Biochem. J. (1968) **108**, 169
Printed in Great Britain

Some Properties of Galactokinase in Developing Rat Liver

By D. G. WALKER and H. H. KHAN*
Department of Biochemistry, University of Birmingham

(*Received* 1 *December* 1967)

1. The nature of the galactokinase present in the livers of foetal, newborn and adult rats was examined by the application of several separation procedures and by measurement of a range of kinetic parameters. 2. No evidence of enzyme heterogeneity at any stage of development was found during gel filtration on Sephadex G-100, column chromatography on DEAE-cellulose or a variety of electrophoretic procedures. 3. The K_m values, inhibition characteristics and other kinetic parameters appear to remain constant during development. 4. Rat liver galactokinase activity does not adapt to dietary changes in either the adult or the newborn rat; hence it is unlikely that the presence of galactose in milk controls the enzymic activity profile during development. 5. On the present evidence it is concluded that only one form of galactokinase is present in rat liver and that the enzymic activity is controlled by non-dietary factors.

Lactose is essentially the only carbohydrate nutrient in milk ingested by the neonatal animal. In the rat, two β-galactosidases occur in the intestinal mucosa for catalysing the hydrolysis of this lactose and the activity of these enzymes is high during the neonatal period, though there seems to be no correlation between feeding with lactose and induction of the enzyme (Doell & Kretchmer, 1962; Koldovský & Chytil, 1965; Hsia, 1966). The galactose formed is then absorbed and either phosphorylated by galactokinase (ATP–D-galactose 1-phosphotransferase, EC 2.7.1.6) or oxidized by galactose dehydrogenase (D-galactose–NAD oxidoreductase, EC 1.1.1.48) in the liver.

Galactokinase activity in liver tissue was described by Cardini & Leloir (1953) and the enzyme has been partially purified from pig liver (Ballard, 1966*a*). Cuatrecases & Segal (1965) reported that peak maximum activity occurred in rat liver 5 days after birth. Walker, Khan & Eaton (1965–66) found the peak to occur 8 days after birth in their rats and also reported that no significant changes in activity occurred during the late-gestational, neonatal and weaning periods in guinea-pig liver. At an early stage of our investigations Cuatrecases & Segal (1965) also reported evidence that they interpreted as suggesting that two forms of galactokinase may exist in rat liver and that hepatic galactokinase activity responded to dietary changes in young developing rats. These results were at variance with our own preliminary findings (Walker *et al.* 1965–66) and much of the present paper is concerned with an examination of these differences. We have found no evidence of heterogeneity of rat liver galactokinase or of its adaptive behaviour.

* Present address: Department of Chemistry, Government College, Lyallpur, West Pakistan.

MATERIALS AND METHODS

Animals. The rats were an albino Wistar strain, and the normal dietary and weaning regimens and the method for assessing gestational age were as described by Vernon & Walker (1968*a*). Unless otherwise stated, adult animals were normal males about 3 months old. Adult animals to be starved were placed in wire-bottomed individual cages. Alloxan-diabetes was induced as described by Walker & Rao (1964).

Special diets were prepared and administered by using the general procedures described by Walker & Eaton (1967) and Vernon & Walker (1968*b*). Further details about the diets are given in the Results section. For the experiments involving modification of the milk diet of neonatal rats, the pups were removed from their mother at 9a.m., kept in an incubator at 30°, fed by intubation into the oesophagus (Walker & Eaton, 1967) at 3hr. intervals and returned to their mother after the last artificial feed at 9p.m.

Chemicals. ATP (disodium salt), NADH, ADP, phosphoenolpyruvate (sodium salt), galactose 1-phosphate (potassium salt), triethanolamine hydrochloride, pyruvate kinase (EC 2.7.1.40) (125units/mg.) and muscle lactate dehydrogenase (EC 1.1.1.27) (360units/mg.) were from Boehringer Corp. (London) Ltd. (London, W. 5). 2-Deoxy-D-galactose was from Sigma (London) Chemical Co. Ltd. (London, S.W. 6). Bovine plasma albumin was from Koch–Light Laboratories Ltd. (Colnbrook, Bucks.). Galactosamine hydrochloride and all other chemicals were from British Drug Houses Ltd. (Poole, Dorset). Sephadex G-100 was from Pharmacia (G.B.) Ltd. (London, W. 13). DEAE-celluloses (DE22, fibrous, and DE32, microgranular as dry powder) and DEAE-cellulose paper (DE81) were from

Whatman (H. Reeve Angel and Co. Ltd., London, E.C. 4). Starch (hydrolysed) was from Connaught Medical Research Laboratories (Toronto, Ont., Canada). Components of the diets were from the sources given by Walker & Eaton (1967). D-[1-^{14}C]Galactose (2–4mc/m-mole) was from The Radiochemical Centre (Amersham, Bucks.).

Tissue preparations. The use of phosphate buffer as homogenizing medium was found to give higher enzymic activities than the use of media based on KCl or tris buffer. 2-Mercaptoethanol or *N*-acetylcysteine in the medium largely prevented an otherwise rapid loss of activity with time, and the addition of bovine plasma albumin largely prevented a loss of activity due to dilution of the enzyme preparation.

Homogenates (20%, w/v) were prepared as described by Walker (1963) in homogenizing medium containing KH_2PO_4 (50mM), EDTA (5mM), mercaptoethanol (10mM) and bovine plasma albumin (0·1mg./ml.) adjusted to pH 7·5 with N-NaOH. The homogenate was centrifuged at 105000*g* for 1hr. in an MSE Super-Speed 40 centrifuge (8 × 10ml. head) to obtain the supernatant fraction. Bovine plasma albumin was omitted from the homogenizing medium and *N*-acetylcysteine (4mM) was used as the thiol-protecting agent in the kinetic experiments. This modified medium is referred to below as 'phosphate buffer'.

Assay of galactokinase. Both spectrophotometric and radioactivity assay methods for galactokinase were first examined and then used as follows.

(1) Spectrophotometric method. This was similar to that used by Ballard (1966*a*) except that the final pH was 8·0 and the galactose concentration was 1mM. The NaF was omitted when enzyme preparations previously subjected to gel filtration were used.

This assay was used for the studies on substrate specificity in which given concentrations of galactosamine or 2-deoxygalactose replaced the galactose in the above incubation medium. The method was of limited value when applied to the crude supernatant fraction because of the high blank values in the absence of a phosphoryl acceptor, due mainly to adenosine triphosphatase activity; the addition of NaF had only a limited effect in lowering the blank value.

(2) Radioactivity assay. The formation of [1-^{14}C]-galactose 1-phosphate from [1-^{14}C]galactose was measured essentially by the procedure of Sherman & Adler (1963) and Sherman (1963). Unless otherwise stated, the incubation mixture (total volume 0·25ml.) contained: [1-^{14}C]galactose (1mM for all routine assays on crude supernatant fractions and 0·5mM for the kinetic studies, approx. $5{\cdot}5 \times 10^5$ counts/min. in both cases), glycylglycine buffer, pH 8·0 (250mM), *N*-acetylcysteine (4mM), ATP (5mM) and $MgCl_2$ (7·5mM). When kinetic experiments required the use of a range of galactose concentrations the [^{14}C]galactose was diluted in a manner such that the initial radioactivity (counts/min.)/reaction mixture was approximately the same as that given above. Control incubations minus ATP were always performed and treated in the same way. The total initial radioactivity was counted in an identical manner in the spotted area of the DEAE-cellulose paper strips that had not been chromatographed before being dried. The phosphor used contained 4g. of 2,5-diphenyloxazole and 100mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l. of reagent-grade toluene.

All enzyme activities calculated from the radioactivity in the galactose 1-phosphate area were corrected for both zero-time controls and the blank incubations containing no ATP. Activities, expressed in μmoles of galactose 1-phosphate formed/min., were measured at two different enzyme concentrations and two different incubation times. The mean of these four rates, which agreed to within ±5%, was used for the calculation; the maximum conversion of galactose into galactose 1-phosphate was 17% and the rate of galactose phosphorylation was linear under these conditions.

Gel filtration and column chromatography. (1) Gel filtration. Sephadex G-100 was suspended in 'phosphate buffer' [containing KH_2PO_4 (50mM), EDTA (5mM) and *N*-acetylcysteine (4mM) and adjusted to pH 7·5 with NaOH] and poured into a column to give a bed size 32cm. × 3·5cm. Crude liver supernatant (3ml.) from a 50% (w/v) homogenate was washed into the column, which was then eluted with more 'phosphate buffer'. The contents of the tube containing maximum galactokinase activity were used for many of the kinetic studies. In some cases the contents of all the tubes containing galactokinase activity were combined and applied to a DEAE-cellulose column.

(2) Column chromatography. Chromatography on DEAE-cellulose was performed with either the fibrous (DE22) or the microgranular (DE32) form. The bed sizes, flow rates and volumes of the fractions collected for DE22 and DE32 DEAE-cellulose columns were respectively 16cm. × 3·5cm. and 25cm. × 3·5cm., 2ml./min. and 1ml./min., and 6ml. and 8ml. The pooled samples containing galactokinase from gel filtration above were applied to the column and washed into the exchanger with 150ml. of 'phosphate buffer'. The galactokinase was then eluted with 600ml. of a linear gradient of 'phosphate buffer' containing 0–0·6M-KCl.

Electrophoretic procedures. (1) Horizontal electrophoresis on starch gel. This was performed by standard procedures in 10mM-tris buffer containing $MgCl_2$ (4mM), EDTA (4mM) and *N*-acetylcysteine (4mM) and adjusted to either pH 8·0 or 7·0. Various running times ranging from 2 to 18hr. at a voltage of 5v/cm. length and 0·4mA/cm. width of gel were tested. Various concentrations of barbitone and imidazole buffers (10–100mM) over a range of pH values (7·0–8·6) were also tried. After electrophoresis, the gel was either stained for protein or treated to detect enzymic activity by immersing it in a medium at 37° containing (final concentrations): triethanolamine buffer, pH 8·0 (100mM), galactose (1mM), ATP (5mM), $MgCl_2$ (7·5mM), KCl (100mM), phosphoenolpyruvate (0·75mM), NADH (0·15mM), lactate dehydrogenase (25μg./ml.) and pyruvate kinase (50μg./ml.). A blank containing no galactose was performed on an identical gel strip. The gel was examined several times during the course of about 2hr. by viewing under a u.v.-light source.

(2) Electrophoresis on cellulose acetate strips. This was attempted with a range of tris, barbitone and imidazole buffers similar to those employed in the starch-gel electrophoretic studies over a range of pH values (7·0–9·0). ADP-forming enzymic activity was detected as described above.

RESULTS

Properties of galactokinase in rat liver at various ages

The activity–age relationship in developing rat liver determined by using the radioactivity assay

method and 1mM-galactose showed (Walker *et al.* 1965–66) that the activity increases during late gestation to about the adult level at birth; this increase continues during the first week after birth to a maximum at 8 days, and thereafter the activity slowly decreases, reaching adult levels at about 35 days of age. Because of the extremely limited amount of liver tissue available from the neonatal rat no attempt was made to prepare a quantity of partially purified galactokinase from livers of rats of different ages, but it was decided rather to examine certain properties including kinetic parameters in fresh tissue preparations from a significant number of animals at various stages of development.

Basic properties. Preliminary experiments with both the radioactivity and spectrophotometric assay methods revealed (*a*) that the optimum pH at all ages was pH 8·0 and that there was a rapid decrease in activity either side of this value, (*b*) that the Mg^{2+}/ATP ratio could be varied over the range 1–5:1 with no significant variation of activity and only higher ratios resulted in inhibition of galactokinase activity by excess of Mg^{2+}, and (*c*) that inhibition by excess of substrate occurred above about 2mM-galactose but that no such inhibition was observable with 1mM-galactose. These results applied to supernatant preparations of livers of foetal, neonatal and adult rats. The routine assay procedure was therefore standardized as described in the Materials and Methods section and the next studies were designed to apply several separation techniques to the liver supernatant preparations to seek evidence for enzyme heterogeneity.

Gel filtration. Passage of the supernatant fraction of liver homogenates through the Sephadex G-100 column permitted the galactokinase activity to be freed of all the hexokinase and most of the adenosine triphosphatase activity. Essentially identical results, similar to that shown by Ballard (1966*a*; Fig. 2*a*), were obtained with liver preparations of neonatal (1–3 days and 8 days) and adult rats. In nine separate experiments, with animals ranging from a gestational age of 21 days to adult (male and female) and with the same column of Sephadex G-100 under an identical set of conditions, the elution volumes at which peak colour or activity was found were always within the following ranges: blue dextran, 66–68ml.; hexokinase, 95–96ml.; adenosine triphosphatase activity, 115–116ml.; haemoglobin, 130–134ml.; galactokinase, 132–134ml. The only differences in the elution patterns were in the heights of the peaks. The preparations of adult livers showed, in addition, another peak in later tubes corresponding to the high-K_m glucokinase (EC 2.7.1.2). The galactokinase elution profile was virtually superimposed on that of haemoglobin and corresponded to mol.wt. about 70000, in agreement with the result of Ballard (1966*a*) for pig liver galactokinase. The total recovery of galactokinase activity after gel filtration was always over 80% of that applied to the column.

Chromatography on DEAE-cellulose columns. The combined fractions containing galactokinase activity from gel filtration on Sephadex G-100 were applied to columns of both fibrous and microgranular DEAE-cellulose as described in the Materials and Methods section. Galactokinase activity was eluted in a single peak with no signs of heterogeneity by the increasing potassium chloride gradient in a manner similar to that shown by Ballard (1966*a*; Fig. 2*b*). In five experiments (starting with liver from an adult male, an adult female and 8-day-old, 2-day-old and foetal rats) with a column of the fibrous form of exchanger (DE22) under as near identical conditions as possible, the peak galactokinase activity was found at an elution volume of 316 ± 6ml. (mean ± S.E.M.). In four experiments (on liver from an adult female and 8-day-old, 3-day-old and foetal rats) with the granular form (DE32) similarly the range of elution volumes for peak galactokinase activity was 410 ± 11ml. The difference in elution volumes between the two sets of experiments was due to the difference in size of the columns (see the Materials and Methods section). Galactokinase activity was eluted over the concentration range between $0{\cdot}12 \pm 0{\cdot}01$M- and $0{\cdot}27 \pm 0{\cdot}02$M-potassium chloride from both columns. The recovery of galactokinase activity in terms of that initially applied to the Sephadex G-100 column was 40–57%.

Electrophoretic experiments. Both starch gel and cellulose acetate strips were used with a range of different buffers at various pH values as described in the Materials and Methods section. Detection of galactokinase activity, which depends on detection of ADP formation by coupling enzymically to the oxidation of NADH, was not too satisfactory owing to the interference that could be seen in blanks in which galactose was omitted from the medium used to detect activity. It was clear, however, that under all these conditions galactokinase activity migrated only a very small distance (not more than 1cm.) from the line of application. There was no evidence of heterogeneity when the material applied to the supporting medium was either the crude supernatant fraction or the tube containing peak galactokinase activity after gel filtration as described above. Livers from rats of many ages were examined in these ways, all with negative evidence for heterogeneity.

Kinetic parameters of galactokinase in rat liver at various ages

Most of the results described below were obtained on the galactokinase activity present either in the

Table 1. *Affinities for substrates of rat liver galactokinase*

Enzyme activity was determined by the radioactivity method as described in the text and with either the crude supernatant fraction or the tube containing peak galactokinase activity after gel filtration through a Sephadex G-100 column. Adult animals were males 3 months old, neonatal animals were 2–5 days old and the foetal animals were of gestational age 21–22 days. The apparent K_m values were obtained by extrapolation of Lineweaver–Burk double-reciprocal plots. Results are given either individually (where only two determinations were made) or as means ± S.E.M. with the numbers of separate observations in parentheses.

Type of enzyme preparation	Type of animal	K_m for galactose (mM)	K_m for ATP (mM)
Crude supernatant	Adult	0·14 ± 0·01 (6)	0·10, 0·08
	Neonatal	0·15 ± 0·01 (4)	0·13, 0·12
	Foetal	0·14 ± 0·01 (4)	0·07, 0·10
Peak tube from gel filtration	Adult	0·16 ± 0·01 (10)	0·08, 0·12
	Neonatal	0·14 ± 0·02 (7)	0·11 ± 0·02 (3)
	Foetal	0·14 ± 0·02 (6)	0·09, 0·10

Table 2. *Substrate specificity of rat liver galactokinase*

Enzyme activity was determined by the spectrophotometric method as described in the text and with as source of enzymic activity the tube containing peak galactokinase activity after gel filtration through a Sephadex G-100 column. Both K_m and $V_{max.}$ were determined by extrapolation of Lineweaver–Burk double-reciprocal plots; this extrapolation was necessary because substrate inhibition was observed above 1 mM-galactose, approx. 10 mM-galactosamine or approx. 10 mM-2-deoxygalactose. Values for $V_{max.}$ are expressed relative to that for galactose (= 100). The phosphorylation coefficient is defined (Sols & Crane, 1954) thus:

$$\text{Phosphorylation coefficient} = \frac{V_{max.}\ (\text{substrate})}{K_m\ (\text{substrate})} \times \frac{K_m\ (\text{galactose})}{V_{max.}\ (\text{galactose})}$$

Results are the means of two observations on different enzyme preparations. The ages of the animals were as given in Table 1.

Type of animal	Substrate	K_m (mM)	Relative $V_{max.}$	Phosphorylation coefficient
Adult	Galactose	0·135	(100)	1
	Galactosamine	1·53	118	0·104
	2-Deoxygalactose	1·43	150	0·142
Neonatal	Galactose	0·108	(100)	1
	Galactosamine	1·38	114	0·089
	2-Deoxygalactose	1·55	154	0·107
Foetal	Galactose	0·130	(100)	1
	Galactosamine	1·66	127	0·100
	2-Deoxygalactose	1·90	152	0·104

crude supernatant fraction of liver homogenates or in the tube containing peak activity after gel filtration on Sephadex G-100. Preparations were made fresh for each experiment. For gel filtration the animals were killed and the homogenates and supernatants prepared in the evening and the filtration was allowed to proceed overnight; the kinetic studies were then made the next morning.

Affinity of substrates. No inhibition by excess of galactose was observable up to 1 mM-galactose. Marked substrate inhibition was seen with all liver preparations above 2 mM-galactose, but no essential differences between the concentrations giving rise to this inhibition were ever noted between enzyme preparations of rats of different ages. Table 1 gives no indication of variation of the apparent K_m values depending on the age of the animal. Four determinations of the K_m for galactose with the galactokinase present in the tube containing peak activity after DEAE-cellulose (DE 32) chromatography gave the following results (mM): adult, 0·12, 0·16; neonatal, 0·13; foetal, 0·13.

Substrate specificity. Hepatic galactokinase catalysed the phosphorylation of galactosamine and 2-deoxygalactose (cf. Ballard, 1966*a*) as well as galactose, and the variations between the results in Table 2 for substrate specificity are all within the limits that can reasonably be expected from experiments with different enzyme preparations that are not highly purified. No consistent variations depending on the age of the animal were seen.

Product inhibition. The inhibition patterns due

Table 3. *Product inhibition of rat liver galactokinase*

Enzyme activity was determined by the radioactivity assay method as described in the text and with sources of enzymic activity similar to those used in Table 1. Values for K_i were determined from Lineweaver–Burk double-reciprocal plots. Results are given either individually or as means ± S.E.M., and the ages of the animals were all as given in Table 1.

Inhibition constant		K_i for galactose 1-phosphate (mM)		K_i for $MgADP^-$(mM)
Varying substrate		Galactose	$MgATP^{2-}$	Galactose
Type of inhibition		Non-competitive	Non-competitive	Non-competitive
Type of enzyme preparation	Type of animal			
Crude supernatant	Adult	5·6, 5·2	6·4, 7·0	23, 19
	Neonatal	4·5, 5·0	5·2 ± 0·6 (3)	21, 21
	Foetal	4·2 ± 0·1 (3)	4·0, 4·5	19, 18
Peak tube from gel filtration	Adult	5·4, 7·0	8·0	23
	Neonatal	6·0	6·0, 3·9	22·0 ± 0·6 (3)
	Foetal	7·0	5·7	23·5 ± 0·4 (3)

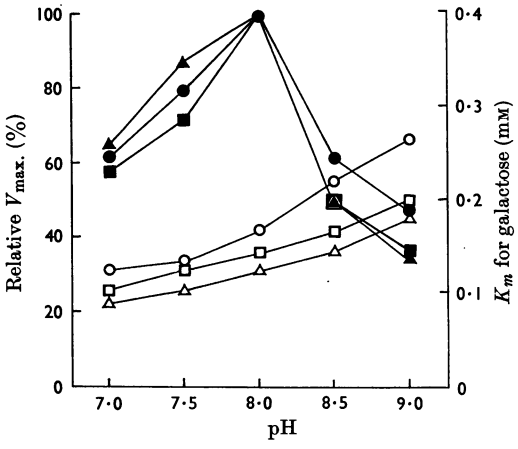

Fig. 1. Effect of pH on the relative $V_{max.}$ (●, ▲ and ■) and K_m for galactose (○, △ and □) of the galactokinase activity in preparations of adult, neonatal and foetal rat liver. A portion (20 µl.) of the peak tube from gel filtration on Sephadex G-100 of the supernatant fraction, prepared as described in the text, was used in each case. Velocities were measured by the radioactivity method and both $V_{max.}$ and K_m were determined by Lineweaver–Burk double-reciprocal plots. The relative $V_{max.}$ values were then calculated as percentages of the activity determined at pH 8·0. ○ and ●, adult; △ and ▲, neonatal; □ and ■, foetal.

to both $MgADP^-$ and galactose 1-phosphate were found to be very similar to the results of Ballard (1966*b*) for the more purified pig liver galactokinase. The results in Table 3 indicate no differences in the apparent K_i values for the galactokinase present in liver tissue at various stages of development.

Effect of pH *on* $V_{max.}$ *and* K_m. Both of these parameters were deduced from studies in which results were plotted in the form of Lineweaver–Burk double-reciprocal plots and with galactose concentrations between 0·1 and 1 mM. The optimum pH was 8·0 in all cases, and Fig. 1 again shows that, within the limits of normal variation, there were no differences between the enzyme preparations of animals of different ages.

Physiological aspects

Table 4 presents the activities of hepatic galactokinase in adult rats under various conditions. On a basis of activity/g. wet wt. no significant differences between the groups were seen and only in alloxan-diabetic animals and those starved for 2 or more days did the specific activity (per 100 mg. of supernatant nitrogen) decrease. In other experiments (not shown) groups of adult rats were fed on diets containing 20%, 40% or 60% (w/w) of galactose for 3 or 5 days. On comparison with control animals fed similarly but with glucose instead of galactose or with animals fed on the normal diet, no statistically significant differences in hepatic galactokinase activities expressed on several different bases were recorded (for details see Khan, 1967). Cuatrecases & Segal (1965) reported slightly increased liver galactokinase activity after 20 (but not 5) days on a 40%-galactose diet. Our results for adult animals are therefore in agreement with those authors, but our results for young growing rats are different as follows.

A series of experiments was performed on immature rats in which animals were fed on various liquid (Expts. 1–4) or solid (Expts. 5–8) diets (for details see Walker & Eaton, 1967; Vernon & Walker, 1968*b*) for the given periods before they were killed and enzyme assays performed. The results, none of which showed any effects of galactose treatment, are given in detail elsewhere (Khan, 1967) and summarized here.

Table 4. *Effect of sex, age, starvation and alloxan-diabetes on the galactokinase activities in adult rat liver*

Activities were measured in the supernatant fraction of liver homogenates by the radioactivity method as described in the text. Results are given as means ± S.E.M. All the animals except those 2 years old weighed 200–250g. before any treatment.

	No. of observations	Galactokinase activity (μmoles/min.) (per g. wet wt.)	(per 100mg. of supernatant N)
Adult, males	10	0·85±0·09	6·2±0·16
Adult, normal females	6	0·80±0·02	6·0±0·18
Adult, pregnant females	6	0·79±0·02	5·4±0·17
Adult, lactating females	3	0·78±0·02	5·3±0·10
Adult, males, starved for 1 day	3	0·90±0·03	5·8±0·40
Adult, males, starved for 2 days	3	0·87±0·02	4·9±0·17
Adult, males, starved for 3 days	3	0·80±0·03	4·5±0·15
Adult, males, alloxan-diabetic	3	0·84±0·07	4·3±0·17
Adult, males, 2 years old	4	0·73±0·04	5·7±0·10

Expt. 1. Half of the litter were removed from the mother during the daytime and fed on a liquid diet (Walker & Eaton, 1967) containing 20% of galactose; the duration was from day 1 to day 5 after birth. Feeding with galactose did not enhance the normal rapid increase in galactokinase activity over that period.

Expt. 2. This was as described for Expt. 1, but for the period 8–12 days after birth.

Expt. 3. This was as described for Expt. 2, except that the liquid diet contained 10% of galactose and the period was 8–16 days after birth.

Expt. 4. This was as described for Expt. 3, except that the liquid diet contained 15% of galactose and the period was 9–17 days after birth.

Expt. 5. Suckling rats were weaned when 18 days old and fed on a diet consisting of 40% of galactose + 60% of powdered rat cake or 40% of glucose + 60% of powdered rat cake, and a second control group were left to wean in the presence of their mother on to normal rat cake. During the experimental period of 18–26 days of age, the average gains in body weight in the three groups were 3, 13 and 35g. respectively, but the galactokinase activities per g. wet wt. of liver or per 100g. body wt. were not different and the activities per 100mg. of supernatant nitrogen showed that feeding with the galactose diet did not prevent the normal decrease in liver galactokinase activity during that time.

Expt. 6. This was as described for Expt. 5, except that suckling rats were weaned when 16 days old and killed for assay when 24 days old.

Expt. 7. Weanling rats were removed from the mother when 24 days old and fed on a 40%-galactose or 40%-glucose diet; these diets were prepared as described by Walker & Eaton (1967). The animals were killed and liver galactokinase assays performed when 36 days old.

Expt. 8. This was as described for Expt. 7, except that the animals were completely weaned when 20 days old and killed for assay 10 days later.

DISCUSSION

Nature of galactokinase. Because of the extremely small quantities of liver tissue in the foetal and neonatal rat, insufficient starting material was available for as much purification to be achieved as in the detailed study by Ballard (1966*a*,*b*) on the pig liver enzyme. Gel filtration separated the galactokinase activity from that of the other hexokinases and from most of the adenosine triphosphatase activity, thus excluding any possible interference from such enzymes.

No evidence of heterogeneity of the galactokinase present in foetal, neonatal and adult rat liver was noted during gel filtration on Sephadex G-100, during chromatography on DEAE-cellulose (which separates, for example, the isoenzyme forms of mammalian hexokinase; González, Ureta, Sánchez & Niemeyer, 1964; Grossbard & Schimke, 1966) or during the wide range of conditions and supporting media under which electrophoresis was used. The recovery of enzymic activity after the column operations was always sufficiently high to make loss of a labile form of galactokinase an unlikely if not impossible occurrence. Ballard (1966*a*) observed no signs of enzyme heterogeneity of the pig liver enzyme during similar procedures.

The kinetic parameters of the galactokinase in foetal, neonatal and adult rat liver were qualitatively similar to the results of Ballard's (1966*a*,*b*) studies on pig liver galactokinase and to the K_m values for galactose and ATP of adult rat liver galactokinase reported by Cuatrecases & Segal (1965). Our results differ from the latter authors

(who used the supernatant fraction without further treatment) in that, first, they obtained much higher K_m values for galactose in newborn (0·65mM) and foetal (0·91mM) rat livers, whereas we found no difference with the age of the animal, and, secondly, we have found no altered inhibition characteristics, e.g. by galactose 1-phosphate on the enzyme of immature liver. Our results argue against the presence of more than one form of hepatic galactokinase as suggested by Cautrecases & Segal (1965) to explain their results. This does not rule out the possibility, of course, that other separation procedures or analytical methods might not reveal signs of heterogeneity, but our evidence suggests that the measurable changes in activity around birth are concerned with one molecular form only.

Physiological aspects. Not dissimilar developmental profiles to that of hepatic galactokinase (Cuatrecases & Segal, 1965; Walker *et al.* 1965–66) have been reported for both UDP-glucose–α-D-galactose 1-phosphate uridylyltransferase (EC 2.7.7.12) (Bertoli & Segal, 1966) and galactose dehydrogenase (Cuatrecases & Segal, 1966) in rat liver. These activity increases in the neonatal rat permit utilization of the galactose present in milk (as lactose). It is unlikely that this galactose controls the enzymic activity because, first, galactokinase activity in adult rats is essentially non-adaptative and changes occur only in extreme conditions under which the activities of a wide spectrum of enzymes also alter. Secondly, the increase in galactokinase activity commences several days before birth in the rat (Cuatrecases & Segal, 1965; Walker *et al.* 1965–66), i.e. before exposure to galactose occurs. Thirdly, the hepatic galactokinase activity in neonatal and weanling rats has been shown not to be influenced by dietary modifications of a type that, by comparison with the case of several other adaptive enzymes (Walker & Eaton, 1967; Vernon & Walker, 1968*b*), should be likely to cause marked changes. The changes that occur around birth and during the first postnatal month must be controlled primarily by mechanisms other than the diet itself. In this respect galactokinase is similar to the intestinal β-galactosidases.

We thank the Medical Research Council for a grant towards the expenses of this research.

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