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Metabolism of lipoproteins in Rodent malaria,

Relationship between lipolysis, steatosis and increased biosynthesis of V.L.D.L.

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Métabolisme des lipoprotéines du paludisme de Rongeur. Relation entre la *lipolyse, la stéatose et l'augmentation de la biosynthèse des V.L.D.L.*

RESUME. L'étude cinétique des acides gras libres sériques, des lipides totaux et des triglycérides hépatiques, nous a conduit à conclure que la biosynthèse des lipoprotéines riches en triglycérides devrait être augmentée au cours du Paludisme.
Il semble que le parasite induise une lipolyse du tissu adipeux afin d'assurer ses besoins en acide gras, notamment en acide palmitique, et que l'excès d'acides gras libéré par la lipolyse induise une synthèse accélérée des lipoprotéines riches en triglycérides.
L'acide cis-vaccénique a aussi été dosé en fonction de la parasitémie ; il paraît peu probable que ces variations puissent expliquer l'hémolyse extra-parasitaire.

SUMMARY. The kinetic study of the seric free fatty acids, total lipids and hepatic triacyglycerides had led us to conclude that the biosynthesis of T.A.G.-rich lipoproteins increases during malaria.
It seems that the parasite induces a lipolysis of adipose tissue in order to meet its own needs for fatty acids and that the excess of the latter taken by the liver involves an increased synthesis of the V.L.D.L.

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The cis-vaccenic acid has also been analysed during the evolution of parasitaemia; these variations by themselves cannot explain the extra parasitic hemolysis.

Introduction

In a previous report (1) we described major modifications of the lipoprotein metabolism during experimental rodent malaria. They are characterized by a transient increase of triacylglycerides (T.A.G.) rich lipoproteins, chylomicrons, Very Low Density Lipoproteins (V.L.D.L.) and Low Density Lipoproteins (L.D.L.) and at the same time a decrease in High Density Lipoproteins (H.D.L.). Simultaneously we observed a transient increase in liver weights *P. chabaudi* model shows this observation perfectly. An almost similar phenomenon was observed in man (2).

This led us to put forward the following hypotheses as regards the lipoprotein metabolism during malaria (1):

1 — A lowered clearance of chylomicrons and V.L.D.L. by endothelial lipoprotein lipase;

2 — An increased synthesis of V.L.D.L., related to increased lipolysis and the subsequent catabolism of V.L.D.L. into I.D.L. and L.D.L.;

3 — A metabolic regulation of plasma H.D.L. concentration by increased levels of triacylglycerides-rich lipoproteins (mainly V.L.D.L.).

The classic mechanism of V.L.D.L. hepatic biosynthesis being the following one: adipose tissue lipolysis associated with release of free fatty acids, uptake of the latter by the liver converting them into T.A.G., then in V.L.D.L. A rise in the V.L.D.L. rate should then be preceded by a sudden increase in seric free fatty acids (FFAs) followed by an increase in hepatic T.A.G.

Many authors have studied seric FFA acid and hepatic TAG rates (3) (4) (5) (6). Their works using various strains of *Plasmodium* were generally carried out only at the time of the parasitaemia peak.

The present paper deals with the measure in relation to parasitaemia evolution, the rate of seric FFAs and hepatic TAG; the results obtained would led us to conclude that the biosynthesis of endogenous TAG-rich lipoproteins is accelerated.

Material and methods

We used 3 months-old female Swiss mice weighing 20 to 25 g (Janvier Farm). They were fed with a diet containing 5% of lipids, non deficient is essential fatty acids. The composition in fatty acids of this food was follows: 16:0 20%, 18:0 9%, 18:2 30%, 18:1 (n—7) 1.7%, 18:2 32%, 18:3 (n—3) 3% for the more frequent fatty acids.

The *P. chabaudi* strain, chosen for its non fatal evolution was inoculated by intraperitoneal injection of 10^6 parasites per mouse. Parasitaemia was measured on blood smears fixed and stained with methanol-Giemsa.

Preparation of sera and livers.

5 mice were daily killed by bulb shock and after beheading their blood immediately collected on adequate media. This method was the best to avoid an additional release of fatty acids in blood at the times when the mice were killed. Then, the plasma were gathered to carry out the different biochemical titrations. Livers were also collected.

Seric fatty acid titration.

Seric fatty acids were titrated by gas liquid chromatography (G.L.C.) according of Fruchart's procedure, slightly modified (7).

200µl of serum were extracted by 2 ml of Trout solvent (heptan-iso-propanol-H_2SO_4 N, 1/4/0.1, V/V/V). That solvent contained a known quantity of free fatty acids 17:0 as internal standard. After 10 mn rest, 1.2 ml of heptan and 0.8 ml of water were added. The mixture was kept at rest in order to obtain a good demixing.

The upper organic layer was dried over nitrogen stream, diluted in a minimum volume of chloroform and purified by preparative thin-layer chromatography on silicagel, developed with the traditional solvent of neutral lipids (petroleum ether, ethyl ether, acetic acid 9/1/0.1, V/V/V). The fraction corresponding to FFAs was collected. This ultimate purification is crucial if one want to measure only free fatty acids. Thus collected fatty acids were converted into methyl esters by sulfuric methanol according to Nouvelot (8). After methanolysis, FFA methyl esters were extracted by heptan and injected in the chromotograph.

The G.L.C. was carried out on 50 m long capillary column, full of carbowax 20 M or OV 101. A temperature programmation between 150-220 °C at the rate of 2 °C per minute gave a good separation of fatty acid methyl esters and especially of cis-vaccenic acid (C.V.) (18:1, n—7) (*fig. 1*). The equipment used was a Girdel type 300 which could be coupled with a mass spectrometer.

Other methods.

1 — Glycemia: glycemia was daily determined by glucose oxydase method on a centrifuge analyser.

2 — Liver lipid determination: total lipids were extracted by Folch's technique (9), weight determination of total lipids, TAG colorimetric determination were carried out according to Fruchart (7).

3 — Determination of total free fatty acids in adipose tissue and free parasites: total lipids were also extracted by Folch's classic technique (9), then treated as previously after methanolysis. Free parasites were isolated according to Charet *et al.* (10) then treated in the same way for the determination of fatty acids.

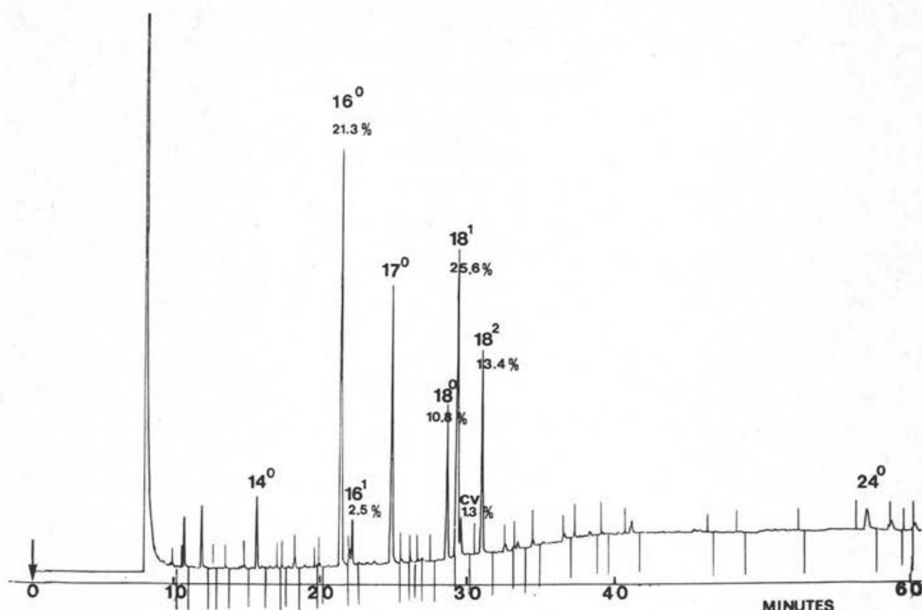

Fig. 1. — Gas chromatography of seric free fatty acids (*P. chabaudi*, day 9) on Girdel 300, 50 m glass capillary column coated with Carbowax 20 M. Conditions : Carrier gas : N_2, pressure 0,5 bar, temperature programmed 150°C - 225°C, 2°C/min.

4 — Determination of urinary catecholamines: by pressing daily the bladders of 150 infected or non infected mice, at fixed time, it was possible to obtain 10 to 20 ml of urine.

Catecholamines were determined after extraction by fluorimetry according to Natelson (11).

The urinary creatinine has been determined by Jaffe classical method on a centrifuge analyser (Boehringer kit).

Results

1 — *Variation of seric FFAs.*

The evolution of total seric free fatty acids in relation to parasitaemia is reported in *figure 2*. These experiments have been carried out three times, the results in each case are similar and here we have chosen to report the most significant experiment. This evolution, at first is characterized by a sudden increase (day I) as early as the beginning of the infection whereas the parasitemia is lower than 1%.

Then after a decrease the rate of the free fatty acids varies strongly (day 5-day 7) while the parasitemia increases.

The evolution of the main fatty acids is shown in *figure 3*. We can also observe three peaks. But between day 4 and day 8, two peaks of increasing FFAs appear. We can note that the fatty acids in C 18:2 and C 18:1 increase again, whereas the rate of 16:0 varies: it increases (day 5), then collapses at day 6 and increases again at day 7.

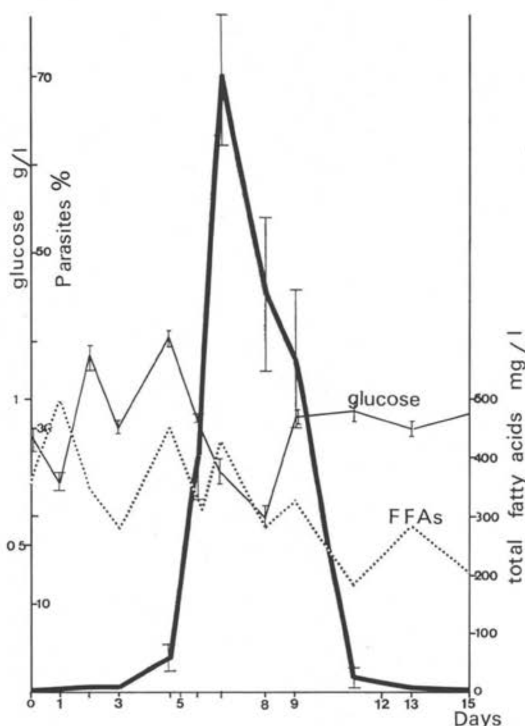

Fig. 2. — Evolution of total seric free fatty acids (··········) and glycemia (———) in relation to parasitaemia (━━━). Each determination corresponds to the average of 5 titrations (± standard deviation) on serum pool from 5 mice.

2 — *Clycemia.*

As expected, glycemia has often an opposite evolution of that of FFAs (*fig. 2*).

3 — *Total lipids and hepatic TAG.*

Total lipids and hepatic TAG show a noticeable increase preceding slightly the peak of parasitaemia, which coincides with the second increase in seric free fatty acids (*fig. 4*).

4 — *Composition in fatty acids of free parasites,* non infected red blood cells and adipose tissue.

Table I shows the composition in total fatty acids of these different elements.

We shall note the characteristic composition of the adipose tissue in comparison to that of the free parasite and the non infected red blood cell which have almost identical compositions.

Table I. — *Composition in F.As of the adipose tissue, normal mouse red blood cells and* P. chabaudi *(per cent of the main F.F.As).*

F.A.	*P. chabaudi*	R.B.C.	Adipose tissue
16 : 0	30,8	37	22,3
16 : 1	0,8	< 1	1,9
18 : 0	10,8	14.9	6,3
18 : 1 (n - 7)	0,7	< 1	2,5
18 : 1 (n - 9)	14,35	19,3	33,8
18 : 2	10,4	13,5	28,9

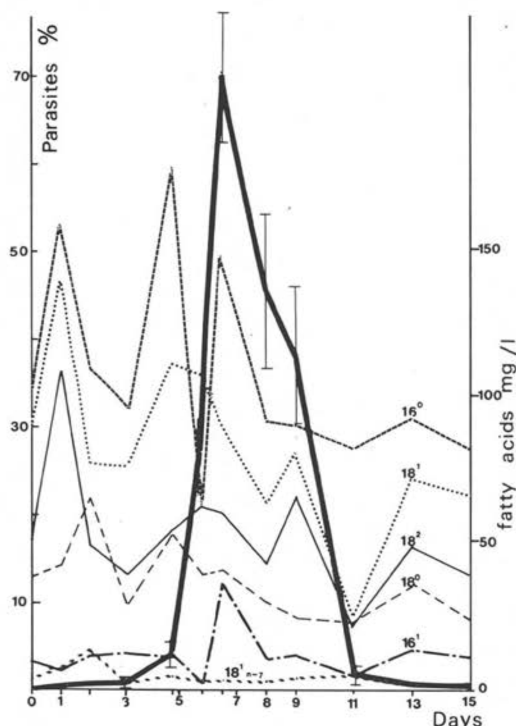

Fig. 3. — Evolution of the rate of the main seric free fatty acids during the evolution of the parasitaemia (———) : 16 : 0 (-----), 16 : 1 (-·—·-), 18 : 0 (— — —), 18 : 1 (n - 7) (C.V. ------), 18 : 1 (n - 9) (··········), 18 : 2 (———).

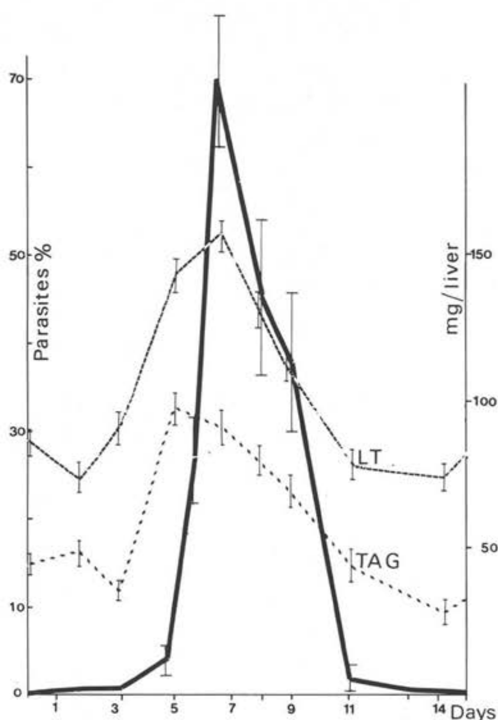

Fig. 4. — Evolution of the rate of total lipids (-------) and hepatic TAG (------) in relation to the parasitaemia (———). Eeach point corresponds to the average of 5 determinations (± standard deviation) out of a pool of 5 livers.

5 — *Determination of urinary catecholamines.*

Table II shows the evolution of the content in catecholamines of mouse urines during the first 7 days of the infection.

We can point out that catecholamine rate notably increases only at day 4 when parasitaemia starts increasing; it becomes again normal whereas parasitaemia is high, in spite of a decrease in the urinary volume.

Table II. — *Determination of catecholamines and the urinary creatinine during an experimental infection carried out on 150 mice. The coefficient of variation of the method is 4.4 %.*

Days	0	1	2	3	4	5	6	7
% parasitaemia	—	—	0,1	1	5	12	40	50
Urinary volume : ml	18,7	16	14	13	11	9,3	9,5	10
Creatinine : mg/l	560	552	547	559	565	584	866	825
Catecholamine : μg/l	101,6	85,6	105	113	145	100	101	108

6 — *Determination and identification of cis-vaccenic acid.*

Some fatty acids and especially the cis-vaccenic acid 18:1 (n—7) (*fig. 3*) drew particulary our attention, considering the hemolytic role that some authors reported about it [Laser (12), Holz (13)].

Besides identification by direct separation in gas liquid chromatography, the coupling whith mass spectrometry enables us to obtain identical mass spectra of standard and experimental cis-vaccenic acids, corresponding to those described by Ryhage (14), which identifies this acid (*fig. 5*). Determination of the cis-vaccenic acid rate during parasitic evolution shows that it first doubles as early as the beginning of the infection.

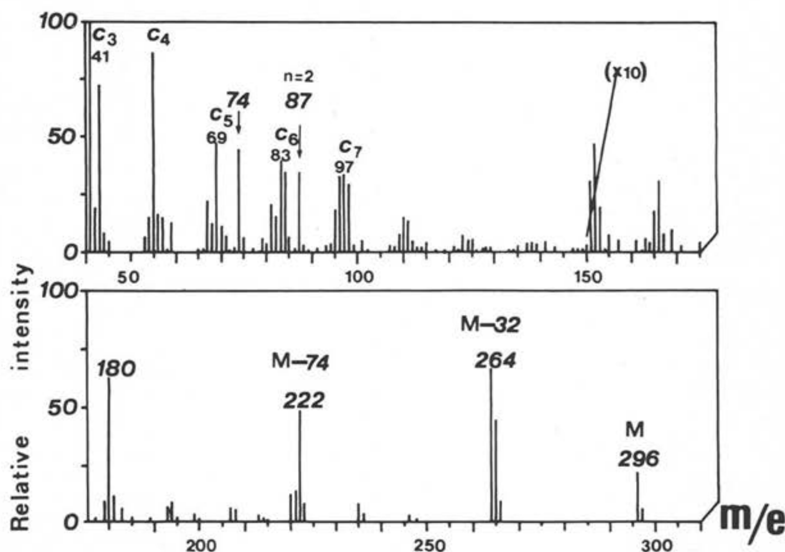

Fig. 5. — Mass spectra of the methyl (n - 7) octadecenoate obtained by coupling gas chromatography and mass spectrometry (*P. chabaudi*, day 9).

Discussion

In a previous paper, Maurois *et al.* (1), we showed an important increase in VLDL, endogenous origin lipoproteins and chylomicrons whose origin is exogenous, during experimental malaria. We expressed the hypothesis that these facts resulted from an inhibition of lipoprotein lipase activity (L.P.L.).

In order to show this hypothesis, we have estimated the activity of adipose tissue L.P.L. (15), and we have found a decrease of this enzyme activity during the infection.

If indeed the enzyme inhibition is enough to explain the increase of chylomicrons; as regards the VLDL, the chronology of the facts which have been observed, leads us to conclude that irrespective of this inhibition of L.P.L., V.L.D.L. synthesis must be highly stimulated according the following system:

1 — Acceleration of the release of fatty acids from the adipose tissue (day 1-day 2 and day 4-day 7 mainly); the adipose origin of fatty acids freed at the beginning of the infection is certain, for we note an increase in the 18:2, an essential fatty acid which can only come from reserve lipids (Table 1).

2 — Captation of seric free fatty acids by the liver and synthesis of hepatic TAG whose increase is noted mainly about day 4-day 8 at the same time as the second increase of lipolysis.

3 — *Release of TAG as seric VLDL.*

This variation of the rate of fatty acids is indeed borne out by the opposite evolution of the glycemia as Randle described it as early as 1963 (16).

This sudden acceleration of the release of fatty acids from the adipose tissue is likely due to a classic mechanism, Eisenberg (17): activation of membrane adenylcyclase of adipocytes, synthesis of cyclic AMP and increase in triacylglyceride lipase activity. Simultaneously, the high level of c AMP inhibits lipoprotein lipase activity which delays the breakdown of lipoproteins rich in TAG (chylomicrons and VLDL) and can explain the excessive seric accumulation of the latter.

The adenylcyclase activation can be carried out by various factors: by stress with release of catecholamines of by other activators such as ACTH, glucagon, TSH... The most likely hypothesis being the stress, we have titrated urinary catecholamines. Unfortunately the stress can not be taken into consideration until the 4th day and can not explain the first flow of free fatty acids.

But the other factors can act on the synthesis of cAMP. Indeed as early as 1973, in malaria, Shoemaker (18) showed an early increased activity of the thyroid in the mouse infected by *P. berghei*. Finally, more recently Hertelendy *et al.* (19) showed that the synthesis of cAMP was increased in parasitized red blood cells. The factors inducing this stimulation in the red blood cell must likely cause the same effect in the adipocyte.

When the parasitaemia increases strongly (day 5) a new flow of seric free fatty acids occurs (*fig. 2*). The analysis fatty acid by fatty acid (*fig. 3*) shows important changes of the main fatty acids that we try to explain as following:

— from day 5 to day 7 there is always a great lipolysis as the increase in fatty acid in particulary in 18:2 and 18:1 shows it;

— at day 6 the parasitaemia keeps on increasing highly and synchronously, we note that the rate of 18:1 and 18:2 fatty acids are high again, and that the 16:0 collapses. This might be explained by the fact that the 16:0 would be the most important FFA used by the *Plasmodium* during its growth (*table I*);

— at day 7 the relative and absolute composition of free fatty acids is indeed different and seems quite similar to that of the parasite and red blood cells (*table I*). At that time

of intense lysis, the release of fatty acids might likely come from lysed red blood cells and parasites.

At the same time (day 5-day 8) we have previously noted (Maurois *et al.*, 2) that the liver weight doubled while the rate of hepatic TAG almost trebled (*fig. 4*). This increase in hepatic TAG represents the main part of that of total hepatic lipids. It would be likely that apoprotein synthesis necessary for the future metabolism of hepatic TAG as VLDL is not sufficient. This could explain partly why this transient steatosis was observed.

Finally, let us note that, in malaria, the cis-vaccenic acid had never been identified directly. Although its rate obviously varies during our experiment, these variations remain insufficient to explain by themselves so marked and hemolysis which persists, as we demonstrated (20) a long time after the disappearance of parasites.

In conclusion we can assert that the parasite induces from the beginning of infection to day 8-9 a lipolysis of the adipose tissue, and an increase of FFAs rate in circulation.

Also in relation to the nutritional needs of the parasite for same particular FFAs and to release of FFAs from lysed cells, the excess of seric FFAs being taken by the liver induces a transient storage of TAG which is used progressively for an increased VLDL synthesis.

Finally this work enabled us to show that the study of changes in lipid metabolism of the host, absolutely must be carried out kinetically.

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