Platelet-Activating Factor (PAF) Antagonists in Foods: A Study of Lipids with PAF or Anti-PAF-like Activity in Cow's Milk and Yogurt

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Yogurt, a food with indisputable nutritional value, is also used for therapeutic purposes. Milk derivatives are blamed for some pathological effects of yogurt noted in selected subjects such as sensitive newborns or infants. In this study, we investigated the probable existence of platelet-activating factor (PAF) and lipids inhibiting PAF action in raw and incubated milk and yogurt. Detection of these substances may explain the controversial properties of these milk products. The in vitro biological study of lipids in washed platelets showed little production of PAF in incubated milk (0.3−0.8 ng/100 mL sample) although the concentration of PAF in milk fat remained constant during the majority of the incubation time. Yogurt lipids of intermediate polarity presented stronger inhibitory activity against PAF than lipids corresponded to raw or incubated milk. Our data demonstrated that *Streptococcus thermophilus* and *Lactobacillus bulgaricus* biosynthesize important quantities of PAF inhibitors, whereas random contamination of milk leads to the production of small amounts of PAF and PAF inhibitors.

Keywords: *Platelet activating factor (PAF); PAF inhibitors; lipids; yogurt; milk bacteria*

INTRODUCTION

Platelet activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is one of the most potent inflammatory phospholipid mediators (Demopoulos et al., 1979) that is synthesized by and that acts on most proinflammatory cells (Hanahan and Kumar, 1987). The role of PAF in modulation of the immune response has been well documentated, but it also exhibits profound effects on the physiology and pathology of lung, kidney, blood vessels, heart, and digestive tract, including lather gastric ulcers and intestinal damage (Koltai et al., 1990). Two metabolic steps are involved in the biosynthesis of PAF. The action of phospholipase A_2 on membrane alkyl-acyl phosphorylcholine lipids results in the production of lyso-PAF, and acetylation of the lyso-compound by an acetyltransferase yields the biologically active molecule. In addition to mammals, a lot of bacteria, including pathogenic ones, are able to produce PAF with exogenous lyso-PAF (Denizot et al., 1989).

The antibacterial action of fermented milk and lactic drinks is known to cure mild cases of diarrhea in patients who have consumed pathogenic bacteria, and yogurt has been used since ancient times to prevent diarrhea and intestinal problems. Yogurt is also very effective against lactose intolerance, and many studies since 1973 have shown an antitumor activity of this product. It is believed that all these actions are due to the live bacteria of the yogurt, mainly *Lactobacillus* species, and to the highly digestible nutrients that cause a normalizing of the intestinal microflora and an increase in the *Bifidobacterium* population (Yukushi et al., 1992).

Denizot and Benveniste (1989) detected PAF and large amounts of immediate PAF precursors, such as alkyl-acylglycerophosphocholine and lyso-PAF, in commercial cheese and yogurt samples. The presence of PAF in these products (1 ng/100 g of sample) is less than the necessary amount for any pathological effects in humans (10 ng/kg). However, considering the immunoregulation effects of PAF and the putative effect of yogurt on the immune response (De Simone et al., 1986), we believe that a relation between these two properties could be postulated. It remains to be ascertained whether or not the presence of PAF in dairy products can explain some of the pathological effects of milk derivatives in selected subjects, such as sensitive newborns or infants.

In this work, which is part of a study to investigate the relation between PAF and foods (Koussisis et al., 1993, 1994), lipid fractions of cow's milk and yogurt were tested for PAF or anti-PAF like activity in washed rabbit platelets. The presence of these substances was related to the random infection and the increasing bacterial population in the dairy samples.

MATERIALS AND METHODS

Solvents and Reagents. All solvents used were of analytical grade and were purchased from Merck (Darmstadt, Germany). Standard lipids were purchased from Sigma (St. Louis, MO), Merck, or Serva (Heidelberg, Germany). Semisynthetic PAF was synthesized in our laboratories (Demopoulos et al., 1979).

Thin-Layer Chromatography (TLC) Plates. The 20 × 20-cm glass TLC plates were coated with silica gel G and activated by heating at 130 °C for 30 min. The thickness of the TLC plates was between 0.5 (analytical) and 1.0 mm (preparative). Up to 50 mg of lipids per TLC plate were used for fractionation; every fraction was scraped off, extracted with various mixtures of chloroform and methanol, and centrifuged, and the liquid phase was evaporated under a nitrogen atmosphere.

Sample Characteristics. A total of four milk samples were examined, each one in triplicate. The raw cow's milk samples used were collected within the first 48 h after milking

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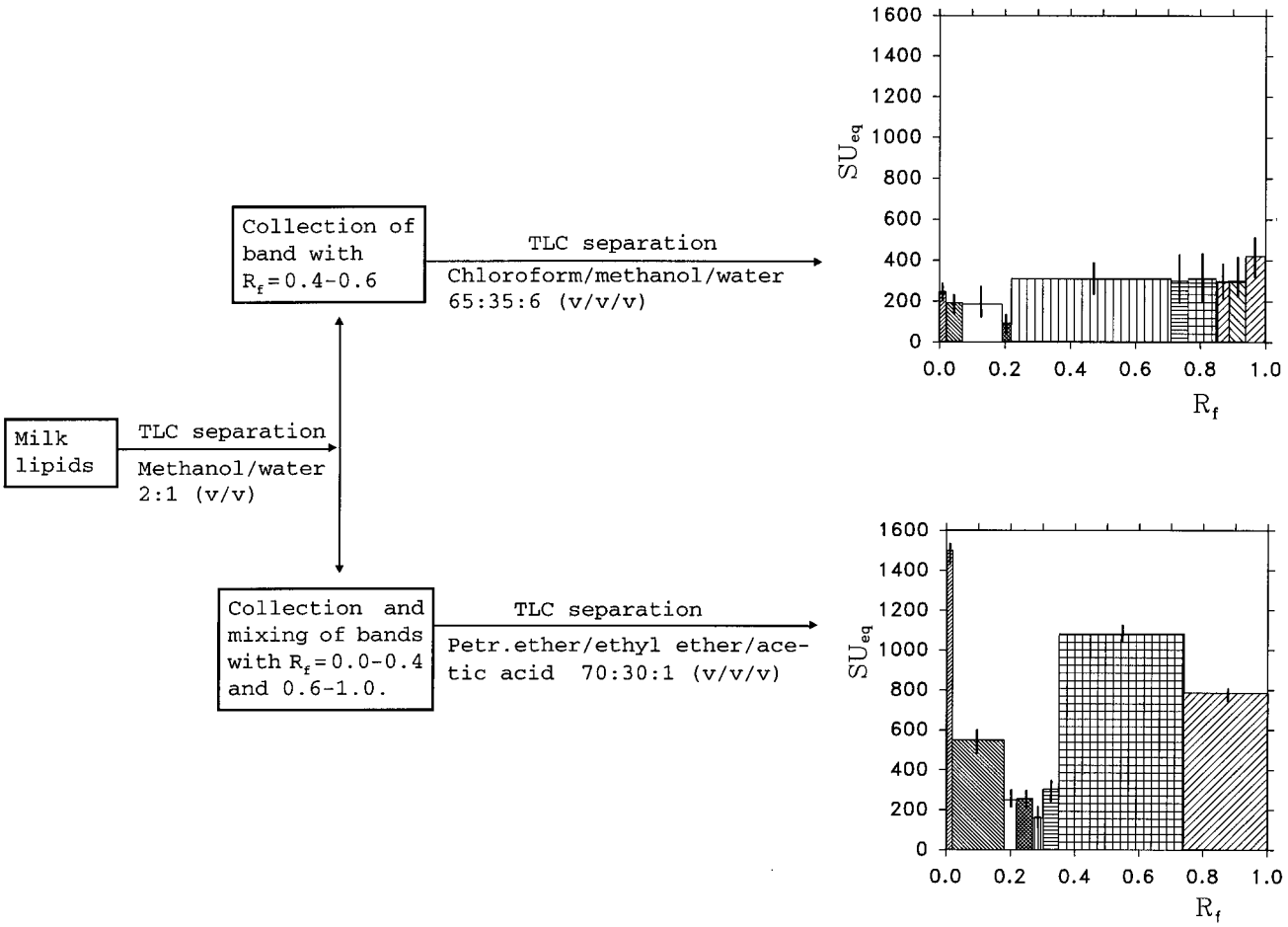

Figure 1. Inhibitory effect (expressed as SU_{eq} versus R_f) of milk lipids separated by TLC on PAF-induced platelet aggregation, where SU_{eq} is the equivalent units of the activity of the fraction per milligram of total milk lipids causing 50% inhibition (IC_{50}).

to insure they had a high microbial content. The organoleptic characteristics (color, odor, taste) and the proximate analysis results (pH, specific gravity, acidity, moisture fat, protein contents) of the milk samples did not deviate from the normal values. The yogurt produced with the aforementioned milk also had normal characteristics (no whey separation, good cohesiveness, fat and moisture contents within limits).

Microbial Counting. The standard plate count method (Meeser et al., 1985) was used for the determination of the mesophyllic bacteria in milk. The incubation took place at 32 °C for 48 h.

Preparation of Starter Culture. Skim milk powder of 10% total solids was pasteurized at 85 °C for 30 min. A few milligrams of freeze dried yogurt culture, containing *Streptococcus thermophilus* and *Lactobacillus bulgaricus* (1:1), were added to 150 mL of the heat-treated milk (1st culture), and the inoculated milk was incubated at 43 °C until its acidity expressed as lactic acid reached 0.8%. A 150-mL sample of the skimmed milk powder solution was inoculated with the 1st culture at a concentration of 2.5% (v/v) and incubated at 43 °C until its acidity reached 0.8% within 3−4 h (2nd culture). The same procedure was repeated to produce the final culture (3rd culture), with the difference being that the acidity had to reach 0.8% within 2-3 h.

Yogurt Production. The raw milk samples, after heat treatment at 85 °C for 30 min, were inoculated with the starter culture at a concentration 2.5% (v/v) and incubated at 43 °C for 24 h.

Isolation and Fraction Separation of Milk and Yogurt Lipids. The lipids of 100-g samples were extracted by the technique of Bligh and Dyer (1959), with a mixture of chloroform, methanol, and water. The separation of the total lipid fractions was achieved by successive TLC on silica gel plates. The solvent systems was methanol:water (2:1, v/v) for the total lipid extract. The band with R_f = 0.4−0.6 was subjected to a second chromatography procedure with chloroform:methanol:water (65:35:6, v/v/v to detect the presence of PAF-like activity. The previously obtained bands of the methanol:water system, with R_f = 0−0.4 and 0.6−1.0, were subjected to a second TLC development with petroleum ether: ethyl ether:acetic acid (70:30:1, v/v/v). Chloroform:methanol: water (65:35:6, v/v/v) was used for the rechromatography of polar lipids obtained from the previous TLC systems.

Isolation and Fraction Separation of Incubated Milk Lipids. Samples of the raw milk (200 mL) were incubated at 37 °C from 0 up to 48 h to increase the mesophyllic microorganisms population. Every sample, after microbial counting, was centrifuged under chill conditions (4000 rpm for 5 min in a constant angle refrigerated centrifuge of 17 cm radius). Three layers were collected; namely, the upper (U; fat), the intermediate (I), and the lower (D, microorganisms). The lipids of every layer were extracted by the Bligh-Dyer technique and fractionated by preparative TLC. The developing system was methanol:water (2:1, v/v). The band with R_f = 0.4−0.6 was rechromatographed with chloroform:methanol: water (65:35:6, v/v/v) on silica gel, and the three collected bands, with R_f values of 0−0.19 (a), 0.19−0.23 (b), and 0.23−1.00 (c), were tested for biological activity.

Biological Activity in Vitro. Aliquots of the isolated fractions were evaporated to dryness under a nitrogen stream, and the residue was dissolved in 200 μL of a solution containing 2.5 mg of bovine serum albumin (BSA) per milliliter of saline. Each isolated fraction was tested for its ability to aggregate washed rabbit platelets or to inhibit PAF- and thrombin induced aggregation. The biological activity of each fraction was measured in a chronolog aggregometer (Demopoulos et al., 1979).

Data Processing. The TableCurve program of Jandell Scientific and a series of mathematical operations were used to calculate the amount of sample that caused 50% inhibition

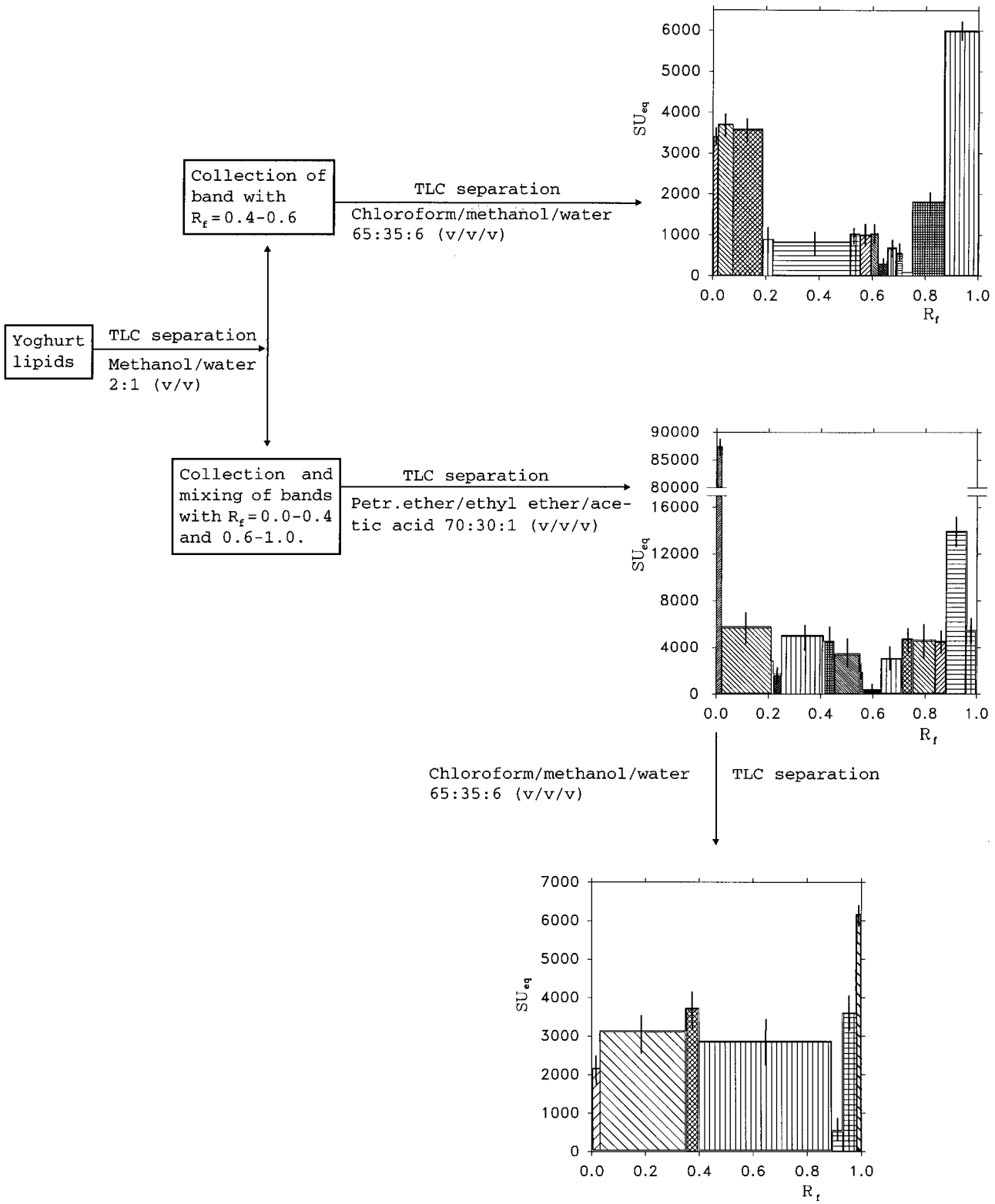

Figure 2. Inhibitory effect of yogurt lipids on PAF-induced platelet aggregation. The fraction with $R_f = 0.0-0.02$ (solvent system, petroleum ether:ethyl ether:acetic acid, 70:30:1, v/v/v) was rechromatographed with chloroform:methanol:water (65:35:6, v/v/v).

for a given concentration of PAF (1.25×10^{-10} M, final concentration) and thrombin (0.01 U/cuvette 0.5 mL), causing submaximal reversible aggregation. The activity of the sample is expressed by SU_{eq}, which represents the equivalent units of the sample causing 50% inhibition (IC_{50}) per milligram of total lipids. The same treatment was followed for each TLC plate and the results are presented in bar charts.

RESULTS

Presence of Biologically Active Lipids in Milk. None of the isolated fractions, including the one corresponding chromatographically to PAF, induced aggregation in washed rabbit platelets. Some of the isolated fractions inhibited PAF-induced aggregation (Figure 1). Finally, none of the fractionated lipids inhibited thrombin-induced aggregation.

Presence of Biologically Active Lipids in Yogurt. As in the case of milk, none of the isolated lipid fractions induced aggregation in washed rabbit platelets. Some of the lipid fractions caused potent inhibition of PAF-induced aggregation (Figure 2). None of these fractions

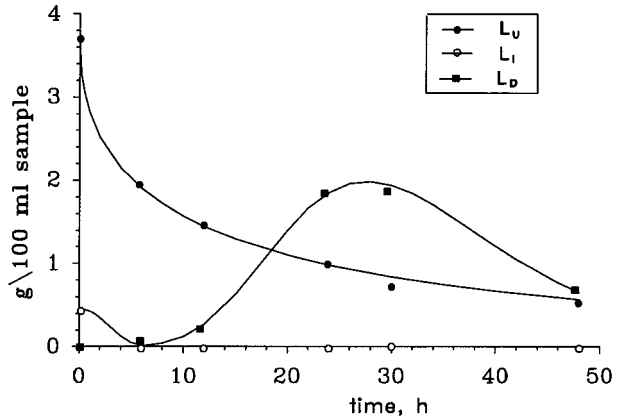

Figure 3. Concentration change of milk lipids during incubation in the three phases taken after centrifugation. (U) upper phase (milk lipids); (I) intermediate phase; (D) down phase (bacterial lipids). (Figures 3−6 include data from the same experiment.)

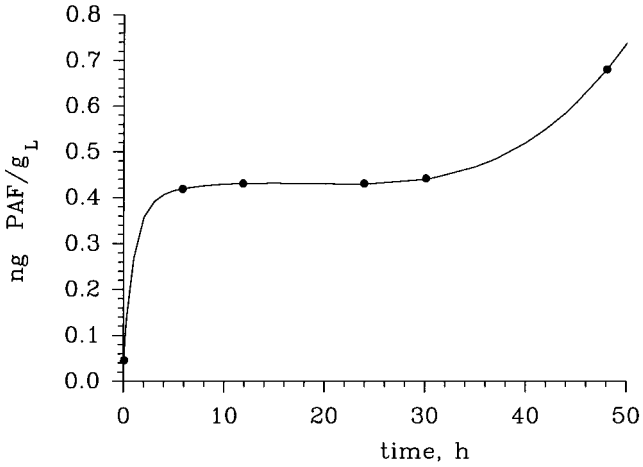

Figure 4. Aggregation activity of fraction U_b (ng PAF/g phase lipids) versus incubation time.

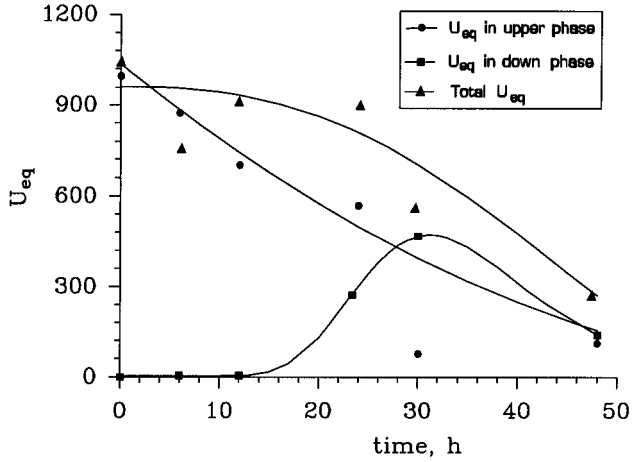

Figure 5. Effect of incubation time on equivalent units (U_{eq}) of inhibitor from fraction c for upper and down phases and for total sample.

inhibited thrombin-induced aggregation, so they are specific antagonists of PAF.

Effect of Incubation on the Biological Activity. The growth curves of bacteria population versus incubation time showed that the microorganisms present in the milk samples, under these experimental conditions, were characterized by a rapid logarithmic growth phase followed by a short stationary one. The growth of acid-producing bacteria resulted in a drastic reduction of pH

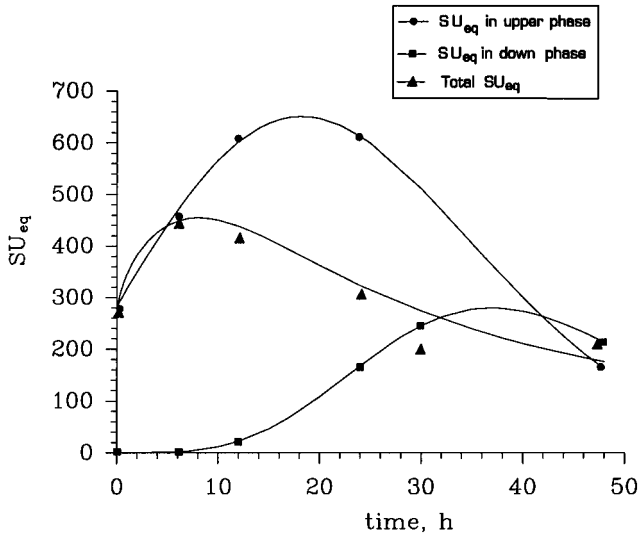

Figure 6. Effect of incubation time on specific equivalent units (SU_{eq}) of inhibitor from fraction c (expression of concentration of lipidic fraction in active compound).

from 6.4 to 4.5 and coagulation (at pH 4.9−4.7) after 7 ± 2 h from the start of incubation. In addition to this, the active biosynthetic process during population growth causes a decrease of the original lipids of milk (upper phase, U) and an increase of the bacterial ones (lower phase, D) that finally decreases after the death of the microorganism (Figure 3).

Most of the b fractions (with R_f = 0.19−0.23 as described in Materials and Methods) of the upper phase (U) corresponded chromatographically to PAF, and caused a small reversible aggregation in platelets equivalent to PAF values of 0.3−0.8 ng per 100 mL of sample during the incubation time. As shown in Figure 4, the concentration of the PAF-like fraction in the lipids of the upper phase remained almost constant and equal to 0.4 ± 0.1 ng of PAF per gram of lipids for most of the incubation time. None of the fractions of the lower phase D (microorganisms) exhibited an ability to induce platelets aggregation.

Although fractions c presented a moderate inhibition of PAF-induced aggregation, it was much lower than the one caused by the TLC bands in yogurt. The total quantity (U_{eq}) of antagonists in upper and lower phases decreased during incubation time (Figure 5) because their decrease in milk lipids U (Figure 5) was more intense than their increase in microorganisms lipids D (Figure 5), which had a bell-like shape. Nevertheless, the concentration of active compounds (SU_{eq}) always presented a maximum that for the whole sample corresponded to time of maximum population or to maximum biosynthetic activity (Figure 6).

DISCUSSION

PAF has been detected into a great number of biological fluids, so its presence in cow's milk is probable. The fact that no PAF-like activity was detected in milk may be because the PAF concentration was lower than the detection limit of the method used (10^{-11} M in aggregometer's cuvette or 0.15 ng per 100 g of sample) or because of incomplete separation of the lipids in the samples with the resulting presence of PAF antagonists.

Some of the isolated fractions obtained from milk inhibited PAF-induced aggregation. Nevertheless, the low value of SU_{eq} and the poor reproducibility of TLC bands indicate that the inhibition to PAF induced

aggregation is a result of the large amount of lipids (e.g., fatty acids).

As in the case of milk, none of the isolated lipid fractions obtained from yogurt induced aggregation in washed rabbit platelets, and conclusions analogous to those drawn from the milk results can be made. Some of the lipid fractions from yogurt caused a potent inhibition to PAF-induced aggregation. Although SU_{eq} represents a semiquantitative index of the concentration of the active compound in the sample, its high values for yogurt in comparison with those for milk (two or three orders of magnitude larger) and the better reproducibility of the TLC bands indicate that these lipid fractions contain strong antagonists of PAF, which structurally belong to lipids of intermediate polarity.

To what extent the excess of acetic acid will produce PAF or structural analogs with antagonistic activity to PAF depends on the presence of other substrates (e.g., alkyl-acyl-glycero-phosphocholine, Lyso-PAF, etc.) and mainly on the existence of appropriate enzymes, which in turn depends on the presence of bacteria. In this way, the biochemical processes of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* in yogurt, resulted in the formation of lipids with medium polarity and strong inhibitory activity on PAF-induced platelet aggregation. On the contrary, in the case of random contamination of milk, PAF was probably produced by the action of exoenzymes (given that PAF was detected in lipids and not in the microorganisms) and PAF antagonists. However, the PAF produced by random contamination of milk had lower activity than that biosynthesized in yogurt. Although the maximum content of each compound corresponds approximately to the maximum activity of microbial population, the lipid fraction of milk contained a constant and low amount of PAF (Figure 4) for considerable time.

Further study of the random contamination of milk is required, especially during the first hours of microbial growth because milk becomes unsuitable for consumption, no matter what the PAF content is, because of the poor organoleptic characteristics and the high microbial count.

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