Fatty Acid Utilization during Development of the Rat

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1. In the absence of added carnitine pronounced changes occur during development, in the degradation of [U- 14 C]palmitate to 14 C-labelled acid-soluble material (predominantly ketone bodies) and 14 CO $_2$ by liver homogenates. The formation of both products by liver homogenates of 5-day-old rats is considerably higher than in foetal livers. This high value is maintained during the suckling period and falls after weaning to adult values which are similar to those of the foetus. Addition of carnitine stimulates production of acid-soluble material throughout development. The effect of added carnitine on CO $_2$ production varies with age, stimulating in foetal and post-weaning age groups but inhibiting during the suckling period. 2. The degradation of [U- 14 C]palmitate to acid-soluble material and CO $_2$ by heart homogenates also varies during development. Formation of both products is highest 5 days after birth and thereafter decreases steadily to adult values. Addition of carnitine stimulates the production of both CO $_2$ and acid-soluble material at all ages. 3. The activity of carnitine palmitoyltransferase in liver increased after birth to reach maximum activity at 2–5 days of age. Thereafter the activity of the enzyme decreased until day 15 of life and then remained constant until day 30, decreasing after this time to adult values which were about one half of the foetal liver activities.

Recent work has shown that the hepatic metabolism of the rat adapts to the nutritional changes that occur during development (Taylor, Bailey & Bartley, 1967; Ballard & Hanson, 1967; Yeung, Stanley & Oliver, 1967; Vernon & Walker, 1968; Lockwood, Bailey & Taylor, 1970). At birth there is a sudden change from a predominantly carbohydrate diet (transplacental) to a high-fat diet (milk). At weaning this is replaced by the laboratory diet which usually has a high-carbohydrate and low-fat content. Although changes in hepatic gluconeogenesis and lipogenesis at birth and at weaning have been well documented, changes in the utilization of lipids during development have been little investigated. During the suckling period the neonatal rat derives most of its energy from the fat-content of the milk (Drahota, Hahn & Honova, 1966) and so we have investigated changes in the utilization of [U- 14 C]palmitate by liver and heart homogenates from foetal life to maturity in the normal laboratory rat. Since Shepherd, Yates & Garland (1966) have proposed that the rate-limiting step in palmitate degradation is the formation of palmitoylcarnitine we have studied the effect of carnitine on palmitate degradation at various ages and also measured the activity of hepatic carnitine palmitoyltransferase throughout development.

EXPERIMENTAL

Materials. All reagents used were of analytical grade or the purest available. The following chemicals were obtained from C. F. Boehringer Corp. (London) Ltd., London W.5, U.K.: ATP, NAD $^+$, NADH, CoA and β -hydroxybutyrate dehydrogenase (grade II). Bovine serum albumin was purchased from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex, U.K. and made fat-free by the method of Chen (1967). DL-Carnitine was obtained from Sigma (London) Chemical Co. Ltd., London S.W.6, U.K. Palmitoyl-DL-carnitine was prepared by the method of Bremer (1966). [U- 14 C]Palmitic acid was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., and converted into potassium [U- 14 C]palmitate as described by Björntorp (1966). [1- 14 C]-DL-Carnitine was obtained from Tracerlab (GB) Ltd., Weybridge, Surrey, U.K. NE221 gel scintillator and NE220 liquid scintillator were purchased from Nuclear Enterprises (G.B.) Ltd., Edinburgh 11, U.K. Other chemicals were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Animals. Male Wistar rats from the Sheffield University Animal House Colony were used throughout. The animals were killed at about the same time of day by a blow on the head. Foetuses were removed from the mother near term, i.e. 19–20 days as estimated from foetal body weights (Lang, 1965). The young were weaned at 21 days on to a rat-cube diet [Oxoid diet 86 obtained from Herbert C. Styles (Bewdley) Ltd., Bewdley, Wores., U.K.]

Preparation of homogenates. For studies on the degradation of [$U\text{-}^{14}\text{C}$]palmitate, liver and hearts were homogenized with 4 vol. of ice-cold 0.25M-sucrose adjusted to pH 7.0 with 1M-NaOH. Nuclei and debris were removed by centrifugation at 600g for 5 min and the supernatant solution was used in the assays. For the assay of carnitine palmitoyltransferase livers were homogenized in 4 vol. of ice-cold 10% (w/v) sucrose containing 10mM-tris-HCl buffer, pH 7.5. Nuclei and debris were removed by centrifugation for 5 min at 600g and a crude mitochondrial preparation was obtained by centrifuging the supernatant solution at 12000g for 15 min. The mitochondria were resuspended in a suitable volume of 10% (w/v) sucrose containing 10mM-tris-HCl buffer, pH 7.5, to give approximately 8mg of mitochondrial protein/ml; 0.1 ml of this fraction was used in each assay.

Measurement of [$U\text{-}^{14}\text{C}$]palmitate degradation. Liver and heart homogenates (0.5 ml) were incubated for 60 min at 37°C in single-side-arm Warburg flasks sealed with Suba-seal stoppers, with the following reaction mixture: sucrose (100mM), EDTA (0.4mM), L-malate (0.01mM), KCl (80mM), sodium phosphate buffer, pH 7.4 (8mM), ATP (1mM), CoA (0.04mM), NAD^+ (2.5mM), MgCl_2 (5mM), fat-free bovine plasma albumin (0.17mM) and DL-carnitine (when present) (1mM). The final reaction volume was 2.5 ml. The reaction was started by adding 0.5 ml (1 μCi) of potassium [$U\text{-}^{14}\text{C}$]palmitate (1mM final concentration) from the side arm and stopped by injecting 0.1 ml of 50% (v/v) HClO_4 through the rubbersealing cap. Evolved $^{14}\text{CO}_2$ was absorbed in 0.2 ml of 20% (w/v) NaOH in the centre well of the flask. Preparation and radioassay of $\text{Ba}^{14}\text{CO}_3$ and ^{14}C -labelled acid-soluble material were as described by Taylor *et al.* (1967). Rates of palmitate oxidation were linear with time for 90 min. Determinations were carried out in random order, three or more age groups being included in any one batch of determinations.

Measurement of ketone bodies in acid-soluble material. Ketone bodies in the acid-soluble material were determined enzymically by the method of Williamson, Mellanby & Krebs (1962). The acid-soluble material was neutralized with 10% (w/v) KOH. After keeping the solution at 0°C for 1 h the KClO_4 was removed by centrifugation and the β -hydroxybutyrate and acetoacetate content of the supernatant solution was determined. Endogenous ketone bodies, i.e. those present in or formed during incubation of tissue in the absence of [^{14}C]palmitate were always determined.

Determination of carnitine palmitoyltransferase activity. The enzyme activity was determined by the isotope-exchange method of Norum (1964). Incorporation of [^{14}C]carnitine (0.22 $\mu\text{Ci}/\mu\text{mol}$) into palmitoyl[^{14}C]carnitine was found to be proportional to time for the first 10 min and so an incubation time of 8 min was used as a routine. Enzyme activity is expressed in terms of radioactivity incorporated into palmitoylcarnitine/min per mg of mitochondrial protein.

Protein determination. Protein was measured by the method described by Lowry, Rosebrough, Farr & Randall (1951).

RESULTS

Degradation of [$U\text{-}^{14}\text{C}$]palmitate by liver homogenates. In the absence of added carnitine the oxidation of [$U\text{-}^{14}\text{C}$]palmitate to $^{14}\text{CO}_2$ increases

Fig. 1. Production of $^{14}\text{CO}_2$ from [$U\text{-}^{14}\text{C}$]palmitate by liver homogenates from male rats during development. The details are as described in the Experimental section. Each point represents the mean of five determinations $\pm$ s.e.m. except the values for foetal and 2-day-old rats which are the mean of three determinations $\pm$ s.e.m. each carried out on pooled tissue from six to ten animals. F, Foetal rats; S, adult stock rats. ●, 1 mM-DL-Carnitine present in the incubation mixture; ○, DL-carnitine omitted from the incubation mixture.

Table 1. Effect of carnitine on the production of $^{14}\text{CO}_2$ from [$U\text{-}^{14}\text{C}$]palmitate by liver homogenates

The ratios are calculated from the determinations of $^{14}\text{CO}_2$ production when 1 mM-DL-carnitine was included in the incubation mixture and when DL-carnitine was omitted from the incubation mixture (see the Experimental section).

Age (days)	Ratio of $^{14}\text{CO}_2$ production in presence and absence of added carnitine
Foetal	2.1
2	0.74
5	0.48
15	0.51
20	0.61
25	1.3
30	1.8
40	1.4
Adult stock	2.0

sharply after birth and then remains at a reasonably constant value during suckling (Fig. 1). At the weaning time there is a sharp decline in the oxidation of the fatty acid with a minimum at 30 days and then a slight increase to the adult value. This pattern is in contrast with the results of Taylor *et al.* (1967)

who found that the oxidation of [^{14}C]palmitate to $^{14}\text{CO}_2$ by liver slices decreased after the first few days of life to reach adult values at about 10 days of age. In the presence of added carnitine a different developmental pattern is observed. The production of $^{14}\text{CO}_2$ is similar from late foetal life until day 2 of life and then declines to a minimum at day 5. There is little change during suckling or at the weaning time but the formation of $^{14}\text{CO}_2$ increases slightly

between day 40 and maturity. Previous reports have always found that carnitine stimulated the oxidation of palmitate to CO_2 but our results show that during the suckling period addition of carnitine actually inhibits CO_2 production. A stimulation in $^{14}\text{CO}_2$ production from [$\text{U-}^{14}\text{C}$]palmitate is found at all other ages. The effect of carnitine on CO_2 production is clearly seen in Table 1.

Developmental changes in the formation of radioactive acid-soluble material from [$\text{U-}^{14}\text{C}$]palmitate are shown in Fig. 2. Again the formation of these products shows that the degradation of palmitate is higher during the suckling period than at all other ages. The effect of adding carnitine to the incubation medium is to stimulate the production of acid-soluble material, to a similar extent, at all stages of development. Enzymic assay of the radioactive acid-soluble material formed at different stages of development both in the presence and absence of carnitine has shown that it is composed almost entirely of the ketone bodies, acetoacetate and β -hydroxybutyrate (Table 2).

Degradation of [$\text{U-}^{14}\text{C}$]palmitate by heart homogenates. The degradation by heart homogenates of [$\text{U-}^{14}\text{C}$]palmitate to $^{14}\text{CO}_2$ is shown in Fig. 3(a) and the acid-soluble material in Fig. 3(b). In contrast with liver homogenates the acid-soluble material produced by heart homogenates contain virtually no ketone bodies (E. A. Lockwood, unpublished work) and presumably consists of other compounds derived from acetyl-CoA, such as tricarboxylic acid-cycle intermediates. The formation of both $^{14}\text{CO}_2$ and radioactive acid-soluble material is higher during suckling than after weaning. This difference is found both in the presence and absence of carnitine, the addition of which stimulates the formation of both products of palmitate breakdown at all ages. The results for CO_2 production are in marked

Fig. 2. Production of ^{14}C -labelled acid-soluble products from [$\text{U-}^{14}\text{C}$]palmitate by liver homogenates from male rats during development. The details are as in the Experimental section. Each point represents the mean of five determinations $\pm$ S.E.M. except the values for foetal and 2-day-old livers which are the mean of three determinations $\pm$ S.E.M. each carried out on pooled tissue from six to ten animals. F, Foetal rats; S, adult stock rats. ●, 1 mM-DL-Carnitine present in the incubation mixture; ○, DL-carnitine omitted from the incubation mixture.

Table 2. Ketone-body composition of the labelled acid-soluble products formed from the degradation of [$\text{U-}^{14}\text{C}$]palmitate by liver homogenates

β -Hydroxybutyrate and acetoacetate were determined enzymically as described in the Experimental section. The values given are of two separate determinations and mean values are indicated in parentheses. The total ketone-body composition of the acid-soluble products was calculated assuming that the ketone bodies were uniformly labelled and is the mean of two values. n.m., Not measurable.

Age (days)	DL-Carnitine	Ketone bodies in total acid-soluble products (μmol)			Ketone-body composition of acid-soluble products (%)
		β -Hydroxybutyrate	Acetoacetate	Total	
5	+	1.02, 1.23 (1.12)	0.22, 0.74 (0.48)	1.6	91
	—	0.38, 0.11 (0.25)	0.23, 0.61 (0.42)	0.67	94
15	+	0.62, 1.53 (1.08)	0.27, 0.53 (0.4)	1.48	81
	—	0.31, 0.49 (0.4)	0.25, 0.27 (0.26)	0.66	95
25	+	0.36, 0.37 (0.37)	0.23, 0.17 (0.2)	0.57	79
	—	0.11, 0.05 (0.08)	0.09, 0.05 (0.07)	0.15	78
Adult stock	+	0.14, n.m. (0.07)	0.35, 0.15 (0.25)	0.32	93
	—	0.13, n.m. (0.06)	0.34, 0.13 (0.24)	0.3	90.5

Fig. 3. Production of $^{14}\text{CO}_2$ (a) and ^{14}C -labelled acid-soluble products (b) from $[\text{U-}^{14}\text{C}]$ palmitate by heart homogenates from male rats during development. The details are as described in the Experimental section. Each point represents the mean of three determinations $\pm$ S.E.M. and each determination was carried out on pooled tissue from at least six animals. S, Adult stock rats. ●, 1 mM-DL-Carnitine present in the incubation mixture; ○, DL-carnitine omitted from the incubation mixture.

contrast with those of Wittels & Bressler (1965), who found low activities of palmitate oxidation in newborn-rat hearts with adult activities being reached at 10 days of age.

Activity of carnitine palmitoyltransferase. Changes in the activity of hepatic carnitine palmitoyltransferase during development are shown in Fig. 4. After birth the activity of the enzyme increases to a maximum at 2–5 days of age. Thereafter the activity decreases until day 15 and then remains constant until day 30. After day 30 the enzyme activity decreases to adult values which are about one-half of the foetal-liver activities.

DISCUSSION

During development the changes that occur in degradation of palmitate by liver and heart homogenates indicate that these organs adapt in such a way as to enable them to deal most efficiently with alterations in the diet of the rat. Thus the ability to degrade fatty acids is greater during suckling

Fig. 4. Activity of hepatic carnitine palmitoyltransferase during development in male rats. Enzyme activity was measured by an isotope-exchange method as described in the Experimental section. Each point represents the mean of five determinations $\pm$ S.E.M. except the value for foetal rats which is the mean of three determinations each carried out on pooled tissue from ten animals. S, Adult stock rats; F, foetal rats. Enzyme activity is expressed as c.p.m. incorporated into palmitoylcarnitine/min per mg of mitochondrial protein.

when a high-fat diet (milk) is being consumed than at other stages of life, foetal and post-weaning, when a predominantly carbohydrate diet is available. The results bear an inverse relationship to developmental changes in lipogenesis reported by Taylor *et al.* (1967). Since the concentrations of endogenous palmitate in heart and liver are highest during suckling (Dobiasova, Hahn & Koldovsky, 1964) the observed changes in $[\text{C}^{14}]$ palmitate are probably not due to isotope-dilution effects as these would lead to a relative decrease in formation of radioactive products during suckling.

The high value of ketone-body production during suckling is in agreement with the work of Drahota, Hahn, Kleinzeller & Kostolanska (1964) and Drahota *et al.* (1966), who used liver slices. However, these workers found that at all stages of development most of the ketone bodies were acetoacetate. Our results indicate that with liver homogenates from the suckling rat in the presence of added carnitine the major product is β -hydroxybutyrate, whereas the greater part of ketone bodies produced by homogenates of the adult rat liver is acetoacetate. Krebs, Wallace, Hems & Freedland (1969) using the perfused adult rat liver found that the $[\beta\text{-hydroxybutyrate}]/[\text{acetoacetate}]$ ratio produced in the presence of long-chain fatty acid was in the range 2–3.

It is not possible to interpret the results obtained in the absence of carnitine in an unequivocal fashion since the concentration of carnitine in the tissues may change during development. The results of Pearson & Tubbs (1967) indicate that our reaction mixtures for adult liver and heart homogenates would contain final concentrations of $10\mu\text{M}$ - and $20\mu\text{M}$ -carnitine respectively. Slight variations in the concentration of carnitine during development could lead to changes in the activity of palmitoyl-transferase and hence of palmitate breakdown. Little is known of variations in tissue carnitine concentration during development, but Wittels & Bressler (1965) found lower concentrations in foetal rat heart than in adult heart, indicating that changes may occur. However, developmental changes often reflect dietary changes and Pearson & Tubbs (1967) have shown that liver and heart carnitine concentrations of the adult rat vary very little under a variety of dietary conditions.

The addition of carnitine stimulates the degradation of [^{14}C]palmitate to ketone bodies by the liver at all stages of development whereas the production of $^{14}\text{CO}_2$ is only stimulated in foetal and post-weaning periods and is inhibited during suckling. It appears that during suckling carnitine stimulates the degradation of palmitate to acetyl-CoA (and thus to ketone bodies) by liver homogenates, but inhibits the oxidation of acetyl-CoA via the tricarboxylic acid cycle. Thus in the presence of added carnitine a similar rate of $^{14}\text{CO}_2$ formation is observed at all ages. *In vivo*, when the concentrations of carnitine are presumably not limiting, acetyl-CoA not required for oxidation in the tricarboxylic acid cycle would be directed into the formation of ketone bodies. A possible mechanism for the carnitine effect is suggested by the results of Garland, Shepherd, Nicholls & Ontko (1968), who have shown that palmitoyl-carnitine degradation in rat liver causes stimulation of ketone-body formation and inhibition of the tricarboxylic acid cycle. The formation of palmitoyl-carnitine is affected by the activity of carnitine palmitoyltransferase which has been proposed by Shepherd *et al.* (1966) to be the rate-limiting step in palmitate degradation. This enzyme has the highest activity during suckling and has also been shown to increase in activity in response to fat feeding (Norum, 1965). Thus, with homogenates from suckling rats in the presence of added carnitine, there might be a higher rate of palmitoylcarnitine formation resulting in stimulation of ketone-body production and inhibition of acetyl-CoA oxidation. In the absence of added carnitine the formation of palmitoylcarnitine would be limited by the concentration of endogenous carnitine, thus leading to a decrease in palmitate degradation and less ketone-body formation, but greater conversion of acetyl-

CoA into CO_2 . It is noteworthy that in the heart, an organ that does not produce ketone bodies, the addition of carnitine stimulates $^{14}\text{CO}_2$ production at all ages, indicating that a different mechanism exists in liver and heart to control the utilization of acetyl-CoA.

Although the developmental pattern of liver carnitine palmitoyltransferase tends to support the proposal that this enzyme may be the rate-limiting step in fatty acid oxidation, it must be noted that Van Tol & Hülsmann (1969) have suggested that the enzyme is not rate-limiting as they found the enzyme activity far in excess of the rate of β -oxidation of fatty acids. If this is so, our results suggest that carnitine may be involved in the control of hepatic mitochondrial acetyl-CoA utilization either by the ketone-body-forming enzymes or by the tricarboxylic acid cycle.

Augenfeld & Fritz (1970) have reported studies very similar to those presented here. They find qualitatively similar changes in carnitine palmitoyl-transferase activity during development but report much lower enzyme activities in foetal liver. It is possible that the different strains of rats used (Bartley, Dean, Taylor & Bailey 1967; Eggleston & Krebs, 1969) and variations in maternal diet could explain these differences. Augenfeld & Fritz (1970) also obtain lower rates of palmitate oxidation by foetal liver than those reported in this paper. However, our results agree with those obtained by Taylor *et al.* (1967), who used liver slices. Further, Augenfeld & Fritz (1970) get little stimulation of $^{14}\text{CO}_2$ production in the presence of added L-carnitine by liver homogenates from newborn and adult rats. This can probably be explained by the different incubation mixture which contained no added malate, NAD or CoA. Fritz (1963) found that the extent of carnitine stimulation of CO_2 production by heart homogenates depends on the presence of these cofactors.

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