CHAPTER V

SUMMARY AND CONCLUSION

The present study comprises three main experiments: 1) the preparation of platelet concentrates (PC) and 4 kinds of lecithin-rich fat emulsions (LRFE); 2) the establishment of condition for the incubation between PC and LRFE in order to yield good production; 3) the study of fatty acid transferred to and from platelets after the incubation. All results of 3 experiments are summarized as follows.

A. The preparation of platelet concentrates (PC) and 4 kinds of lecithin-rich fat emulsions (LRFE).

All PC's were prepared and provided for us under the supervision of the Chief of Plasma and Fractionation Section, Thai Red Cross Society: Mrs.Aroonrat Chantanakajornfung. All specimen were donated from healthy subjects with HIV-free and hepatitis-free.

1. One hundred and twenty two units of PC with various blood groups (A, AB, B and O) were employed in the study.
2. The volunteers as separated according to gender were 100 for male (M) and 22 for female (F).

3. PC bags prepared from bloods of both genders contained not different amounts of various blood cells, either platelets, white blood cells and red blood cells, specific gravity and volumes. Thus the gender provided no effect to the preparation of PC.
4. When PC were separated according to blood groups: B, 97; O, 11; AB, 8; A, 6. There was no difference observed among characteristics of PC bags classified according to blood groups.
5. In PC bag, platelets were vast majority of blood cells. The contamination of white blood cells in PC was not exceed to 0.03% where as that of RBC was trace and ignoreable.
6. Two kinds of fish meal were selected for the present study: the grade 1 Thai fish meal and Danish fish meal. The latter was produced from marine fish of the North Atlantic ocean. The former was from gulf of Thailand.
7. Lecithin of Danish fish meals had DHA content of 27-29% whereas Thai fish meals had 20-23%. Danish fish meal was chosen for the present study according to its prominent in lipid characteristics as well as availability and accessibility in comparison to Thai fish meal.
8. In addition, the contents of polyenes, EPA, DHA, total n-3 PUFA's and the ratio of n-3 to n-6 PUFA of Danish fish meal were significantly higher than those of Thai fish meal.
9. Danish fish meal had proximate contents of moisture 7.52, protein 64.55, fat 8.08 and ash 15.60 g/100 g sample.
10. The present experiment recovered fat content in fish meal by the procedure of three consecutive extractions employing 3 different organic solvents: acetone, methanol,

n-hexane. Fat content found was 17.4 with lecithin content of 10.2 g/100 g sample. This higher fat content than ever expected is probably explained by several reasons: the higher recovery of fats by our procedure; the partial contamination of polar proteins following methanol extraction and/or; the remaining of water droplets derived from the moisture.

11. Lecithin extracted from Danish fish meal comprised 24.5 PL and 75.5 TG in weight % which provided PL:TG ratio of approximately 1:3.
12. Danish fish meal lecithin had EPA and DHA contents of 7.51 and 21.68 g/100 g total fatty acids, respectively. TG-FA had DHA content of 17.56 whereas PL-FA had DHA content of 28.32g/100 g total fatty acids. Danish fish meal lecithin was thus a good source of n-3 PUFA's especially DHA and the latter present majorily in PL subfractions.
13. All polyenoic fatty acids present majorily in PL fractions in comparison to TG fraction (44.18 vs 32.84 for polyenes, 38.49 vs 27.88 for n-3 and 5.69 vs 4.96 for n-6 PUFA's of PL-FA vs TG-FA, respectively).
14. PL of fish meal had lower content of MUFA ($p < 0.05$) but higher content of PUFA's in both groups of n-3 and n-6 polyenoic fatty acids ($p < 0.05$). Therefore, n-3 PUFA were major polyenes in fat of marine animals. The contents of n-3 and n-6 PUFA's obtained from fish meal used in the study were 5-6 times different. DHA dominated in moieties of both TG and PL in much higher amount in PL than in TG. This confirms the fact that DHA of marine fish distributed favorably in PL.
15. Four fat emulsions with high content of lecithins, i.e., FM-LRFE, SY-LRFE and SL-FOFE were prepared in the present study whereas 20% Lipofundin (EY-

LRFE) was commercially available. Lecithin extracted from Danish fish meal had PL content of 24-25 g/100g. TG was as high as nearly three-fourth of weight content of fish meal lecithin. Low PL to TG ratio of fish meal lecithin thus limited the composition of PL and TG in all other 3 fat emulsions used in the experiment to be 1:3 (w/w) except for EY-LRFE so as to the results of incubation would be compared.

16. Three fat emulsions have ratio of PL and TG physically similar with different in the chemical property of their fatty acid composition in moieties of both surface (PL) and core lipids (TG). The content of DHA in the lecithin fraction (surface) of FM-LRFE was approximately 61% higher than that found in TG fraction (core) (28.32 vs 17.56 for PL-DHA vs TG-DHA).
17. Total n-3 PUFA of the surface of FM-LRFE was nearly 38% higher than that of the core (38.49 vs 27.88). By contrast, SY-LRFE and SL-FOFE show their rich of n-6 FA's expecially LA in the surface (57.77 vs 57.61) whereas 20% Lipofundin (commercial EY-LRFE) exhibits high content of LA and AA in the surface as 15.24 and 2.87%, respectively.
18. The ratio of n-3 to n-6 PUFA of FM-LRFE's surface lipids was much greater than those of SY-LRFE, of SL-FOFE and of 20% Lipofundin (6.76 vs 0.13, 0.13 and 0.27, respectively).
19. The highest content of DHA found in FM-LRFE's surface whereas small amount was found in 20% Lipofundin's surface but none in SY-LRFE's and SL-FOFE's surfaces.

20. The result confirms that fish meal was the rich source of DHA-containing lecithins which exhibited predominantly at the surface of the prepared emulsion. Markedly higher contents of n-3 PUFA and DHA present in the surface of FM-LRFE in comparison to other 3 emulsions: SY-LRFE, SL-FOFE and 20% Lipofundin, were obviously impressive and statistically significant ($p < 0.05$). By contrast, when n-6 PUFA contents in the emulsions' surface were considered, FM-LRFE possessed the lowest value whereas SY-LRFE and SL-FOFE surfaces contained majorily of n-6 PUFA exclusively LA to the value of approximately 58 g/100g total PL-FA.

B. <u>The establishment of incubation condition for PC and LRFE.</u>

1. To study the effect of presentation of autologous plasma on fatty acid exchanges, the incubation of platelets in incubation mixture with and without autologous plasma.
2. The alteration of individual fatty acids of platelets incubating wihtout plasma were higher than that of platelets incubated with autologous plasma.
3. At lecithin concentration of 600 mg/dl, DHA increased 31% in the presence of plasma (from 2.44 rose upto 3.2) but soared 236% in the absence of plasma (from 2.47 rose upto 5.82).
4. The incubation of platelets with FM-LRFE were performed for 1 h at 22°C in lecithin concentration at 600 mg/dl. Incubation without plasma yielded platelets with higher content of DHA and n-3 PUFA than that incubated with plasma (5.82 vs 3.2 and 8.31 vs 4.9 for DHA and n-3 PUFA's, respectively).

5. Without plasma, the content of n-3 of platelets increased markedly higher than that of the incubation with plasma in contrarily to the results of n-6 PUFA's. Both ratios of EPA to AA and n-3 to n-6 PUFA's of platelets incubated without plasma increased to the levels significantly higher than those of platelets incubating with plasma.
6. Incubation of platelets in fat emulsions without plasma induced the marked increment of n-3 PUFA of platelets upto 4.45 g/100 g total FA's whereas incubating with plasma affected much less (1.12 g/100 g total FA's). Subsequently with the prominent effect of incubation without plasma, the incubation of platelets with all LRFE's were established without plasma in all experiments of the present study.
7. To investigate the stability of fatty acids in platelets after the incubation, platelets were subjected for incubation with lecithin at highest concentration and after stopping the incubation, platelets were soaked in NSS for certain periods of time before processing for fatty acid analysis.
8. After soaking in NSS for 0, 1, 3 and 5 h, all altered fatty acids were found to remain unchanged after long period of time. There was no significant alteration of platelet FA at the various times prolonged. The results demonstrated that fatty acids changed on platelet membranes still remained stable for a period of time at least 5 h.

C. The study of fatty acid transfere to and from platelet membranes after incubation.

1. PC at the concentration of 1.86 x 10^9 cells/cm^3 were incubated with 4 types of fat emulsions at various concentrations of PL ranging from 0-600 mgPL/100 ml incubation mixyure.
2. The alteration of individual fatty acids of platelets after incubating with FM-LRFE: 3 platelet fatty acids obviously affected by FM-LRFE were investigated: AA, EPA and DHA. There was marked rise of n-3 PUFA ($p<0.001$). N-3 polyene increment is higher than the proportion of n-6 polyene drop. The consequent rise of n-3 to n-6 ratio was then shown ($p<0.001$).
3. The saturated and monoenoic fatty acids were not much affected by SY-LRFE. The obvious alterations were observed in polyenoic fatty acids in both groups of n-3 and n-6. N-6 fatty acid shows its significant increment in relation to the contents of PL in incubation mixture.
4. When platelet incubated with 20% Lipofundin at various concentrations, the results were likely to demonstrate the maintenance of membrane fatty acid composition. Only minor alteration in fatty acid composition was observed.
5. Incubating platelets with FM-LRFE, the six major membrane fatty acids were markedly affected and changed in their contribution in platelets. Two fatty acids demonstrated their mark increments: DHA and palmitic acid (C 16:0). Among all fatty acids, DHA exhibited its tramendous increment whereas C 16:0 rose with much less extent.

6. Four fatty acids decreased, 2 in 4 were saturated: C 18:0 and monoene : C 18:1 n-9, other two were polyenes: LA and AA. Among them, C 18:0, C 18:1 n-9 and LA showed their gradual drop whereas AA reached the plateau at the lowest concentration of PL at 100 mg/dl incubation mixture.
7. Platelets after incubated with SY-LRFE, LA rose markedly reaching the relative value of approximately 155%. Rationally, the huge amount of LA present in SY-LRFE's surface was responsible for the rise in membrane. The losses of membrane fatty acids were found in three major fatty acids especially C 18:0, AA and DHA. The replacemant of LA for C 18:0, AA and DHA at sn-2 position of membrane PL was probably the answer of this exchanges.
8. The effect of SL-FOFE on platelet membrane was investigated. The results of 5 major platelet fatty acids affected by SL-FOFE changed resemblingly with SY-LRFE except for C 18:1n-9 which showed its gradual increment with SY-LRFE but maintained with SL-FOFE.
9. After the incubation of platelets with 20% Lipofundin, 6 major platelet fatty acids were slightly affected. The steady decrement of AA and DHA were probably responsible by the rise of LA. Noticeably, slight drop of DHA shows that it was probably affected by the incubation with 20% Lipofundin.
10. The alterations of membrane fatty acids after incubation with 4 fat emulsions were summarized herewith. Saturated fatty acids rose upto 1.25 and 0.37 of platelet incubated with respective FM-LRFE and 20% Lipofundin whereas SAFA of those incubated with SY-LRFE and SL-FOFE decreased. The decrement of monoenoic fatty acids were found in platelets incubated with 4 fat emulsions.

11. Incubating with SY-LRFE and SL-FOFE induced the marked increment of polyenes in platelets upto 4.32% and 4.72%, respectively whereas FM-LRFE and 20%Lipofundin affected much less (1.46 and 0.19, respectively, $p<0.05$). FM-LRFE induced the marked increase of n-3 FA upto 4.45% with DHA contributed for 75% (3.35 in 4.45) whereas no alteration was observed with SY-LRFE, SL-FOFE and 20%Lipofundin. Marked decrease of n-6 FA was found in FM-LRFE group in contrary to the result observed in SY-LRFE and SL-FOFE which show their rise upto respective 3.52% and 3.87%. Differently from two former groups, 20%Lipofundin shows consistency of n-6 FA. All results of n-3 FA and n-6 FA as described earlier lead to much higher ratio of n-3 to n-6 PUFA of FM-LRFE in comparison to those of SY-LRFE, SL-FOFE and 20%Lipofundin.
12. Slope and r values were obtained from linear regression of equation. All platelet PUFA's except for LA were affected in relation to the concentrations of PL in FM-LRFE: n-3 PUFA's increased whereas n-6 PUFA's (except for LA) decreased. The correlation between the ratio of n-3/n-6 PUFA's and the concentration of FM-LRFE's PL was impressive at the value as high as 0.95 ($p<0.001$).
13. High correlation to nearly 1 between n-3 PUFA's: EPA and DHA, and FM-LRFE 's PL concentrations (0.93, 0.94 and 0.95 for r values of EPA, DHA and n-3 PUFA, respectively) imply that FM-LRFE was effective donor of n-3 PUFA's to platelets.
14. The concentrations of SY-LRFE's PL yielded high correlation with PUFA's of platelets especially ALA and two major n-6 PUFA's: LA and AA (0.93 and 0.88 for r of LA and AA, respectively). The correlation was low and non-significant

with n-3 PUFA's. The results imply that SY-LRFE behaves like a good carrier of n-6 PUFA's as well as of ALA for platelets. The alteration of n-3 to n-6 PUFA ratio was, however, not dramatically affected with SY-LRFE as found with FM-LRFE (r equal to 0.65 vs 0.95 for n-3 to n-6 PUFA ratio of SY-LRFE vs FM-LRFE).

15. The effectiveness of SL-FOFE as the carrier of PUFA to platelets was similar to SY-LRFE except that there was no correlation in n-3 to n-6 PUFA ratio with SL-FOFE (r equal to 0.38 vs 0.65 for SL-FOFE vs SY-LRFE). SL-FOFE is thus a good carrier of n-6 PUFA's as well as of ALA for platelets but not for EPA and DHA.
16. Among 4 fat emulsions, 20% Lipofundin affected the least alteration of platelet FA. The statistically significant p values were found at rather low values of r not exceed to 0.65 for ALA (r, 0.61), EPA (r, 0.48) and LA (0.45). The results of correlation imply that commercial fat emulsion with egg yolk PL do not provide fatty acids to blood cells. It is thus considered as highest stable emulsion in comparison to other emulsions prepared in our experiment.

Implementation of The Results of The Present Study

We have demonstrated in our previous experiment that our novel fat emulsions with lecithin rich in n-3 PUFA especially DHA could induce the augmentation of n-3 PUFA in erythrocyte membranes after brief incubation for 1 h (Chatnilbandhu, 1996). We then extrapolated the effectiveness of the previous study to

other blood cells and we selected platelets as the genuine active cells for the present experiment. While erythrocytes are considered as inert cells without nucleus, platelets are recognized in the opposite aspect. Platelets are highly active and the way to handle them for the experiment are delicate and need much more sophisticate procedure. After having tried several trial and error techniques for dealing with platelets, we succeeded with a low-temperature incubation procedure as described in the method.

We not only confirmed the increment of n-3 to n-6 PUFA ratio of the platelets and speculate their alteration of various membrane functions especially in eicosanoid production, but also confirmed our hypothesis for the first time that emulsion's core TG yields barely effect to the exchange of fatty acid between the platelets and the surface of fat emulsion particles. The results of the present study as well as from our previous one encourage us to perform advanced experiment dealing with *in vivo* study initiating with animal model in order to evaluate the novel liposomes and/or emulsions with tailored n-3 to n-6 PUFA ratio appropriate for correcting abnormal cell n-3 to n-6 PUFA ratio. The application is also speculated for further investigation in the field of clinical biochemistry in the future.