4. The fractionation of fibroin by alkali is compared with that obtained by hydrolysis with trypsin, and the relation of the fractions to the fibroin molecule is discussed.

The skilled technical assistance of Miss D. E. Pinion is gratefully acknowledged.

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Acetoacetate Formation by Liver Slices from Adult and Infant Rats

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(Received 13 January 1964)

It has become evident in recent years that fat is the main energy source for most infant mammals, particularly the rat. In that animal milk represents in fact a high-fat diet (Glass, 1956), and fat is not only rapidly utilized but is also laid down during the first few days after birth (Hahn & Koldovský, 1960). Reflexions of this state are high lipid concentrations in the blood of fed infant rats (Grafnetter, Hahn, Koldovský & Viktora, 1960) and a rapid suppression of lipogenesis in the liver very soon after birth (Vilée, 1958).

Since the end product of fatty acid oxidation in the liver is acetoacetate (Lehninger, 1945; Quastel & Wheatley, 1933; Edson, 1935), it might be expected that more of this substance is formed in the liver during the suckling period than later in life, when the fat content of the diet and of the liver is low. Surprisingly, this has not previously been examined. Even concentrations of ketone bodies in the blood of young rats have been determined only after weaning (Wick & McKay, 1940).

In the present work an attempt has been made to follow developmental changes in acetoacetate formation by liver slices and to correlate these with changes in the ketone-body concentration in the blood. A preliminary report of part of this work has appeared (Drahota, Hahn, Kleinzeller & Kostolánská, 1963).

MATERIALS AND METHODS

Animals. Male white Wistar rats fed on a standard laboratory diet (Fábry, 1959) were used throughout. Rats younger than 23 days were employed regardless of sex. Litters were always reduced to eight on the second day after birth. When animals were starved, rats younger than 23 days were kept at 30° without the mother animal in an incubator. Adult animals were kept at room temperature (about 22°) and were deprived of both food and water.

Tissue preparations. Rats were decapitated and the livers were rapidly removed and chilled. Tissue slices were cut by hand. They were gently dried on filter paper and weighed on a torsion balance, and 50–80 mg. portions placed in chilled Warburg vessels containing 3 ml. of saline medium (see below). The slices were incubated at 37° in oxygen in a Warburg apparatus. The central well of each flask contained 0.2 ml. of 20% (w/v) KOH solution. Since high concentrations of phosphate inhibit acetoacetate formation (Quastel & Wheatley, 1933), the composition of the saline medium was adjusted to the following: NaCl, 134 mM; KCl, 5.6 mM; CaCl₂ 2.25 mM; potassium phosphate, 4.2 mM; MgSO₄, 1.4 mM; the final pH was 7.2. Other additions were made as indicated in the text. The Q_{O_2} and respiratory quotient (R.Q.) values were determined by the direct Warburg method (see Dixon, 1943).

Analytical methods. Acetoacetate was determined colorimetrically according to the method of Walker (1954). Sodium acetoacetate used for calibration was prepared according to the method of Ljunggren (1924) and standardized according to the method of Edson (1935).

Determination of acetoacetate in blood. Samples of blood (0.2–0.5 ml.) were deproteinized with 10% (w/v) trichloroacetic acid in such a way that the final volume was 2.5 ml. and the resultant concentration of trichloroacetic acid was 3%. After centrifugation the supernatant was neutralized with KOH, and the acetoacetate was determined.

Determination of acetoacetate formation by liver slices. After termination of incubation the vessels were cooled to 0°. Portions of the medium were deproteinized with 40% (w/v) trichloroacetic acid to give a final concentration of 3%. After centrifugation the supernatant was neutralized and the acetoacetate was determined. Acetoacetate formation is expressed in $\mu\text{moles/g. wet wt. of tissue/hr.}$

Determination of total ketone bodies. Blood (0.1 ml.) was transferred to 1.9 ml. of water and deproteinized with Ba(OH)_2 and ZnSO_4 . After centrifugation the ketone bodies were determined in the supernatant according to the method of Bessman & Anderson (1957).

Chemicals. All common chemicals were of reagent grade and were used without further purification. Solutions were prepared from glass-redistilled water.

Calculations of results. All determinations were made in duplicate and the means were used for calculating the mean $\pm$ S.E.M. for the given group of animals.

RESULTS

Time-course of acetoacetate formation by liver slices.

The rate of acetoacetate formation from endogenous substrate by liver slices of adult and infant rats is linear during 2 hr. of incubation. The values in adult animals correspond to those found by Quastel & Wheatley (1933). In further experiments a 60 min. incubation period was used.

Respiration of liver slices from 10-day-old and 90-day-old rats. Table 1 shows the values for oxygen consumption by liver slices from rats of different age groups. It is apparent that endogenous respiration rates are higher in 10-day-old than in 90-day-old rats. The addition of sodium succinate (final concn. $13\mu\text{M}$) to the incubation medium increases the respiration rate, and the age difference is no longer in evidence.

Acetoacetate formation by liver slices during postnatal development. Acetoacetate formation by liver slices increases between 0 and 24 hr. after birth

and decreases again after day 10 (Table 2). If the rate of acetoacetate formation is expressed per unit dry wt. the differences are even more evident. In 1-day-old and 10-day-old rats the rate of formation is five times that found in 90-day-old animals.

The higher rate of acetoacetate formation by liver slices from infant rats may be due to more fatty acids being oxidized, and this should be reflected in the R.Q. In fact, the R.Q. for liver slices from 10-day-old fed rats was 0.74 ± 0.025 (seven animals) compared with 0.89 ± 0.025 (eight animals) for slices from adult fed male rats.

It is well known that the rate of acetoacetate formation from endogenous substrate in the liver is increased during starvation (Edson, 1935). It was therefore decided to investigate whether the high rate of acetoacetate formation in infant rats could be further raised by starvation.

Effect of starvation on the rate of acetoacetate formation by liver slices. In agreement with Edson (1935) the acetoacetate formation by liver slices is increased after 24 hr. without food in adult rats (Table 3). This is not the case in 10-day-old rats, where the high rate of acetoacetate formation is not raised any further by starvation. Even so, the rate

Table 1. *Respiration of rat-liver slices of 10-day-old and 90-day-old rats*

Slices were incubated for 60 min. in a Warburg apparatus at 37° in 3 ml. of saline medium, pH 7.2 (defined in the Materials and Methods section). The centre well contained 0.2 ml. of 20% KOH and the gas phase was O_2 . Where indicated, $40\mu\text{moles}$ of sodium succinate were added to each flask. The results are given as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats (days)	Condition of rats	O_2 uptake ($\mu\text{l./100 mg. wet wt./hr.}$)	
		Endogenous	With succinate added
10	Fed	169 ± 6.9 (6)	336 ± 31.7 (6)
	Starved for 24 hr.	168 ± 9.5 (5)	286 ± 8.0 (5)
90	Fed	139 ± 5.8 (8)	317 ± 18.9 (8)
	Starved for 24 hr.	136 ± 8.7 (4)	282 ± 10.2 (4)

Table 2. *Acetoacetate formation by rat-liver slices during postnatal development*

Slices were incubated as indicated in Table 1. The values for liver dry wt. were taken from Hahn & Koldovský (1960). The results for acetoacetate formation/g. wet wt./hr. are given as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats	Dry wt. (% of wet wt.)	Acetoacetate formation		
		($\mu\text{moles/g. wet wt./hr.}$)	(% of adult values)	($\mu\text{moles/g. dry wt./hr.}$)
0–2 hr.	—	2.6 ± 0.3 (4)	100	—
12 hr.	—	4.2 ± 0.3 (4)	162	—
24 hr.	24.3	10.2 ± 1.8 (6)	397	42.0
10 days	26.2	10.3 ± 0.6 (15)	400	39.1
23 days	27.5	6.6 ± 0.5 (6)	254	24.1
90 days	31.0	2.6 ± 0.5 (15)	(100)	8.4

of acetoacetate formation in 10-day-old rats is still twice that in 90-day-old rats (Table 3).

These differences between adult and infant rats might be due to: (a) a higher concentration of endogenous substrate in the infant liver, and (b) a different pattern of enzyme activity in that organ. To investigate this question the following experiments were performed.

Factors affecting the rate of acetoacetate formation by liver slices. The addition of acetate increases the rate of acetoacetate formation in both 8-day-old and 90-day-old rats, whether fed or starved (Table 4). This is less pronounced in the younger age group, but per unit weight the rate of formation in the infant rat is still twice that in the adult

animal. Starvation does not appear to affect this phenomenon. Table 4 also shows that the rate of formation of acetoacetate from added palmitate is less pronounced than that from added acetate, excepting only the 8-day-old starved animals. In all cases, however, the rate of formation is much higher in infant rats.

Acetoacetate formation is suppressed by added glycerol, glucose or succinate (Edson, 1935). Table 5 shows that succinate depresses the rate of acetoacetate formation in both 8-day-old and 90-day-old animals. The inhibition is less pronounced in starved than in fed rats, and the percentage decrease is about the same in both age groups. Succinate has a smaller effect in 1-day-old rats, and this

Table 3. *Acetoacetate formation by liver slices of 10-day-old and 90-day-old fed and starved rats*

Slices were incubated as indicated in Table 1. The results for acetoacetate formation are given as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats (days)	Acetoacetate formation (μ moles/g. wet wt./hr.)		$\frac{B}{A} \times 100$ (%)
	Fed rats (A)	Rats starved for 24 hr. (B)	
10 (a)	10.3 $\pm$ 0.6 (15)	12.4 $\pm$ 0.6 (17)	120
90 (b)	2.6 $\pm$ 0.5 (15)	5.7 $\pm$ 0.6 (19)	220
$\frac{a}{b} \times 100$ (%)	400	220	—

Table 4. *Activation of endogenous acetoacetate formation by acetate and palmitate in liver slices from 8-day-old and 90-day-old rats*

Slices were incubated as indicated in Table 1. Where indicated, 40 μ moles of sodium acetate, pH 7.2, or sodium palmitate (added together with bovine serum albumin, giving a final concn. of palmitate of 0.5 mM and of serum albumin 2%) were added to each flask. The values for acetoacetate formation are given as means, and those for the percentage activation as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats (days)	Condition of rats	Acetoacetate formation (μ moles/g. wet wt./hr.)			Activation of acetoacetate formation (%)	
		Endogenous	With acetate added	With palmitate added	By acetate	By palmitate
8	Fed	13.4	27.1	19.3	203 $\pm$ 13 (4)	145 $\pm$ 6 (4)
	Starved for 24 hr.	13.5	23.2	22.3	172 $\pm$ 11 (8)	165 $\pm$ 19 (8)
90	Fed	3.3	9.7	5.5	294 $\pm$ 31 (8)	167 $\pm$ 16 (8)
	Starved for 24 hr.	5.4	13.2	9.4	245 $\pm$ 31 (4)	174 $\pm$ 22 (4)

Table 5. *Influence of succinate on acetoacetate formation by rat-liver slices*

Slices were incubated as indicated in Table 1. Where indicated, 40 μ moles of sodium succinate were added to each flask. The values for acetoacetate formation are given as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats	Condition of rats	Acetoacetate formation (μ moles/g. wet wt./hr.)		Inhibition by succinate (%)
		Endogenous	With succinate added	
2 hr.	—	2.6 $\pm$ 0.3 (4)	1.4 $\pm$ 0.2 (4)	54
10 days	Fed	10.8 $\pm$ 0.8 (3)	3.1 $\pm$ 0.2 (3)	26
	Starved for 24 hr.	11.0 $\pm$ 0.2 (8)	6.5 $\pm$ 0.4 (8)	59
90 days	Fed	1.8 $\pm$ 0.3 (4)	0.5 $\pm$ 0.2 (4)	28
	Starved for 24 hr.	5.7 $\pm$ 0.8 (5)	2.4 $\pm$ 0.2 (5)	42

Table 6. *Blood concentration of acetoacetate in fed and starved rats*

The values are given as means $\pm$ S.E.M., with the numbers of animals used in parentheses.

Age of rats	Concn. of acetoacetate (μ moles/100 ml. of blood)	
	Fed rats	Rats starved for 24 hr.
0-2 hr.	7.9 ± 1.8 (3)	—
24 hr.	56.5 ± 7.5 (6)	3.9 ± 2.0 (4)
10 days	31.1 ± 1.5 (7)	38.0 ± 1.8 (14)
15 days	30.6 ± 2.2 (6)	—
23 days	15.4 ± 1.0 (6)	—
90 days	2.8 ± 0.4 (9)	33.4 ± 6.3 (5)

might be related to the lower succinoxidase activity in the liver of newborn rats (Dawkins, 1959).

The above findings *in vitro* showed elevated rates of acetoacetate formation by the liver of infant rats. Do similar conditions prevail in the whole animal? If so, one would expect a raised concentration of acetoacetate in the blood.

Acetoacetate concentration in blood during postnatal development. Table 6 demonstrates that changes in acetoacetate formation by the liver are reflected by changes in the blood concentration of this substance. Within 24 hr. of birth the concentration of acetoacetate in the blood has increased sevenfold and returns to normal adult values after day 23 (see Walker, 1954). Surprisingly, in newborn animals aged 24 hr. that have never received food there is no rise in the acetoacetate concentration in the blood, although *in vitro* the rate of formation of this substance has already increased by 50 %.

As described by Rosenthal (1949), the acetoacetate concentration in the blood is raised in starvation in adult rats, and thus no differences between 10-day-old and 90-day-old animals are found in starvation, since in the former age group starvation does not raise the acetoacetate concentration in the blood.

Total ketone-body formation. Since acetoacetate is transformed into β -hydroxybutyrate and acetone, total ketone bodies were determined in some experiments to make sure that the differences found were not due to a different ratio between acetoacetate and hydroxybutyrate in different age groups. In 8-day-old fed rats the rate of total ketone-body formation by liver slices was $20.2 \pm 0.7 \mu$ moles/g. wet wt. of tissue/hr. compared with $4.0 \pm 0.9 \mu$ moles/g. wet wt./hr. in adult animals. Acetoacetate always represented about 60 % of the total ketone bodies.

DISCUSSION

The greater rate of acetoacetate formation by liver slices from infant rats has been shown not to be due to a change during development in the ratio of

acetoacetate to other ketone bodies formed. This, however, applies to endogenous acetoacetate formation only, and it is possible that this ratio is affected differently in infant and adult rats after the addition of exogenous substrates. This will have to be further elucidated.

Acetoacetate formation in liver tissue is the result of the activity of two enzymic complexes. Acetoacetate is formed: (a) as a terminal C_4 remnant during oxidative degradation of fatty acids and some amino acids (cf. Lehninger, 1952), and (b) by the condensation of two acetyl-CoA molecules, and evidently mainly by the condensation of acetyl-CoA and acetoacetyl-CoA via hydroxymethylglutaryl-CoA (Caldwell & Drummond, 1963).

The fact that age differences remain clearly in evidence even after the addition of palmitate or acetate indicates that these differences are not due only to the fact that more lipid substrate is available to the infant liver than to the adult organ. Oxidation of fatty acids probably proceeds more rapidly in infant rats, and since the enzymes responsible for fatty acid oxidation are localized in the mitochondria (Lehninger, 1945) the cause of this age difference might be situated in that cell particle. There is some indirect evidence for this contention. Schneider & Potter (1943) found higher cytochrome-oxidase activity in infant mammals, and the work of Van Rossum (1961) and the present experiments show that there is a higher rate of respiration in liver slices from infant rats than in those from adult animals. Dawkins (1959) demonstrated a rapid increase in the activity of several oxidative enzymes after birth. In addition, the higher oxygen consumption of liver slices from infant rats might indicate the oxidation of fatty acids, as also borne out by the R.Q. values.

The greater rate of formation of acetoacetate by liver slices from infant rats than by those from adult animals may, of course, also be due to lower activities of enzymes of the citric acid cycle. Kiessling (1962, 1963) and Kiessling & Lundquist (1962) reported a lower rate of oxidation of pyruvate and succinate by liver and muscle mitochondria from 10-day-old rats than in older animals; Freedmann & Nemeth (1961) showed that pyruvate in suckling rats is incorporated into dicarboxylic acids to a much greater extent than in adult or foetal animals. Thus lower utilization of acetate in the citric acid cycle and a higher rate of fatty acid oxidation would lead to the accumulation of acetate and thus to a greater rate of acetoacetate formation. This could be linked with the finding of Garland, Randle & Newsholme (1963) in heart muscle that fatty acids and ketone bodies inhibit the activity of fructokinase, and this in turn would lead to decreased oxaloacetate formation and diminished introduction of acetate from fatty acids

into the citric acid cycle. Moreover, in adult rats in which ketosis has been induced (diabetes or a high-fat diet) the concentrations of acetyl-CoA and acyl-CoA in the liver are increased (Wieland & Weiss, 1963; Borst & Lynen, 1963).

Finally, it must be asked whether the higher rate of acetoacetate formation in the liver and the higher concentration in the blood of infant rats also affects the peripheral utilization of this substance. Only one paper in this field is known to us. Gould & Coleman (1962) report a lower utilization of acetoacetate by muscle of infant mice than by that of adult animals, but their results cannot be accepted without reserve. They determined only the fall in endogenous acetoacetate content of muscle homogenates during incubation. Although the blood concentration of acetoacetate most probably was higher than in adult animals, the acetoacetate content in the muscle at the start of incubation was lower in the infant than in the adult mice.

In preliminary experiments with heart-muscle slices we found that acetoacetate utilization was usually higher in infant than in adult hearts. Apparently, however, heart muscle may easily be injured during preparation, and hence more attention to the preparative procedure and the incubation conditions is needed before a final conclusion is reached. It is known, however, that the glycogen content of the heart of 10-day-old rats is twice as high as that of adult animals (Hahn & Koldovský, 1959), and this might be explained by the greater utilization of fatty acids or ketone bodies (Randle, Garland, Hales & Newsholme, 1963).

The results presented above show that there is a good correlation between the fat content of the diet (milk and the adult diet) and the rate of acetoacetate formation in the liver. Further work is required to distinguish between the diet-induced changes and endogenous developmental changes.

SUMMARY

1. Acetoacetate formation by liver slices from young (0–23-day-old) and adult (90-day-old) rats was studied.

2. The rate of endogenous acetoacetate formation at birth is the same as in adult rats, i.e. $2.5\mu\text{moles/g. wet wt./hr.}$ It is raised to $10.2\mu\text{moles/g. wet wt./hr.}$ within 24 hr. of birth and remains elevated beyond day 10.

3. A 24 hr. period of starvation increases the rate of acetoacetate formation by 120 % in 90-day-old rats and by only 20 % in 10-day-old rats, yet acetoacetate formation is still twice as great in the infant rats.

4. Palmitate or acetate raises the rate of acetoacetate formation in both 10-day-old and 90-day-

old fed or starved rats. In all cases the rate of acetoacetate formation is higher in the infant rats. Succinate elevates the respiration rate and decreases the endogenous formation of acetoacetate in both age groups.

5. The blood concentration of acetoacetate increases within 24 hr. of birth and remains elevated beyond day 10, after which it decreases again.

6. The results are discussed in relation to the character of the diet of infant and adult rats.

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