7.4 with 2N-NaOH). The combined eluates were adjusted to pH 7.7 with 2N-NaOH and the solution was dialysed in viscose tubing (previously soaked in 0.2% EDTA solution, pH 8.0, with 2N-NaOH) against 0.05 M- NaH_2PO_4 adjusted to pH 7.7 with 2N-NaOH.

(7) Chromatography on DEAE-cellulose (Srere, Cooper, Tabachnik & Racker, 1958). The enzyme was adsorbed on a column of DEAE-cellulose that had been equilibrated with 0.05 M- NaH_2PO_4 adjusted to pH 7.7 with 2N-NaOH (15 g. of exchanger, 0.43 m-equiv./g., is sufficient for 400 units of enzyme at this stage). The column was washed with 0.05 M- NaH_2PO_4 adjusted to pH 7.7 with 2N-NaOH until the filtrate had extinction at 280 m μ of less than 0.020 (1 cm. cell). The enzyme was eluted with 0.1 M-NaCl in 0.05 M- NaH_2PO_4 adjusted to pH 7.7 with 2N-NaOH at a flow rate of 80 ml./hr. The fractions of highest specific activity were combined. The enzyme is stable in solution at 0° for at least 1 month and may be freeze-dried in this solution or after dialysis against water in viscose tubing treated as described previously.

The enzyme was purified about 400-fold from the yeast autolysate and the yield was 12–15%. It contained less than 1 part in 10000 of glucokinase, phosphogluconate dehydrogenase (EC 1.1.1.43), phosphoglucosmutase (EC 2.7.5.1), glucose 6-phosphatase (EC 3.1.3.9) and glucose phosphate isomerase. The final specific activity was 77 μ moles of NADP reduced/mg. of protein/min. at 30° under the specified assay conditions.

Assay of glucose 6-phosphate dehydrogenase. (Modified from Kornberg, 1950.) Reaction mixtures contained 4 mm-glucose 6-phosphate, 0.5 mm-NADP, 10 mm- MgSO_4 and 50 mm-tris buffer, pH 7.4, adjusted with 2N-HCl in a final volume of 0.8 ml. in 1 cm. silica micro-cuvettes. The rate of change of extinction at 340 m μ and 30° was determined after addition of enzyme to the cuvettes in a Beckman DU spectrophotometer. A unit of enzyme was defined as that amount which reduced 1 μ mole of NADP/min. under these conditions.

Preparation of liver extract. Liver samples were homogenized in 5 vol. (w/v) of ice-cold 0.15 M-KCl containing 5 mm- MgSO_4 and 5 mm-EDTA, adjusted to pH 7.0 with 0.01 N-KOH. They were centrifuged for 2 hr. at 25000g at 0° in an International refrigerated centrifuge model PR2 with rotor 296. The supernatant was used for the estimation of ketohexokinase, but for the assay of glucokinase it was necessary to dialyse the supernatant against the medium used for homogenization to remove endogenous glucose. Because of the instability of the enzyme the dialysis was carried out for a standard time of 4 hr. at 0° on a rocking dialyser.

Assay of glucokinase. Glucokinase was assayed 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. The cuvettes contained 44 mm-tris buffer adjusted to pH 7.5 with N- H_3PO_4 , 0.75 mm-NADP, 7.5 mm- MgSO_4 , 3 mm-ATP

adjusted to pH 7.5 with 0.01N-KOH, 0.1M-glucose and excess of glucose 6-phosphate dehydrogenase. The reaction was started by the addition of liver extract, giving a total volume of 3.0 ml., and the rate of reaction at 37° determined by measuring the extinction at 340 m μ at 60 sec. intervals in a Shimadzu spectrophotometer. Blank estimations on liver extracts were carried out in similar reactions mixtures with glucose omitted. The activity was calculated in μ moles of NADP reduced/g. wet wt. of tissue/hr.

Assay of ketohexokinase. Ketohexokinase was assayed by the rate of fructose disappearance. Centrifuge tubes containing 4 mM-ATP, 20 mM-MgSO₄, 20 mM-KCl, 0.1M-tris buffer adjusted to pH 7.3 with N-H₂PO₄, 5 μ moles of fructose and 62 mM-NaF in a volume of 1.5 ml. were incubated with 0.5 ml. of liver extract in a metabolic shaker at 37° for 0, 10, 20 and 30 min. The reaction was stopped by deproteinization with equimolecular amounts of ZnSO₄ and Ba(OH)₂ according to Weichselbaum & Somogyi (1941). We have verified that the precipitate adsorbs phosphorylated hexoses completely. After centrifuging, fructose was determined in the supernatant by the method of Cole, as described by Bacon & Bell (1948).

Isotope experiments. Livers were removed from animals immediately after decapitation and chilled in 10 ml. of oxygenated medium of composition 70 mM-KCl, 20 mM-MgCl₂, 40 mM-KHCO₃ and 10 mM-CaCl₂ adjusted to pH 7.45 with 0.01N-HCl. Slices of 0.3 mm. uniform thickness were cut from the livers with the aid of a transparent plastic block (8 cm. $\times$ 2.5 cm. $\times$ 1 cm.) with a section (8 cm. $\times$ 1.2 cm. $\times$ 0.03 cm. deep) machined from the face. This block, moistened with the fluid described above, was placed on the liver sample and slices were cut by sliding a razor blade against the raised edges of the block. Approximately 100 mg. of liver slices, which had been blotted dry, were immediately transferred to weighed 2.5 cm.-diameter flat-bottomed tubes containing 1 ml. of the incubation medium, which consisted of 70 mM-KCl, 20 mM-MgCl₂, 10 mM-CaCl₂, 40 mM-KHCO₃ and 30 mM-glucose, fructose or galactose adjusted to pH 7.45 with 0.01N-HCl. The appropriate labelled hexose (1 μ C) was also present. The tubes were reweighed to give the weight of the slices added, treated with O₂ + CO₂ (95:5), stoppered and incubated at 100 oscillations/min. at 37° in a Gallenkamp Metabolic Shaker. The pH of the medium fell by approximately 0.1 during the 2 hr. of incubation. The extent of this fall in pH does not vary with the age of animals used. The reaction was stopped by the addition of 2 ml. of 60% KOH and the tissue digested by heating each tube at 100° for 5 min. with

shaking. Glycogen was precipitated with 5 ml. of ethanol. The glycogen was purified as described by Cowgill & Pardee (1957) and washed with ethanol until no radioactivity could be detected in the washings. The purified glycogen was dissolved in water to give a concentration less than 2.5 mg./ml. If the glycogen concentration exceeded this value, the addition of 0.2 ml. of solution to 5.0 ml. of Diotol for counting (Herberg, 1960) resulted in the precipitation of glycogen from the Diotol solution. The Diotol solution consists of 400 ml. of toluene, 400 ml. of dioxan, 240 ml. of methanol, 83 g. of naphthalene and 5.2 g. of 2,5-diphenyloxazole. The radioactivity in the glycogen was determined with 50% efficiency in an EKCO liquid scintillation counter at 20°. The addition of internal glucose standards (1 $\times$ 10⁴ counts/min.) indicated that no quenching corrections were necessary. The glycogen concentration was estimated with the anthrone reagent (Scott & Melvin, 1953).

The results have been expressed as counts/min. incorporated into the glycogen present in 1 g. of liver (counts/min./g. of liver). For an accurate estimate of the glycogen content of the liver slices the purification procedure must result in quantitative glycogen recovery. This occurs only when the glycogen content of incubated slices is greater than 10 mg./g. This value was exceeded in all experiments. The specific radioactivity of the glycogen was not used as a measure of hexose incorporation since its value is dependent on the amount of glycogen present in the liver slices, and the concentration of glycogen in the liver varies considerably with age of the neonatal animal.

The 'incorporation rate' of ¹⁴C into liver glycogen is based on measurements after incubation for 2 hr. Table 1 summarizes an experiment where liver slices from foetal and adult rats have been incubated with ¹⁴C-labelled substrates for various times from 0 to 3 hr. In both foetal- and adult-liver slices the incorporation of ¹⁴C-labelled glucose, fructose and galactose into glycogen continues at a roughly linear rate throughout the incubation.

RESULTS

The incorporation of [¹⁴C]fructose into glycogen of liver slices increases continuously during the late foetal and post-natal development of the rat (Fig. 1a).

Ketohexokinase activity increases from 2.4 μ -moles/g./hr. in 18-day foetal liver to 27.6 μ moles/g./hr. in the adult (Fig. 1b). At birth the activity is

Table 1. *Effect of incubation time on incorporation of ¹⁴C-labelled substrates into glycogen in liver slices*

Methods used are described under 'Isotope experiments' in the Materials and Methods section.

		Incubation time (hr.)						
		0	0.5	1	1.5	2	2.5	3
		$10^{-5} \times$ Radioactivity incorporated (counts/min./g. of liver)						
Source of liver	Labelled substrate							
Foetus (21 days)	Glucose	0	—	1.84	—	4.24	—	6.48
	Fructose	0	—	1.80	—	4.74	—	6.65
	Galactose	0	—	0.94	—	2.70	—	3.74
Adult	Glucose	0	2.47	6.02	11.42	14.3	15.9	18.0
	Fructose	0	—	4.51	—	11.7	—	18.5
	Galactose	0	0.30	1.05	1.64	2.08	3.02	3.50

6.2 μ moles/g./hr. Ketohexokinase activity is depressed in the liver of pregnant rats to give a mean value of 17.5 μ moles/g./hr. No fructose disappeared from incubation mixtures in the absence of ATP.

The incorporation of [14 C]galactose into the liver glycogen increases during foetal and early post-natal growth to a maximal incorporation of 10.5×10^5 counts/min./g. of liver at 8 days after birth (Fig. 2). The incorporation rate remains high at 15 days but, in the adult, the average incorporation rate from [14 C]galactose has dropped to 2.3×10^5 counts/min./g. of liver.

The incorporation of [14 C]glucose into the liver glycogen (Fig. 3) in the foetus and young rat liver is considerably below the rate of incorporation in adult liver. Glucokinase activity, 38 μ moles/g./hr. in 17-day foetal liver, falls to a minimum of 20 μ moles/g./hr. at 15 days after birth and then rises to the adult value of 160 μ moles/g./hr. (Fig. 3). The time-course of development of the enzyme activity could be explained by the replacement of a form of glucokinase specific to the foetal liver by an isoenzyme specific to the adult tissue. The following experiments were designed to test this hypothesis.

Fig. 1. (a) Incorporation of [14 C]fructose into glycogen in liver slices. Slices from neonatal liver were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. (b) Ketohexokinase activity estimated by fructose disappearance at 37° in 25000g supernatants of neonatal rat liver. T, Term; A, adult. Each point represents the mean $\pm$ s.e.m. of at least four duplicate determinations. For each determination on animals younger than 10 days, livers from two litter mates were pooled.

Kinetic experiments. Preliminary experiments on extracts from foetal and adult liver indicated a large difference in the Michaelis constants of the glucokinases for glucose. The K_m of foetal glucokinase was determined by the method of Lineweaver & Burk (1934). The range of Michaelis constants obtained was $5-8 \times 10^{-5}$ M (Fig. 4). As the foetal enzyme was not inhibited by high concentrations of glucose, it was possible to measure the fraction of glucokinase activity due to the foetal isoenzyme in other liver preparations. The reaction velocities were measured over two ranges of substrate concentrations: 10^{-5} – 10^{-4} M- and 4×10^{-3} – 10^{-1} M-glucose. From the former concentration range, a Lineweaver-Burk plot for the foetal component was constructed, given $K_m(f)$ and $V_{max}(f)$ (where f stands for the foetal isoenzyme). The extrapolated values of $1/v$ were used to determine the velocity due to the foetal isoenzyme in the second range, 4×10^{-3} – 10^{-1} M-glucose. Assuming additivity, these velocities (v_f) were subtracted from the total observed velocities (v_t) to give the velocities due to the principal adult isoenzyme (v_a) at any substrate concentration. The resultant velocities due to the adult component were plotted as a Lineweaver-Burk graph from which the values of $K_m(a)$ and $V_{max}(a)$ could be determined by extrapolation (Fig. 5). The range of $K_m(a)$ was $1-3 \times 10^{-2}$ M-glucose.

The ratio $[V_{max}(a)]/[V_{max}(f) + V_{max}(a)]$ expresses the fraction of the adult isoenzyme activity present in any preparation. These values determined at different animal ages are shown in Table 2.

Fig. 2. Incorporation of [14 C]galactose into glycogen in liver slices. Slices from neonatal liver were incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. T, Term; A, adult. Each point represents the mean $\pm$ s.e.m. of at least four duplicate determinations. For each determination on animals younger than 10 days, livers from two litter mates were pooled.

pH *optima*. The variation in glucokinase activity with pH was determined in both adult- and foetal-liver extracts. The extracts from adult liver had maximum activity at pH 7.0. At pH 9.5 only 20 % of the maximum activity remained. The extracts from foetal liver exhibited little pH sensitivity in the range studied, and had maximum activity at pH 9 (Fig. 6).

Reaction product. Since the previous results indicate the existence of different forms of liver glucokinase, the possibility that glucose 1-phosphate might be the initial product of reaction

cannot be dismissed. Although the assay procedure used for glucokinase measures glucose 6-phosphate production, extracts of foetal and adult liver are high in phosphoglucomutase activity. Hence glucose 1-phosphate and glucose 6-phosphate production are not distinguished. This possibility was tested by the use of arsenate as an inhibitor of phosphoglucomutase (DiPietro, Sharma & Weinhouse, 1962). Arsenate inhibits glucose 6-phosphate production from glucose 1-phosphate

Fig. 3. (a) Incorporation of [¹⁴C]glucose into glycogen in liver slices incubated for 2 hr. at 37° as described under 'Isotope experiments' in the Materials and Methods section. (b) Glucokinase activity of neonatal rat liver. Supernatants obtained after centrifuging liver extracts at 25000g were used for the estimation after endogenous glucose had been removed by dialysis. The glucokinase was estimated spectrophotometrically at 340 mμ and 37° by glucose 6-phosphate production from 0.1M-glucose and ATP with NADP and glucose 6-phosphate dehydrogenase. T, Term; A, adult. Each point represents the mean ± s.e.m. of at least four duplicate determinations. For each determination on animals younger than 10 days, livers from two litter mates were pooled.

Fig. 4. Lineweaver-Burk plot of the foetal component of glucokinase. This experiment gives K_m 6×10^{-5} M-glucose. The velocity units are arbitrary.

Fig. 5. Lineweaver-Burk plot of the major glucokinase component of adult liver. This experiment gives K_m 1.2×10^{-2} M-glucose. The method of obtaining this plot is explained under 'Kinetic experiments' in the Results section. The velocity units are arbitrary.

Table 2. Fraction of glucokinase present in rat liver as foetal or adult isoenzyme

Enzymes were assayed as described under 'Kinetic experiments' in the Results section.

Fraction	Foetal liver (17 days)	Neonatal liver		Adult liver
		12 days	20 days	
Foetal form	1.00	0.64	0.30	0.15
Adult form	0	0.36	0.70	0.85

Table 3. *Reaction product of liver glucokinases*

Each cuvette contained excess of glucose 6-phosphate dehydrogenase, 44 mM-tris adjusted to pH 7.5 with $\text{N-H}_2\text{PO}_4$, 0.75 mM-NADP and 7.5 mM- MgSO_4 . Additions listed in the Table gave final concentrations 3 mM-ATP, 0.1 M-glucose, 60 mM-sodium arsenate and 0.1 mM-glucose 1-phosphate. Liver extract (120 μl .) was added to give a final volume 3.0 ml. ΔE over 5 min. at 340 $\text{m}\mu$ was linear for each cuvette.

Enzyme	ATP	Glucose	Glucose 1-phosphate	Arsenate	$\Delta E/5$ min.
Adult	0	0	0	0	0
	0	0	+	+	0.007
	0	0	+	0	0.307
	+	+	0	0	0.190
	+	+	0	+	0.160
Foetal	0	0	0	0	0
	0	0	+	+	0.037
	0	0	+	0	0.500
	+	+	0	0	0.185
	+	+	0	+	0.175

Fig. 6. Plot of relative activity against pH for dialysed glucokinase preparations from adult- and foetal-rat liver. The glucose concentration used in all the assays was 0.1 M. Results are the means of two similar experiments. For each experiment livers were obtained from one male rat (●) and one litter of foetal rats (▲).

in both adult and foetal extracts (Table 3). However, the effect of arsenate on glucose 6-phosphate production from glucose and ATP is only slight in both extracts and therefore glucose 1-phosphate cannot be the initial product of phosphorylation in either case.

DISCUSSION

The glucokinase found in foetal rat liver has K_m 5.8×10^{-5} M for glucose and optimum pH 9. Walker (1962) recently reported that foetal-guinea-pig liver contained a glucokinase with K_m 3×10^{-5} M, which was also present in the adult together with a glucokinase of higher K_m (3×10^{-3} M). Lange & Kohn (1961) reported that adult-rat-liver

glucokinase had K_m 4×10^{-5} M, which could be the foetal enzyme, but they did not find an isoenzyme of higher K_m . The glucokinase of rat liver studied by DiPietro *et al.* (1962) had K_m 1.4×10^{-2} M, in agreement with the value for the adult isoenzyme reported in this paper. The assay method used by DiPietro *et al.* (1962) did not involve the dialysis of liver extracts, resulting in high blank values caused by endogenous glucose present in the extract. It is probable that these blank values are a result of the foetal isoenzyme operating at near maximum activities.

Moore & Angeletti (1961) chromatographed a centrifuged liver extract on DEAE-cellulose and found glucokinase activity in two peaks. It is possible that the peaks represent the two isoenzymes reported in this investigation. Earlier in the progress of this study attempts were made to chromatograph adult-liver extracts on DEAE-cellulose, but the recovery of enzyme was poor and the activity disappeared rapidly either on dialysis or storage in the absence of potassium chloride.

In the adult rat, interchange of glucose between the liver and the blood depends largely upon the relative activities of glucose 6-phosphatase and glucokinase, since the adult liver is freely permeable to glucose (Cahill, Ashmore, Earle & Zottu, 1958). In the foetal liver there is negligible glucose 6-phosphatase activity (Weber & Cantero, 1955; Dawkins, 1961), the glucokinase differs in Michaelis constant and pH optimum and is of low activity. Glyconeogenesis does not occur (Ballard & Oliver, 1962, 1963). If the foetal liver is freely permeable to glucose, the magnitude of the Michaelis constant of glucokinase indicates that the enzyme would promote the phosphorylation of glucose at near maximum rates even at very low concentrations of blood glucose. This system would therefore play little part in the variation of the rate of hepatic utilization of glucose. The change-over of gluco-

kinase from the foetal type to that found in adult liver occurs over the first 20 days after birth. Glucose 6-phosphatase and the glycogenogenic pathway appear when glucokinase activity is at a minimum. Previous results (Ballard & Oliver, 1963) have shown that at about this time pyruvate is more readily utilized for glycogen synthesis in liver slices than is glucose. This may be due to imbalance between the glucokinase and glucose 6-phosphatase activities. As the adult isoenzyme develops, the liver gains in ability to synthesize glycogen from glucose (Fig. 3) and the affinity of the enzyme for glucose is such that large variations in the rate of glucose phosphorylation would occur with variations in the concentration of blood glucose over the normal range of about 3–10 mM (Cole & Harned, 1938). The Michaelis constant of adult glucokinase ($1\text{--}3 \times 10^{-2}\text{ M}$) is thus favourable for the operation of a control mechanism based on the Michaelis relation between substrate concentration and velocity of the enzyme reaction. On the other hand, the foetal-enzyme system could not operate in this way until the concentration of blood glucose fell below about 0.1 mM, since K_m of the foetal isoenzyme is only $5\text{--}8 \times 10^{-5}\text{ M}$.

The similarity between the results with [^{14}C]glucose incorporation into liver slices and the glucokinase assays on extracts indicates that the glucokinase activities accurately reflect a changing capacity of the organized tissue to utilize glucose. The incorporation from glucose in the 15-day liver is only one-fifth of the incorporation from fructose. Since glucose 6-phosphate is an intermediate in the conversion of fructose into glycogen as well as the initial product of glucokinase action, it is probable that glucokinase catalyses the rate-limiting reaction in the conversion of glucose into glycogen in liver slices from young rats.

The increase of ketohexokinase activity and of the incorporation of [^{14}C]fructose into glycogen during the neonatal period indicates the development of a pathway for the utilization of fructose in rat liver. Walker (1962) has demonstrated the development of hepatic ketohexokinase activity in the post-natal guinea pig and rabbit, and Andrews, Britton, Huggett & Nixon (1960) have shown that the isolated perfused liver of the newborn lamb is unable to metabolize fructose, and that utilization cannot be detected before the fifth post-natal day.

The data for incorporation from [^{14}C]galactose into glycogen indicate the availability of this pathway in the post-natal rat liver during the time of suckling. Depressed activity occurs in the adult. No galactokinase (EC 2.7.1.6) assays were undertaken because of the difficulty of measuring the enzyme activity in crude extracts. Alvarez & Sas (1961) have demonstrated a high activity of β -galactosidase (EC 3.2.1.23) in the rat intestine

during the period of suckling. It seems probable that the pathway utilizing galactose and lactose is functional mainly during the period between birth and weaning.

SUMMARY

1. The incorporation of [^{14}C]fructose into glycogen in rat-liver slices indicates the neonatal development of a pathway for the conversion of this hexose into glycogen.

2. The activity of ketohexokinase increases 15-fold from the 18-day foetus to the adult.

3. The rates of incorporation of [^{14}C]galactose into liver glycogen in slices indicate that this system has most activity during the period of milk feeding.

4. The activity of hepatic glucokinase in the adult rat is eight times the value 15 days after birth and four times the value in the 17-day foetus.

5. The incorporation of [^{14}C]glucose into glycogen is also greater in slices from adult and foetal liver than from 15-day liver.

6. There are two isoenzymes of glucokinase in rat liver. The only form in the foetus has a maximum activity at pH 9.0 and K_m for glucose $5\text{--}8 \times 10^{-5}\text{ M}$. This isoenzyme comprises 15% of the liver-glucokinase activity in the adult. An isoenzyme with K_m for glucose $1\text{--}3 \times 10^{-2}\text{ M}$ is the major component in the adult. An extract of adult liver has maximal activity for glucokinase at pH 7.0.

7. These findings have been discussed with reference to the changing pattern of carbohydrate metabolism during neonatal development of liver in the rat.

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Determination of Ubiquinone and Tocopherol in Some Tissues of Shark (*Carcharias ellioti* Day)

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The presence of a quinone-like compound in the non-saponifiable portion of lipids in animal tissues was reported independently by two groups of workers, at Liverpool (Festenstien, Heaton, Lowe & Morton, 1955; Cain & Morton, 1955; Morton, 1958) and at Wisconsin, U.S.A. (Crane, Hatefi, Lester & Widmer, 1957; Lester, Crane & Hatefi, 1958; Lester, Hatefi, Widmer & Crane, 1959). However, it was soon recognized that several homologues of this quinone occurred in Nature; whereas in mammalian tissues the homologue having 10 isoprenoid units predominates, in plants and micro-organisms lower homologues having 6–9 isoprenoid groups have been reported (Lester & Crane, 1959a).

Seven different tocopherols have been found to occur in Nature (Blaxter, 1957), but α -tocopherol is the form most active biologically and is also the form in which tocopherol is preferentially stored in animal tissues. In haddock, the α -form constitutes over 90% of the total tocopherol (Harris, Quaife & Swanson, 1950).

Very little work has been reported on the ubiquinone and tocopherol contents of fish tissues and practically no data are available on the presence of these compounds in shark, although Robeson & Baxter (1943) reported a tocopherol content of 10–40 mg./100 g. of shark-liver oil. The present work was undertaken to determine the ubiquinone and tocopherol contents of the different tissues of shark and the particular homologue present.

EXPERIMENTAL

Saponification and extraction. The liver, heart and muscle tissues of freshly landed sharks (*Carcharias ellioti* Day) were used. Saponification was carried out by a slight modification of the method used by Crane, Lester, Widmer & Hatefi (1959) and Lester & Crane (1959b). About 200–250 g. of the minced tissue was added to 300 ml. of ethanolic 10% (w/v) KOH in which 10 g. of pyrogallol was dissolved, and the mixture was refluxed for 30 min. After cooling, the mixture was extracted thrice with iso-octane (2,2,4-trimethylpentane) and made to volume. This solution was used for the estimation of tocopherol and examined for the spectra of ubiquinone.

Removal of sterols. The volume of the 2,2,4-trimethylpentane extracts was then reduced to about 25 ml. and the solution kept at 5° overnight. A precipitate, which consisted mainly of sterols, was filtered off and washed with ice-cold 2,2,4-trimethylpentane; the solutions and washings were made to volume. The estimation of tocopherol and the spectrum of ubiquinone were carried out once more at this point.

Column chromatography. The method suggested by Mervyn & Morton (1959) and Jayaraman & Ramasarma (1961) was used with slight modifications. The 2,2,4-trimethylpentane was evaporated off under vacuum and the residue was dissolved in a few millilitres of light petroleum (b.p. 40–60°). A column was packed with alumina (Merck, Brockman grade, deactivated with 4% of water), 10 g. of adsorbent being used for each 100 mg. of unsaponifiable matter (Mervyn & Morton, 1959), and washed with light petroleum before use. After addition of the sample, the chromatogram was developed by using pure light petroleum, 4%, 6%, 10% and 20% (v/v) of diethyl ether in light