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Glycerol Metabolism in the Neonatal Rat

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1. The possible role of glycerol as a precursor in neonatal gluconeogenesis in the rat was investigated by recording the activities of glycerol kinase and L-glycerol 3-phosphate dehydrogenase in the liver, kidney and other tissues around birth and during the neonatal period. 2. Blood glycerol concentrations in the neonatal rat are high. 3. There is a marked increase after birth in the ability of both liver and kidney slices to convert glycerol into glucose plus glycogen that correlates with the increase in glycerol kinase activity. 4. High hepatic and renal L-glycerol 3-phosphate dehydrogenase activities are also found in the neonatal period. 5. The marked capacity for neonatal gluconeogenesis from glycerol thus demonstrated and the role of glycerol kinase in its control are discussed.

The stores of glycogen that accumulate in the liver of the rat before birth are rapidly exhausted during the first day *post partum* (Dawkins, 1966). At the same time there is a rapid increase in the capacity of the liver to synthesize glucose from amino acids and other precursors (Ballard & Oliver, 1963; Yeung & Oliver, 1967; Vernon, Eaton & Walker, 1968); the glucose that may thus be formed could supplement the low content of carbohydrate in milk (Dymsza, Czajka & Miller, 1964). Although gluconeogenesis from amino acids is thought to be an important source of glucose in the adult during starvation (Krebs, 1964), the significance of this process in the neonatal animal is uncertain because of the considerable requirement for amino acids in protein synthesis (Hahn, Koldovský, Křeček, Martínek & Vacek, 1961) during this period of very rapid growth.

During the suckling period there is an increased rate of hepatic fatty acid oxidation (Taylor, Bailey & Bartley, 1967) and both endogenous lipid stores and milk with its high fat content provide an abundant supply of substrates. Triglycerides also contain up to about 8% by weight of glycerol, and it has been suggested that glycerol formed during lipolysis may be an important precursor of glucose in the adult during starvation (Steinberg, 1963; Owen *et al.* 1967). In the present work the possible role of glycerol as a precursor of glucose in the developing rat was investigated. That there is an increase in hepatic glycerol utilization for gluconeogenesis in the neonatal period was briefly noted earlier (Vernon *et al.* 1968).

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MATERIALS AND METHODS

Animals. The rats were albino Wistar strain; the normal dietary and weaning regimens and the method for assessing gestational age have been described (Vernon & Walker, 1968). Adult animals were about 3 months old and weighed approx. 200g. All animals were killed between 9 and 11 a.m.

Materials. ATP and glucose oxidase (EC 1.1.3.4) (type II) were obtained from Sigma (London) Chemical Co. Ltd. (London S.W.6, U.K.) and NADH, dihydroxyacetone phosphate (dimethylketal dicyclohexylammonium salt), glycerol kinase, peroxidase and L-glycerol 3-phosphate dehydrogenase from Boehringer Corp. (London) Ltd. (London W.5, U.K.). Dihydroxyacetone phosphate (sodium salt) was prepared from the above derivative by the method described by the manufacturers; solutions of the sodium salt were prepared fresh weekly and stored frozen.

[1-^{14}C]Glycerol was obtained from The Radiochemical Centre (Amersham, Bucks., U.K.). Agidex, a commercial glucoamylase preparation, was a gift from Glaxo Laboratories Ltd. (Ulverston, Lancs., U.K.). DEAE-cellulose paper (DE 81) and Whatman No. 1 chromatography paper were from H. Reeve Angel and Co. Ltd. (London E.C.4, U.K.).

Blood glycerol assay. Whole blood was diluted with 4vol. of 4mM-NaF; this suspension was deproteinized by the method of L. Weiss (personal communication quoted by Wieland, 1963). Glycerol was determined in the supernatant by the method of Wieland (1963).

Measurement of net gluconeogenesis in kidney slices. Slices weighing approx. 20mg were cut free-hand with a razor blade from the kidney cortex of 5-day-old and older rats or from whole kidney in the case of younger animals. The animals were neither starved nor treated with phlorrhizin before being killed. Net gluconeogenesis from glycerol (10mM) was measured as described previously (Vernon *et al.* 1968).

Measurement of gluconeogenesis in liver slices by isotope incorporation. The preparation of liver slices and the incubation conditions were as described previously (Vernon *et al.* 1968) except that 10mM-glycerol was used as substrate. The reaction was stopped and a homogenate in 0.27M-$HClO_4$ was prepared as before. Samples (0.5ml) were taken, the pH was adjusted to approx. 4.0 and the volume to 0.75ml; 0.05ml of 1M-sodium acetate buffer, pH4.4, and 0.2ml of Agidex (5mg/ml) were added and the glycogen was allowed to hydrolyse to glucose by incubation at 50°C for 1h. The whole digest was evaporated to dryness under reduced pressure and redissolved in 1.0ml of methanol. Samples (0.1ml) of the latter were spotted on to Whatman No. 1 paper strips (5cm×36cm). Separation of glucose and glycerol was obtained by descending chromatography for 24h with propan-1-ol–ethyl acetate–water (7:2:1, by vol.), glycerol having R_{Glc} 3.0. After development the chromatograms were cut into 2cm strips and counted at about 56% efficiency in a Nuclear-Chicago Automatic Scintillation Spectrometer (model 725). 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.

The rate of glucose formation was calculated assuming that the incorporation of two molecules of glycerol into glucose was equivalent to the formation of one molecule of glucose.

Enzyme assays. Tissue homogenates (20%, w/v) of liver and kidneys were prepared in 0.15M-KCl containing 1mM-EDTA, pH5.3. Homogenates of whole small intestine were made after the removal of undigested food material. The small intestine from 22-day-old and older rats was cut into strips of 3–5cm before homogenization; complete homogenization of the outer muscular wall was not obtained in the case of the adult, but the whole of the mucosa was extruded into medium. Enzyme assays were performed on the supernatant preparations obtained by centrifugation of the homogenates at 100 000*g* for 40min.

Glycerol kinase (EC 2.7.1.30) was assayed by the method of Newsholme, Robinson & Taylor (1967) except that the radioactivity of the [1-^{14}C]glycerol in the assay mixture was 1μCi/ml and the incubation temperature was 30°C. The assay mixture was preincubated for 2min and the reaction was started by the addition of 0.01ml of supernatant for the liver or 0.02ml for the other tissues. The reaction was terminated after 10min by the addition of 0.1ml of ethanol and by cooling in ice. The L-glycerol 3-phosphate formed was separated from the unchanged glycerol by chromatography on DEAE-cellulose paper (Sherman, 1963); the radioactivity of the resulting strip containing the L-glycerol 3-phosphate was counted at about 56% efficiency in the spectrometer described above. Preliminary experiments with rats of different ages indicated that the loss of glycerol kinase activity during the time that elapsed in routine experiments between the killing of the animals and the assay was always less than 10% of the total activity and that essentially all the glycerol kinase activity was present in the supernatant fraction. An ATP-regenerating system was found not to be necessary.

The assay for L-glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) was based on the method of Beisenherz *et al.* (1953). The incubation mixture contained (total vol. 1.0ml) 100mM-tris–HCl (pH7.3), 16mM-*N*-acetylcysteine, 0.15mM-NADH and 0.5mM-dihydroxyacetone phosphate. The mixture was preincubated for 3min at 30°C and the reaction was started by the addition of 0.05ml of supernatant that had been suitably diluted with homogenizing medium. The change in E_{340} was measured on a Gilford Model 2000 Recording Spectrophotometer.

One unit of enzyme activity is defined as 1μmol of substrate utilized per min and specific activities as units of enzyme activity per 100mg of supernatant protein.

RESULTS

There is a rapid increase in rat blood glycerol concentration immediately after birth, followed by a gradual decline (Fig. 1); this is similar to the changes observed in man (Novák, Hahn, Koldovský & Melichar, 1964). The capacity to incorporate [^{14}C]glycerol into glucose plus glycogen in liver slices also increases during this period from a minimum value in the near-term foetus to a maximum in the adult male (Fig. 2). This developmental pattern is similar to that previously observed when the rate of net synthesis of glucose plus glycogen from glycerol was measured in liver slices from starved phlorrhizin-treated rats (Vernon *et al.* 1968). The capacity for synthesis of glucose plus glycogen from glycerol in kidney slices also increases during the neonatal period (Fig. 2), but, at all ages examined, the rate was lower than that in liver slices.

Enzyme activities. The specific activity of hepatic glycerol kinase showed a fourfold increase during the 48h after birth, whereas that of the kidney-cortex enzyme showed a slower increase that continued until the fifteenth day *post partum* (Fig. 3*a*). When expressed per 100g body wt., the glycerol kinase activity of both tissues paralleled the ability of slices from these tissues to convert glycerol into glucose plus glycogen (Fig. 3*b*). The correlation between glycerol kinase activity and the rate of carbohydrate formation from glycerol was found to be significant in each case, the correlation co-

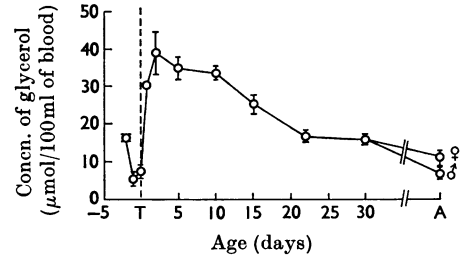

Fig. 1. Glycerol concentration of rat blood as a function of age. Each point represents the means of three to five determinations and the vertical bar shows ±S.E.M. when large enough to record. Details of the method are given in the text. T, Term; A, adult.

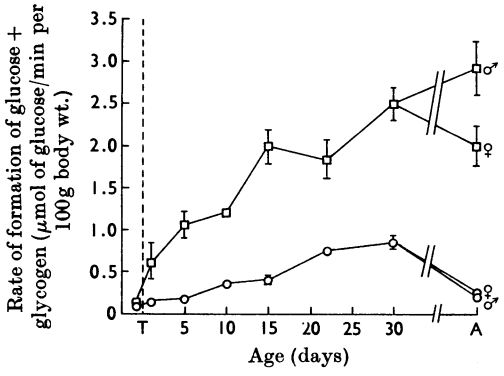

Fig. 2. Rate of formation of glucose plus glycogen from glycerol in rat liver slices (□) and kidney slices (○) as a function of age. The substrate concentration was 10mM in each case. Further details are given in the text. The data for liver slices are based on the use of [^{14}C]glycerol whereas those for kidney slices are based on the net-synthesis experiments. Each point represents the mean of from four to seven determinations and the vertical bar shows ±S.E.M. when large enough to record. T, Term; A, adult.

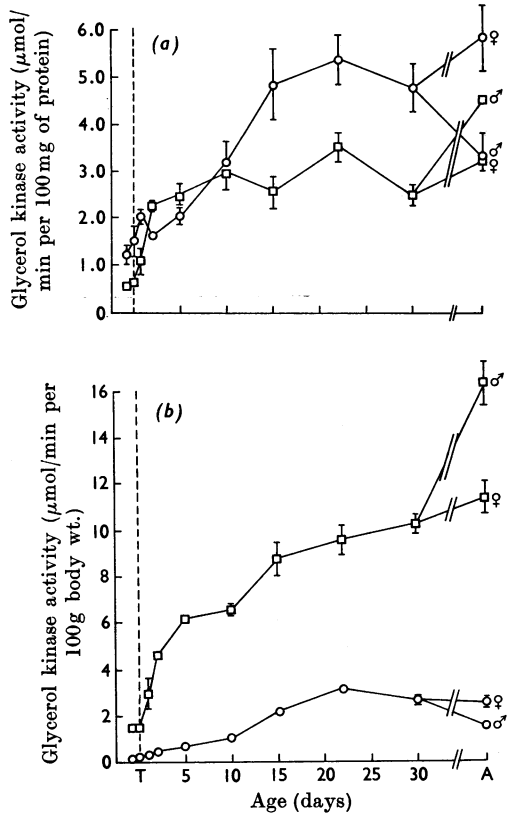

Fig. 3. Activity of glycerol kinase in the rat liver (□) and kidney (○) supernatant fraction as a function of age expressed (*a*) per 100mg of protein and (*b*) per 100g body wt. Each point represents the mean of four determinations and the vertical bar shows ±S.E.M. when large enough to record. Details of the assay procedure are given in the text. T, Term; A, adult.

efficient being 0.99 ($P<0.001$) for liver and 0.87 ($P<0.01$) for kidney.

A tissue survey for glycerol kinase activity in the adult male and in the 5-day-old rat confirmed that there is activity in the small intestine (Haessler & Isselbacher, 1963) and in brown adipose tissue (Treble & Ball, 1963). The method of assay was sufficient to detect an activity of 0.02μmol of L-glycerol 3-phosphate formed/min per g wet wt. of tissue, but no significant activity was found in white adipose tissue, brain, lung, spleen and skeletal and cardiac muscle of rats of both ages, and in testis, pancreas and kidney medulla of the adult rat, thus confirming the observations of Wieland & Suyter (1957), who used a less sensitive assay system. Glycerol kinase activities of about 0.01μmol/min per g wet wt. of tissue have been reported in cardiac muscle and white adipose tissue (Robinson & Newsholme, 1967). The physiological significance of these very low activities is uncertain; when expressed per 100g body wt. they add up to less than 1% of the total liver enzyme activity.

The development profiles of the specific activities of glycerol kinase in intestinal and brown adipose tissue are shown in Fig. 4. When expressed per 100g body wt., the contribution of the brown-adipose-tissue enzyme to the total body glycerol kinase activity is always less than 10% of the total body activity at all ages examined. The peak in activity in the neonatal period can explain the high rate of glycerol incorporation into triglyceride glycerol at this time described by Hahn & Greenburg (1968). The contributions of the liver and kidney enzymes to the total body activity per 100g body wt. is about 70% in the newborn rat and increases to over 95% in the adult animal.

The changes in the specific activity of L-glycerol 3-phosphate dehydrogenase of the liver and kidney are shown in Figs. 5(*a*) and 5(*b*); adult values are reached between the second and fifth day *post partum*. The activity of this enzyme in the brown adipose tissue and intestine was at least as great as that of the adult male at all ages examined. In all the four tissues examined and at all ages, the specific activity of L-glycerol 3-phosphate dehydrogenase was found to be greater than that of glycerol kinase.

Sex differences. Neither the blood glycerol concentrations nor the rates of conversion of glycerol into glucose in kidney and liver slices from adult males and females differed significantly.

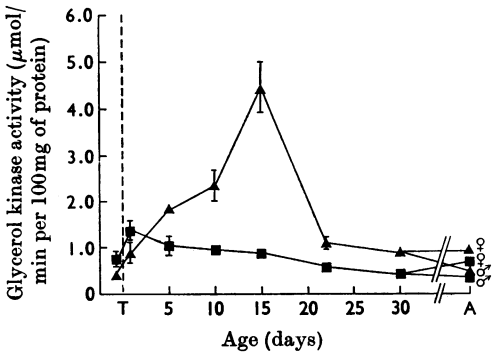

Fig. 4. Activity of glycerol kinase in the rat intestine (■) and brown-adipose-tissue (▲) supernatant as a function of age. Each point represents the mean of four determinations and the vertical bar shows ±S.E.M. when large enough to record. Details of the assay procedure are given in the text. T, Term; A, adult.

Certain sex differences in adult rats (but not in neonatal and weaning rats up to 30 days old) have been noted. Hepatic glycerol kinase activity was significantly greater in the adult male than in the female ($P<0.01$), but the activity in the kidney, intestine and brown adipose tissue was significantly higher ($P<0.05$ or better) in the female (Figs. 3*a* and 3*b*). The activity of L-glycerol 3-phosphate dehydrogenase of the kidney was significantly greater ($P<0.05$) in the female (Fig. 5*b*).

DISCUSSION

Evidence to date suggests that glycerol transport into the cell is not a rate-limiting process in glycerol utilization (Cahill, Ashmore, Renold & Hastings, 1959; Larsen, 1963; Shafrir & Gorin, 1963; Hayashi & Lin, 1965). Of the three enzymes known that could have a role in glycerol utilization only glycerol kinase (K_m for glycerol of 35.0×10^{-6}M to 3.1×10^{-6}M for the hepatic enzyme; Grunnet & Lundquist, 1967*a*; Robinson & Newsholme, 1969*a*) is likely to make a significant contribution because the K_m values for glycerol of glycerol dehydrogenase (EC 1.1.1.6) (0.63M; Moore, 1959) and glycerol pyrophosphate phosphotransferase (3M; Stetten & Rounbehler, 1968) are very high. Robinson & Newsholme (1969*a*) have summarized the evidence suggesting that the glycerol kinase reaction is also a rate-controlling step in glycerol utilization, and there is no evidence in the present results to discredit this view for animals of all ages.

The glycerol concentration in adult rat liver, 0.23mM (Robinson & Newsholme, 1969*a*), is much greater than the K_m for glycerol of glycerol kinase, so that the rate of glycerol phosphorylation should

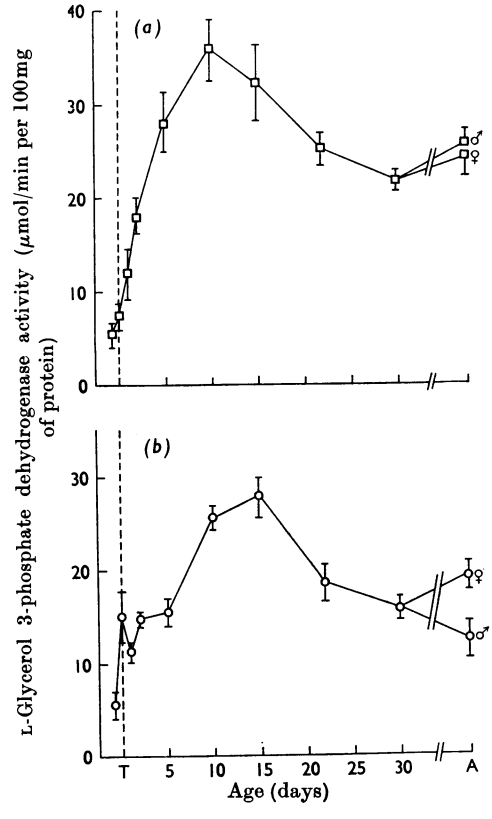

Fig. 5. Activity of L-glycerol 3-phosphate dehydrogenase in (*a*) rat liver and (*b*) rat kidney supernatant as a function of age. Each point represents the mean of four determinations and the vertical bar shows ±S.E.M. Details of the assay procedure are given in the text. T, Term; A, adult.

be independent of changes in glycerol concentration within the physiological range. Robinson & Newsholme (1969*b*), however, using kidney-cortex slices found that the K_m for glycerol utilization was approx. 1.0mM with respect to the extracellular glycerol concentration; their more limited results obtained with liver slices indicated a similar apparent K_m for glycerol utilization in this tissue. As this value is greater than the concentration in plasma, and hence almost certainly higher than that in the hepatic and renal extracellular fluids at all ages also, the rate of glycerol utilization is likely to be dependent on the glycerol concentration. In their studies on the perfused rat liver, Exton & Park (1967) found that decreasing the glycerol concentration of the perfusate from 10mM to 0.3mM resulted in a decrease in the conversion of glycerol into glucose. Glycerol utilization *in vivo*

has also been shown to be dependent on glycerol concentration in the rabbit (Hagen, 1968), the cat (Larsen, 1963) and the sheep (Bergman, 1968).

If the activity of hepatic glycerol kinase *in vivo* is independent of the glycerol concentration, the ability of the liver and kidney to utilize glycerol will increase rapidly after birth, but not reach a maximum until the end of the weaning period. If, on the other hand, the effective K_m for glycerol utilization is about 1mM as indicated by Robinson & Newsholme (1969*b*), and provided that this parameter does not vary significantly during the suckling period, then the maximum possible rates of glycerol phosphorylation by the liver at various ages can be calculated from the results in Figs. 1 and 3. These indicate a rapid increase in the effective physiological activity after birth, reaching adult values by the second day *post partum* and maximum values during the suckling period. Further assessment of the rate of glycerol phosphorylation *in vivo* in the developing rat liver and kidney thus requires a clarification of the K_m of glycerol kinase for glycerol *in vivo*. In the rabbit, the K_m for glycerol utilization *in vivo*, 0.33mM, is very similar to the apparent K_m of hepatic glycerol kinase for glycerol, 0.29mM (Hagen, 1968); the rat, however, differs from the rabbit in that its rate of glycerol turnover *in vivo* is about fivefold greater (Hagen, 1968).

L-Glycerol 3-phosphate has been shown to be a competitive inhibitor of glycerol kinase with respect to glycerol (Robinson & Newsholme, 1969*a*). This inhibition was not observed by Grunnet & Lundquist (1967*b*). There is evidence, however, of an inverse relationship between the rate of glycerol utilization by liver slices and the L-glycerol 3-phosphate concentration (Rawat & Lundquist, 1968). Thus changes in the NAD^+/NADH concentration ratio may influence the rate of glycerol utilization of the liver by altering the L-glycerol 3-phosphate concentration; the rapid increase of hepatic L-glycerol 3-phosphate dehydrogenase activity after birth indicates that this possible means of control of glycerol kinase becomes fully developed between the second and fifth day *post partum*. Burch (1965) reported that the L-glycerol 3-phosphate concentration of the liver is maximal in the foetal rat and decreases to minimal values at birth, and this correlates with the increase in the NAD^+/NADH concentration ratio that occurs at this time (Philippidis & Ballard, 1969). Inhibition of glycerol utilization by L-glycerol 3-phosphate will therefore probably be low during the suckling period.

In the light of the adaptive behaviour of the dehydrogenase in adult rat liver described by Tepperman & Tepperman (1968), the elevated neonatal activities of the enzyme may indicate the quantitative importance of conversion of glycerol into glucose via L-glycerol 3-phosphate and dihydroxyacetone phosphate during the suckling period.

Glycerol metabolism in the whole animal. It has been reported for man (Borchgrevink & Havel, 1963) and the cat (Larsen, 1963) that 80–90% of the blood glycerol is metabolized by the liver and the rest by the kidney. A survey of the glycerol kinase activities per 100g body wt. of rat tissues suggests that a similar situation exists in this animal. The activities of the liver and kidney enzymes contribute at least 80% of the total body activity by 5 days after birth, so that most of the blood glycerol will probably be metabolized by these organs and so could be available for gluconeogenesis during the neonatal period. The relative importance *in vivo* of glycerol, amino acids and lactate as precursors of this glucose and the quantitative requirements for gluconeogenesis remain to be elucidated.

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