CHAPTER III

MATERIALS AND METHODS

Animals

Twenty mid-lactating crossbred Holstein Friesians , 4-5 months after calving and have normally California mastitis test (CMT) were used. All animals were housed in sheds and tethered in individual stall and fed twice daily. Animals individually received amount of concentrate and roughage related to milk production. Cows were milked twice a day in the morning at between 4.30 and 6.30 h and in the afternoon between 16.00 and 18.00 h.

Experimental procedures

Experiments were carried out on twenty cows that prior to the experiment had been assessed their milk quality test with 75%EtOH. Ten cows were assigned to positive EtOH test group, that is, their milk precipitated with 75% EtOH. Other ten cows were assigned to had negative EtOH test group, that is, their milk did not precipitate with 75% EtOH. Both groups, studies were made of the effect of P_i on stability and physicochemical properties of milk both *in vivo* and *in vitro*.

Series 1: *In vivo* study.

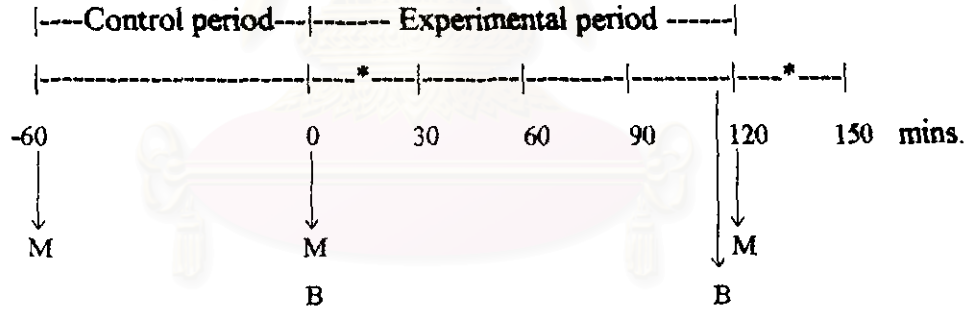
On the day of experiment at 9.00 h, the animal in each group was milked by milking machine and followed by hand milking again until the udder cistern was empty. The animal was kept in stall for 1 hour as the control period. Before the experimental began the animal was local anesthetized by subcutaneous injection of 0.5 ml. xylocaine (Astra, Sweden) through the skin around ear vein. After xylocaine injection, polyvinyl catheter was inserted into ear vein by using a Medicus intravenous cannula (Insyte No.8, Jelco Critikon). This catheter was left in place for the inorganic phosphorus (P_i) solution infusion throughout the experimental period.

At the end of control period prior to P_i solution infusion, milk was collected by hand milking. A venous blood samples were collected from the milk vein under local anesthetized by subcutaneous injection of 0.5 ml. xylocaine by venipuncture with a #21 needle while an arterial blood sample was collected from the cocygeal artery by venipuncture with a #18 needle. Blood samples in heparinized tube were kept in crushed ice for electrolyte determinations. Plasma sample was kept at -20°C until analyzed. Milk sample of the control period was divided into four portions. The first portion was used for determination of casein concentration. The second portion was used for determination of the milk compositions. The third portion was used for in vitro study and the last portion was used for ethanol stability test.

In the experimental period, the primed injection of the solution containing 5ml. of 20% phosphonotonic (Vetoquinal) and 5 ml. of 62.8 g% sodium hydrogen phosphate (BDH) in normal saline (Thai Otsuka pharmacential Co.LtD) was

performed via ear vein and then following by the sustaining infusion of solution of P_i (containing 62.8 g% sodium hydrogen phosphate in normal saline, 20% phophonotonic and normal saline at the ratio 3:2:5) by a peristaltic pump (Model 3, Eyela) at a constant rate of 2.27 ml/min for 120 min. throughout the experimental period.

About 15 minutes before the end of experimental period approached, blood samples were collected from coccygeal artery and milk vein. Milk sample in the experimental period was collected and divided into three portions, the first portion was used for determination of casein concentration, the second was used for determination of the milk compositions and the last portion was used for ethanol stability test. The protocol for sample collections is shown in Figure 4.



M = milking

B = blood

* = EtOH stability test

Figure 4. Diagram of experiment in *in vivo* study

Series 2: *In vitro* study.

On the day of experiment, the third portion of milk after the control period was performed. Three concentrations of inorganic phosphorus (P_i) were added into milk samples with dipotassium hydrogen phosphate solution (Riedel-de Haen). Milk from each animals were allocated into 4 treatments as below. Each level of P_i in the solution was prepared by diluting dipotassium hydrogen phosphate with distilled water as the required concentration. Four treatments were carried out in this series as the following:

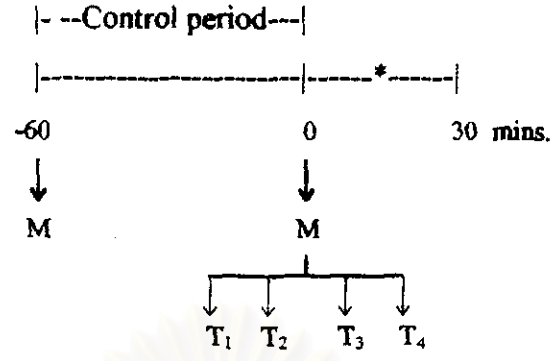
Treatment 1. as 10 ml. of milk +100 µl. of distilled water (control)

Treatment 2. as 10 ml. of milk +100 µl.of P_i solution (10 mg% P_i)

Treatment 3. as 10 ml. of milk +100 µl.of P_i solution (50 mg% P_i)

Treatment 4. as 10 ml. of milk+100 µl.of P_i solution (90 mg% P_i)

All treatments were tested in the stability of milk by ethanol at 6 levels of concentration , 68, 75, 80, 85, 90 and 95% of ethanol and measured the turbidity of milk. The milk sample was let stand about 15 minutes before EtOH stability test. The studied of *in vitro* is shown in Figure 5.



M = milking

T1, T2, T3 and T4 = Treatment 1, 2, 3 and 4 respectively

* = EtOH stability

Figure 5 : Diagram of experiment in *in vitro* study

Determinations of mammary arteriovenous difference and mammary extraction ratio of phosphorus and calcium concentrations

Inorganic phosphorus concentration in plasma was measured by colorimetric method as described by Fiske and Subbarow (1925). Phosphorus ions were determined in a trichloroacitic acid, TCA,(Carlo Erba) by the formation of molybdiphosphate, followed by reduction molybdenum blue with methyl-p-aminophenol sulfate (Elon). The sample solution was let stand about 45 minutes before the measurement of optical density by the spectrophotometer (Model 340,Sequoia-Turner corporation) at wavelength 700 nm.

Calcium in plasma was measured by colorimetric method as described by Moorehead and Biggs (1974). This method used cresolphthalein complexone form red purple with calcium in base solution (2-amino-2-methyl-1-propanol) and prevent interference from magnesium by used 8-hydroxyquinoline. The concentration of sample was read optical density by spectrophotometer (Model 340,Sequoia-Turner corporation) at wavelength 570 nm.

Determination of the stability of milk

The stability of milk was assessed by ethanol (EtOH) test as described by White and Davies (1960). EtOH stability tests were carried out at 6 levels of 68, 75, 80, 85, 90 and 95% of ethanol concentrations.

The stability of milk protein to ethanol was determined by assessing the strength of ethanol solution which added to an equal volume of milk and the formation of clot was observed.

Determination of the precipitation of milk

Two portions of milk were performed.

1. The milk turbidity of normal sample was measured by the method of Abbassy and Wahba (1986). Turbidity was determined at 600 nm in diluted milk

with distilled water (1:10) by the spectrophotometer (Model 340,Sequoia-Turner corporation).

2. The milk sample after EtOH testing was measured for turbidity by the method of Abbassy and Wahba (1986). Turbidity was determined at 600 nm in diluted milk with distilled water and its ethanol concentration (1:4.5:4.5) by the spectrophotometer (Model 340,Sequoia-Turner corporation).

Determination of the protein concentration in milk

The milk protein concentration was measured by micro Kjeldhal method. The total nitrogen content of milk means the nitrogen content expressed in percent by weight obtained by the micro Kjeldhal method.

Total N was corrected by the following equation:

$$\text{Total N} = \frac{\text{ml } H_2SO_4 \text{ in tritration x N of standard } H_2SO_4 \text{ x0.014x100 x 6.38}}{\text{g of sample.}}$$

Determination of the lactose concentration in milk

The milk lactose concentration was measured by the colorimetric method as described by Tele's method (1978). The color development was based on the

combined action of phenol, sodium hydroxide, picric acid and sodium bisulfite with lactose. The concentration of samples were read the optical density at wavelength 520 by spectrophotomer (Model 340,Sequoia-Turner corporation) .

Determination of the fat concentration in milk

The milk fat concentration was measured by the micromethod as described by Chaiyabutr (1994). Milk was filled into the microcapillary tube and was centrifugal for 15 minutes at 12500 rpm. The percentage of fat packed globule was measure by hematocrit reader (Model 150,Baker instrument). The true milk fat concentration was corrected by the following equation:

milk fat concentration (g%) = 0.75 x %fat pack globule - 0.321

Determination of the citrate concentration in milk

The milk citrate concentration was measured by spectrophotometry from tricarboxylic acid as described by White and Davies (1963). Milk sample was precipitate trichloroacetic acid (Carlo Erba). Color development supernatant was performed by action of pyridine (Merck) and acetic anhydride. The concentration of samples were read by spectrophotometer at wavelength 428 nm with a standard curve.

Determination of the α, β and κ-casein concentration

The α, β and κ-casein concentration were determined by dividing each casein by electrophoresis method. It was performed according to the modified method of Laemmli (1970) and Svasti (1977) using running gel of 15% acrylamide and stacking gel of 4% acrylamide. The procedure was described briefly below.

The 15% acrylamide in 3 M Tris-HCl pH 8.8 was prepared in the presence of 0.1% SDS. Ammonium persulfate (0.025%) and 3 µl of N, N, N′, N′,-tetramethylenediamine (TEMED) were added to polymerize the acrylamide. After complete polymerization, the 4% acrylamide in 0.5 M Tris-HCl pH 6.8 containing 0.1% SDS, 0.025% ammonium persulfate and 5 µl of TEMED were added to form stacking gel.

The sample of skim milk was precipitated at pI (4.6) by 1 M HCl and lyophilizing. The sample was used 1 mg/0.5 ml. of buffer. The sample mixture (sample mixed with sample buffer 1:1) was applied to the gel. The sample buffer as same as running buffer was Tris-HCl 3.03 g, glycine 14.4 g, SDS 1 g in distilled water 1000 ml. The electrical power was used the constant current at 30 mA in all experiment period.

The electrophoresed gel was stained by Commassie brilliant blue and destained until the background was clear. The casein concentration was determined by densitometer (Image Master VDS, Pharmacia Biotech).

Determinations of electrolyte concentration in blood and milk samples

Electrolyte concentrations in plasma and milk were measured as follow; the concentration of both sodium and potassium was measured by using a flame photometer (Chemical Flame Photometer 410C, Corning Ltd.), the chloride concentration was measured by using a chloridometer (Chloride Analyser 925,Corning Ltd.) and the magnesium concentration was measured by using an atomic absorption (Spectr AA300,Varian techtron Pty Ltd.).

Calculation:

$$\text{Soluble salt balance (SSB)} = \frac{\text{Ca (mM)} + \text{Mg (mM)}}{\text{P(mM)} + \text{citrate(mM)}}$$

$$\text{Mammary arteriovenous difference of } P_i = A\text{-}V$$

$$\text{Mammary extraction ratio of } P_i = \frac{A\text{-}V}{A} \times 100$$

A : Plasma arterial P_i concentration from coccygeal artery

V : Plasma venous P_i concentration from milk vein

Mammary arteriovenous difference of Ca = A-V

Mammary extraction ratio of Ca = $\frac{A-V}{A} \times 100$

A : Plasma arterial Ca concentration from coccygeal artery

V : Plasma venous Ca concentration from milk vein

Statistic analysis

All the data are presented as the mean $\pm$ SD. Statistical significant difference between period in the same group was determined by the Student's paired t-test. The student's unpaired t-test was used to estimate the statistical significance of the difference between groups.