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Original article

Effects of protein level, methionine supplementation and carbohydrate type of the diet on liver lipid and plasma free threonine contents in the lactating rat

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Summary — Eight groups of 13—15 female rats were fed purified diets after littering. Four groups received a low protein (8% casein) diet (groups 8) and the others, a normal protein (20% casein) diet (groups 20). Carbohydrates were supplied either as starch (groups S) or as starch plus 40% fructose (groups F). Half the animals received a 0.4% methionine supplementation (groups M). Four or five dams per group were sacrificed on days 2, 7 and 14 after littering.

The diet intake was increased by methionine supplementation, substitution of starch for fructose and increased protein content, mainly during the second week of lactation. This influenced weight variation of the dams and litter growth.

On all days, the plasma levels of cholesterol esters, triglycerides and phospholipids were positively correlated with the dietary protein level.

On days 7 and 14, the liver neutral lipid content was increased in rats fed the low protein diets supplemented with methionine (groups 8SM and 8FM) and the normal protein diets containing 40% fructose (groups 20F and 20FM).

The plasma free threonine content was positively correlated with the protein level in the diet. On day 14, rats fed a low protein diet had a threonine deficiency, except those in groups 8S and 8F. The plasma free threonine content of these rats was not reduced, possibly due to an impaired utilization of this amino acid.

The liver lipidosis observed during lactation, in contrast to that observed during growth with a low protein diet, was not due to a threonine deficiency.

lactation — malnutrition — methionine — liver lipids — plasma free threonine

Résumé — Effets de la teneur en protéines, de la supplémentation en méthionine et de la nature des glucides du régime alimentaire de la rate allaitante sur les teneurs en lipides du foie et en thréonine du plasma. *Huit lots de 13 à 15 rates ingérant après la mise bas des régimes purifiés ont été constitués. 4 lots ont eu un régime hypoprotéique à 8% de caséine : lots 8, et 4 autres lots un régime normoprotéique à 20% de caséine : lots 20. La nature des glucides est, soit* *de l'amidon : lots S, soit de l'amidon et 40% de fructose : lots F. La moitié des animaux ont eu, en supplément, 0,4% de méthionine : lots M. 4 à 5 femelles par lots ont été sacrifiées aux jours 2, 7 et 14 après la mise-bas.*

La consommation de nourriture est accrue, principalement au cours de la seconde semaine de lactation, lorsque le régime alimentaire est supplémenté en méthionine, lorsqu'on substitue, l'amidon au fructose, et lorsque la teneur en protéines du régime est augmentée. Cette consommation accrue influe favorablement sur la variation de poids de la mère et sur la croissance du jeune.

Les teneurs du plasma en esters de cholestérol, triglycérides et phospholipides sont corrélées positivement avec la teneur en protéines du régime.

La teneur hépatique en lipides neutres est accrue aux jours 7 et 14 pour les régimes hypoprotéiques lorsque ceux-ci sont supplémentés en méthionine (lots 8SM et 8FM), et pour les régimes normoprotéiques avec 40% de fructose (lots 20F et 20FM).

La teneur plasmatique en thréonine libre est corrélée positivement avec la teneur en caséine du régime. Les rates ingérant les régimes hypoprotéiques présentent une déficience en thréonine, excepté au jour 14 pour les femelles des lots 8S et 8F : la teneur plasmatique en thréonine de ces rates n'est pas abaissée, ce qui est dû probablement à un défaut d'utilisation de cet acide aminé.

La lipidose hépatique observée durant la lactation avec un régime hypoprotéique, contrairement à ce qui se passe au cours de la croissance, n'est pas due à une déficience en thréonine.

lactation — malnutrition — méthionine — lipides du foie — thréonine libre du plasma

Introduction

Methionine supplementation of a low protein diet induces an accumulation of neutral lipids, mainly triglycerides, in the liver of growing (Aoyama and Ashida, 1978) and lactating (Leclerc *et al.*, 1985) rats.

This accumulation is also observed with a low protein diet fed to growing (Wiener *et al.*, 1963) and lactating (Leclerc *et al.*, 1985) rats and with a normal protein diet fed to growing (Bacon *et al.*, 1984) rats, when these diets are rich in sucrose or fructose.

It was demonstrated that during growth, methionine supplementation of the diet reduces the plasma threonine content (Moritoki and Yoshida, 1970; McLaughlan, 1979). This secondary threonine deficiency may be responsible for steatosis (Aoyama *et al.*, 1979). Actually, threonine supplementation suppresses triglyceride accumulation in the rat liver (Aoyama and Ashida, 1978).

In this study, we report the relationship between lipid content of plasma and liver and threonine content of plasma according to protein level, methionine supplementation and carbohydrate type in the diet of the lactating rat.

Materials and Methods

Animals

Female Wistar rats obtained from the animal breeding unit of our laboratory and weighing 200—220 g were mated at the age of 3—3.5 months. At littering (day 0), they were fed semi-synthetic diets (see composition in Table I). Litters were adjusted to 8 pups on day 2. Feed intake was determined daily. Females and their litters (13—15 per group) were weighed at littering, then on days 2, 4, 7, 10 and 14. On days 2, 7 and 14, 4 or 5 females were anesthetized with ether. Blood (4—6 ml) was collected by intracardiac puncture. Plasma was centrifuged, weighed and distributed into 2 tubes for lipid (collected in chloroform—methanol mixture) and threonine analyses. After asphyxia, the liver was excised, weighed and an aliquot was transferred into a chloroform—methanol mixture (2/1, v/v). Plasma and liver samples were stored at −20°C until analysis.

Diets (Table I)

The low protein (groups 8: 8% casein) and normal protein (groups 20: 20% casein) diets were supplemented (groups M) or not with 0.4% methionine. Half the diets included starch as carbohydrate source (groups S) or starch and fructose (groups F). The other dietary components were similar for all the groups.

Methods

The liver and plasma lipids were extracted according to the method of Folch *et al.* (1957). Plasma cholesterol esters were separated on silica cartridges (Wang and Peter, 1983). Liver and plasma neutral lipids (cholesterol esters except for plasma) and phospholipids were also separated on silica cartridges (Juaneda and Rocquelin, 1985). After methylation, fatty acids of fractions were determined by gas—liquid chromatography on carbowax 20 M column using methyl margarate as an internal standard.

Plasma free threonine content was determined by a microbiological micromethod (McLaughlan *et al.*, 1961).

The respective effects of the slaughter date (day 2, 7 or 14), protein level (8 or 20% casein), carbohydrate type (starch or fructose) and methionine supplementation and their possible interactions on the different parameters were studied with the analysis of variance using the contrast method (Dagnelie, 1975). Means were also compared with Duncan's method (Dagnelie, 1975). To simplify the presentation of the results, only data obtained with Duncan's method are reported in the tables, but the interpretation takes into account all findings.

Results

During week two of lactation, the diet intake was increased by methionine

Table I. Composition of experimental diets.

Groups		*8S*	*8SM*	*8F*	*8FM*	*20S*	*20SM*	*20F*	*20FM*
Casein	(g)	8	8	8	8	20	20	20	20
Starch	(g)	81	81	41	41	69	69	29	29
Fructose	(g)	0	0	40	40	0	0	40	40
Methionine [a]	(ml)	0	10	0	10	0	10	0	10
Corn oil	(g)	5	5	5	5	5	5	5	5
Agar-agar	(g)	2	2	2	2	2	2	2	2
Salt mixture [b]	(g)	4	4	4	4	4	4	4	4
Vitamin mixture [c]	(ml)	1	1	1	1	1	1	1	1
Water [d]	(ml)	45	35	15	5	44	34	12.5	2.5

[a] Water solution of methionine (40 g per liter).
[b] Salt mixture of Hübbel *et al.* (1937) with the following differences (in g per kg salt mixture) : $MnSO_4$, H_2O: 3.50; $ZnCl_2$: 1.66; $CaCl_2$, 6 H_2O: 0.08.
[c] Per kg diet: thiamine: 10 mg; riboflavin: 10 mg; vitamin B_6: 10 mg; nicotinic amide: 50 mg; Ca-pantothenate: 10 mg; folic acid: 1 mg; biotin: 0.2 mg; vitamin B_{12}: 0.03 mg; choline chloride: 1 000 mg; inositol: 200 mg; para-aminobenzoic acid: 500 mg; hydrosoluble vitamin A: 6 000 IU; hydrosoluble vitamin E: 100 mg; vitamin K_1: 1 mg; vitamin D_2: 500 IU.
[d] Amount of water added so as to obtain a homogeneous paste.

supplementation, increased protein intake and by the substitution of starch for fructose in the diet (Fig. 1).

The variation of dam and young body weights was positively correlated with the diet intake (Fig. 1).

When lowering the dietary protein content from 20 to 8% casein, the plasma levels of triglycerides, cholesterol esters and phospholipids were reduced from day 2 of lactation and did not change afterwards (Table II).

On day 2 of lactation, the nature of the diet did not affect the liver neutral lipid and phospholipid contents of the dam (Table III). On days 7 and 14, the liver phospholipid content was reduced when the level of casein was lower. In females fed the 20% casein diets, this content was higher when the diet did not contain fructose (Table III).

On days 7 and 14, the liver neutral lipid content was strongly enhanced by methionine supplementation in groups 8 (8% casein) and by the presence of 40% fructose in groups 20 (20% casein) (Table III).

The level of free threonine in the plasma of the dam was reduced with the 8% casein diets on days 2, 7 and 14 of lactation, except for diets not supplemented with methionine (groups 8S and 8F) on day 14. In those cases, the plasma level of free threonine was similar to that observed with the 20% casein diets (Table IV).

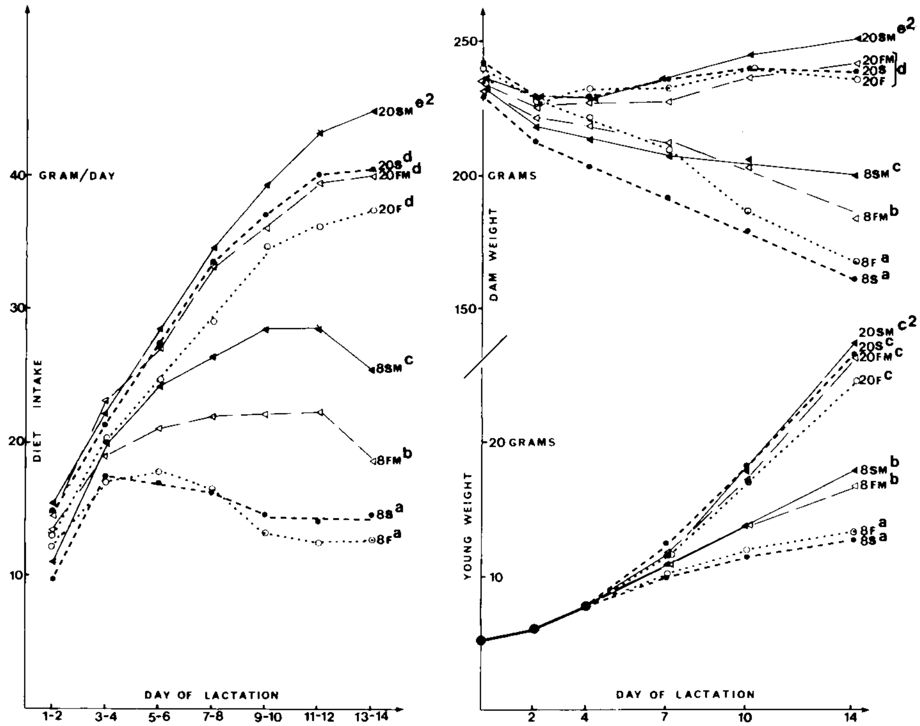

Fig. 1. Diet intake of dam, and body weights of dam and young during lactation 8 or 20: diet with 8 or 20% casein; S: starch-based diet; F: fructose-based diet; M: methionine-supplemented diet. [2] on day 14, means with one same superscript letter are not statistically different ($P > 0.05$).

Table II. Triglyceride (TG), cholesterol ester (CE) and phospholipid (PL) contents of dam plasma (in mg of methyl esters/100 g).

Groups	*8S*	*8SM*	*8F*	*8FM*	*20S*	*20SM*	*20F*	*20FM*	*Pooled SEM*
TG [1]	54 abc	50 abc	46 a	49 ab	64 d	64 d	57 bcd	59 cd	3.2
CE [1]	46 a2	50 a	47 a	57 ab	78 bc	74 bc	83 c	81 c	7.5
PL [1]	124 ab	121 ab	119 a	118 a	148 c	150 c	140 bc	140 bc	6.4

[1] For each group, plasma TG, CE or PL contents are not statistically different on days 2, 7 and 14: also these contents are pooled.
[2] Means with one same superscript letter are not statistically different ($P > 0.05$).

Discussion

Methionine supplementation of a low protein diet (8% casein) stimulated the feed intake of the dam and the growth of young, especially with a starch-based diet; between days 7 and 14 the young gained weight more rapidly, as previously observed (Leclerc, 1984). With a normal protein diet there was no difference in the weight of young between the groups. This confirms former results concerning methionine supplementation (Leclerc, 1980) and the nature of dietary carbohydrates (Bouillon and Berdanier, 1983; Lakshmanan *et al.,* 1983). However, between days 7 and 14 of lactation, the young of group 20SM showed a more rapid growth rate, probably due to the higher feed intake of the dam from this group. Regardless of the casein content of the diet (8 or 20%), addition of methionine to a starch-based

Table III. Neutral lipid and phospholipid contents of dam liver (in g of methyl esters/100 g of diet liver) on day 2 and days 7 and 14.

Groups	*8S*	*8SM*	*8F*	*8FM*	*20S*	*20SM*	*20F*	*20FM*	*Pooled SEM*
Neutral lipids									
day 2	1.9 a2	2.3 a	2.6 a	2.1 a	1.8 a	1.5 a	2.4 a	2.3 a	0.31
days 7 + 14 [1]	1.3 a	9.8 b	1.8 a	11.4 b	2.6 a	2.6 a	10.4 b	8.1 c	0.70
Phospholipids									
day 2	2.4 a	2.1 a	2.4 a	2.3 a	2.4 a	2.6 a	2.4 a	2.4 a	0.12
days 7 + 14 [1]	2.2 ab	2.2 ab	2.2 ab	2.0 a	3.0 c	2.8 c	2.4 b	2.3 b	0.10

[1] For each group, liver neutral lipid or phospholipid contents, not statistically different on days 7 and 14, are pooled.
[2] See footnote 2 under Table II.

Table IV. Threonine content of dam plasma (in µmol/100 g of plasma) on days 2, 7 and 14.

Group	*8S*	*8SM*	*8F*	*8FM*	*20S*	*20SM*	*20F*	*20FM*	*Pooled SEM*
Day 2	15 [a1]	13 [a]	14 [a]	14 [a]	32 [b]	27 [b]	25 [b]	27 [b]	4.6
Day 7	10 [a]	5 [a]	12 [a]	5 [a]	28 [b]	36 [bc]	40 [c]	36 [bc]	3.0
Day 14	33 [a]	9 [b]	35 [a]	9 [b]	26 [a]	35 [a]	33 [a]	35 [a]	5.7

[1] See footnote 2 under Table II.

diet (without sucrose or fructose) increased the feed intake of the lactating rat.

During growth, the plasma threonine content is positively correlated with the dietary protein level (Gustafson *et al.*, 1986). The same phenomenon was observed during lactation from day 2 after littering. However, on day 14, females from groups 8S and 8F showed high plasma levels of this amino acid. Diet intake in dams was clearly decreased, and the growth of the young was sharply reduced; this implies a low milk yield. There was probably an impaired utilization of the free amino acids in the plasma, such as threonine, at the time of protein synthesis in mammary gland : the plasma level of free-threonine was then highly increased in late lactation. In contrast, since milk production increased more markedly than feed intake (Leclerc, 1980) in females of groups 8 SM and 8 FM the utilization of plasma threonine was better and the plasma free-threonine content remained low.

During growth, the plasma triglyceride content is independent of the dietary protein level (Bydlowski *et al.*, 1981). With a low protein diet, this content is increased in late pregnancy (Tournier *et al.*, 1984), whereas during lactation it was decreased according to our observations.

The nature of dietary carbohydrates had no effect on the plasma triglyceride content during lactation. It has been demonstrated that a sucrose- or fructose-rich diet increases this content in male rat or man (Waddell and Fallon, 1973; Hallfrisch *et al.*, 1978). This fact is due to a sex-difference in the response to dietary carbohydrates (McDonald, 1976).

The liver lipidosis observed with some diets (8SM and 8FM, 20F and 20FM) was associated with methionine supplementation for low protein diets and the presence of a large amount of fructose for normal protein diets.

For low protein diets, the results observed on day 14 suggest than this liver lipidosis could be attributed to a secondary threonine deficiency, as during growth (Harper *et al.*, 1954). However, on day 7 of lactation, females from groups 8S and 8F as those from groups 8SM and 8FM exhibited low plasma free threonine contents. Nevertheless, at this stage, in the first two groups they did not exhibit liver lipidosis, whereas they did in the last two groups. Thus, there seems to be no relationship between low plasma free threonine content and liver lipidosis in the lactating rat fed a low protein diet supplemented with methionine: the fact that liver lipidosis was not suppressed during lactation by threonine supplementation (Leclerc *et al.*, 1988) confirms this hypothesis.

As in the growing rat (Cohen and Teitelbaum, 1964; Aoyama *et al.*, 1980), there is an increase in the liver lipid content in the lactating rat when the normal protein diet contains a high proportion of fructose; this is not the case in adult male rat (Herzberg and Rogerson, 1988).

Both the methionine supplementation of a low protein diet and the presence of sucrose or fructose in a normal protein diet cause an increase in liver lipogenesis (Maeda *et al.*, 1975; Herzberg and Rogerson, 1981; 1988). This alone could not account for lipidosis. The transport of triglycerides from the liver to the blood or lipoprotein synthesis was probably depressed. Since nutrient requirements are much higher during lactation, it is suggested that, under our experimental conditions, the need for one or several lipotropic factors was not met.

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References

Aoyama Y. & Ashida K. (1978) Effect of dietary amino acid composition on lipid content of rat liver. *Nutr. Rep. Int.* 17, 463-473

Aoyama Y., Hattori Y., Yoshida A. & Ashida K. (1979) Effect of deficiency in individual essential amino acids in diets on liver lipid content and serum triglyceride level of growing rats. *Nutr. Rep. Int.* 20, 669-675

Aoyama Y., Hattori Y., Yoshida A. & Ashida K. (1980) Effects of glucose and fructose in a diet or a drinking water on body weight gain, liver lipid content and serum triglyceride level in 4 strains of rats. *Nutr. Rep. Int.* 22, 801-810

Bacon B.R., Park C.M., Fowell C.M. & McLaren C.E. (1984) Hepatic steatosis in rats feed diets with varying concentrations of sucrose. *Fundam. Appl. Toxicol.* 4, 819-826

Bouillon D.J. & Berdanier C.D. (1983) Effect of maternal carbohydrate intake on mitochondrial activity and on lipogenesis by the young and mature progeny. *J. Nutr.* 113, 2205-2216

Bydlowski S.P., Stivaletti V.L.G. & Douglas C.R. (1981) Effect of a low protein—high lipid diet on plasma lipid levels of rats. *I.R.C.S. Med. Sci. Libr. Compend.* 9, 834

Cohen A.M. & Teitelbaum A. (1964) Effect of dietary sucrose and starch on oral glucose tolerance and insulin-like activity. *Am. J. Physiol.* 206, 105-108

Dagnelie P. (1975) *In : Théories et Méthodes Statistiques,* Vol. II. Les presses agronomiques de Gembloux, Belgique

Folch J., Lees M. & Sloane Stanley G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissue. *J. Biol. Chem.* 226, 497-509

Gustafson J.M., Dodds S.J., Burgus R.C. & Mercer L.P. (1986) Prediction of brain and serum free amino acid profiles in rats fed graded levels of protein. *J. Nutr.* 116, 1667-1681

Hallfrisch J., Lazar F., Michaelis O.E. & Reiser S. (1978) The effect of sucrose and starch on serum lipids of human subjects. *Fed. Proc.* 37, 628

Harper A.E., Benton D.A., Winje M.E. & Elvehjem C.A. (1954) «Antilipotropic» effect of methionine in rats fed threonine-deficient diet containing choline. *J. Biol. Chem.* 209, 159-163

Herzberg G.R. & Rogerson M. (1981) Dietary corn oil does not suppress the fructose induced increase in hepatic fatty acid synthesis. *Nutr. Res.* 1, 73-82

Herzberg G.R. & Rogerson M. (1988) Hepatic fatty acid synthesis and triglyceride secretion in rat fed fructose or glucose-based diets containing corn oil, tallow or marine oil. *J. Nutr.* 118, 1061-1067

Hübbel R.B., Mendel L.B. & Wakeman A.J. (1937) A new salt mixture for use experimental diets. *J. Nutr.* 14, 273-285

Juaneda P. & Rocquelin G. (1985) Rapid and convenient separation of phospholipids and non phosphorus lipids from rat heart using silica cartridges. *Lipids* 20, 40-41

Lakshmanan F.L., Howe J.C. & Barnes E. (1983) Effect of dietary protein level and kind of carbohydrate on growth and selected pathological and biochemical parameter in female BHE rats. *Nutr. Res.* 3, 719-732

Leclerc J. (1980) Effect of a methionine supplement to 2 diets poor in riboflavin and with different protein concentrations on the riboflavin metabolism of lactating rats. *Ann. Nutr. Aliment.* 34, 641-655

Leclerc J. (1984) Comparative effects of carbohydrate content (fructose or glucose) and methionine supplement in the diet of lactating rat. Feed intake and milk yield of dam, body weight of dam and progeny. *Nutr. Rep. Int.* 29, 769-774

Leclerc J., Hamel O. & Grynberg A. (1988) Methionine and threonine supplementation of a protein poor diet during lactation. *Nutr. Rep. Int.* 38, 635-642

Leclerc J., Miller M.L. & Septier C. (1985) Comparative effects of carbohydrate content (fructose or glucose) and methionine supplement in the diet of lactating rat. Lipid concentrations in the plasma and liver of the dam. *Nutr. Rep. Int.* 32, 1003-1008

Maeda H., Ikeda I. & Sugano M. (1975) Behavior of the liver key lipogenesis enzymes in rat fed threonine imbalanced diet. *Nutr. Rep. Int.* 12, 61-66

McDonald I. (1976) Sex differences in response to dietary carbohydrates. *Proc. Nutr. Soc.* 35, 155-157

McLaughlan J.M. (1979) Fatty liver in rat fed soy protein isolate with added methionine. *Nutr. Rep. Int.* 19, 27-35

McLaughlan J.M., Noel F., Morrison A.B. & Campbell J.A. (1961) Blood amino acid studies. 1. A micromethod for the estimation of free lysine, methionine and threonine. *Can. J. Biol. Physiol.* 39, 1669-1674

Moritoki K. & Yoshida A. (1970) Effect of methionine supplementation to a low casein diet on plasma level of threonine and other amino acids of rats. *J. Jpn. Soc. Food Nutr.* 23, 351-355

Tournier H., Yaryour G., Mercuri O. & De Tomas M.E. (1984) Effect of protein depletion or the VLDL-triacylglycerol secretion and apoprotein synthesis by the perfused liver from pregnant rats. *Lipids* 19, 902-905

Waddell M. & Fallon H.J. (1973) The effect of high carbohydrate diets on liver triglyceride formation in the rat. *J. Clin. Invest.* 52, 2725-2731

Wang S.T. & Peter F. (1983) Gas—liquid chromatographic determination of fatty composition of cholesteryl esters in human serum using silica Sep-Pak cartridges. *J. Chromatogr.* 276, 249-256

Wiener R.P., Yoshida M. & Harper A.E. (1963) Influence of various carbohydrates on the utilization of low protein rations by the white rat. *J. Nutr.* 80, 279-290