III. MATERIALS AND METHODS

A. Materials

1. Specimens

Forty - seven hospitalized patient sera with abnormal liver function test were obtained from Clinical Chemistry Laboratory, Maharaj Nakorn Chiang Mai Hospital (CMH). They were 17 females and 30 males with 49 years mean of ages (range 19-72 years). They were examined and diagnosed to have a kind of liver disease by the physicians from of department of medicine ,CMH. Confirmed by the final hospital records, they were grouped into 5 groups. Three groups of ten were diagnosed as hepatitis , hepatoma and cholangiocarcinoma (CHCA). A group of seven had cirrhosis, the last group with cholestasis composed of six common bile duct stone (CBS) and four gall stone (GS).

Normal control samples

Normal control serum samples were collected from persons who had proved to be normal by parameters specific for blood collection at Blood Bank Section, Maharaj Nakorn Chiang Mai Hospital. They were five female in 36 subjects with 30 years mean of age (range 21-45 years). Their sera were determined for liver function test and serological analysis to clarify from the liver disease.

2. Instruments :

Instruments used in this study were :

- Analytical balance, Mettler H 10, Mettler Instument, Switzerland.
- Double beam Spectrophotometer, Beckman Model 25 UV - Visible.
- Electrophoresis Data Center (EDC), Helena Laboratories, Beaumont, TX., USA.
- Electrophoretic apparatus, Helena Laboratories, Beaumont, TX., USA.

- Gilson adjustable automatic pipette; p20, p200, p1000, Gilson Medical Electronics (France) S.A., France.
- Incubator, Model 800, Memmert GmloH Co, USA.
- Lyophilizer , Lioalpha-10 (Telstar), Spain.
- Magnetic stirrer, Thermolyne Co., USA.
- Microcentrifuge, MSE, USA.
- Microplate reader, Model EL 340, Bio-Tek instrument, USA.
- 96 - well Microtiter plates, Linbro, Flow Laboratories, Mc Lean, VA., USA.
- pH Meter, Model 3560, Beckman, USA.
- Power supply, Duostat, Beckman, USA.
- Stopwatch, Tanita, Fujeribio Inc, China.
- TITAN GEL Chamber, Helena Laboratories, Beaumont, TX., USA.
- UV - Visible recording Spectrophotometer UV-160 A, Shimudzu.
- Vortex mixer, Scientific Industries, Inc. Bohemea, NY., USA.
- Water bath, Hetoberkerqd, Denmark.

3. Chemical and Reagents

All chemicals used were of analytical grade :

- 2 - Amino - 2 methyl - 1 -propanol (Sigma Chemical Co., USA)
- Acetic acid glacial (Merck, Darmstadt, Germany)
- Agarose type V (Sigma Chemical Co., USA)
- Albumin, Bovine, Fraction V (Sigma Chemical Co., USA, No. A 4503)
- ALP Phos iso Control (Helena Laboratories, Beaumont, TX., USA.)
- Barbitone (diethylbarbituric acid) (BDH Chemicals, Poole England)
- Calcium chloride (Merck, Darmstadt, Germany)
- CMP - NeuNAc (Sigma Chemical Co., USA, No. C 8271)

- Concanavalin A (Con A), (Sigma Chemical Co., USA, No. C 7275)
- Copper (II) sulphate (BDH Chemicals, Poole, England)
- DEAE Sepharose (Sigma Chemical Co., USA, No. DCL-6B-100)
- Hydrochloric acid (Merck, Darmstadt, Germany)
- L - Phenylalanine (L-2-Amino-3-phenylpropanoic acid) (Sigma Chemical Co., USA, No. P 2126)
- L(+) Lactic acid (2-Hydroxypropionic Acid) Hemi-calcium Salt (Sigma Chemical Co., USA)
- Lectin from *Triticum vulgaris* (Wheat germ), (Sigma Chemical Co., USA, No. L 9640)
- Levamisole (Sigma Chemical Co., USA, No. L 9756)
- Magnesium chloride-6-hydrate R.G. (Merck, Darmstadt, Germany)
- Mucin Type I-S (Sigma Chemical Co., USA, No. M 3895)
- Neuraminidase Type III from *Vibrio cholerae* (Sigma Chemical Co., USA, No. N 7885)
- Neuraminidase Type VI from *Clostridium perfringens* (Sigma Chemical Co., USA, No. N 3001)
- 4 - Nitrophenyl phosphate, (Merck, Darmstadt, Germany)
- Periodic acid (Fluka A.G., Buchs, Switzerland)
- *Pisum sativum* agglutinin (PSA), (Sigma Chemical Co., USA, No. L 5380)
- Resorcinol (Fluka A.G., Buchs, Switzerland)
- α 2,6- Sialyltransferase (Sigma Chemical Co., USA, No. S 2769)
- Sodium barbital (Merck, Darmstadt, Germany)
- Sodium chloride (Merck, Darmstadt, Germany)
- Sodium dihydrogen phosphate (Merck, Darmstadt, Germany)
- Sodium hydroxide (Merck, Darmstadt, Germany)
- 95% tert-butyl alcohol (Carlo Erba, Germany)
- TITAN GEL Alkaline Phosphatase (HR) Kit (Helena Laboratories, Beaumont, TX., USA, No. 3058)
- Tris [hydroxymethyl] - aminomethane hydrochloride (Merck, Darmstadt, Germany)

B. Methods

Part I. Methods for evaluation of serum specimens for pathological conditions.

[1.] Determination of liver function tests

The liver function tests in healthy donors and patients sera were measured in a Merk-MEGA automate II analyzer (Merk, Darmstadt, Germany). These were included total protein, albumin, alkaline phosphatase, cholesterol, aminotransferase (AST and ALT), bilirubin (total and direct).

[2.] Methods for alkaline phosphatase activity determination

Alkaline phosphatase in serum or from partial purified samples hydrolyzed p-nitrophenyl phosphate (PNPP) substrate with Mg^{++} as an activator under optimal conditions (pH 10.3 at 37° C) at a rate directly proportional to time and enzyme activity to yield p-nitrophenol (PNP). The intensity of resulting yellow color in alkaline was measured at 404 nm.

The procedures for analysis of alkaline phosphatase activity were as follow :

2.1 Standard method (Tietz *et al.*, 1982) **measured with Double - beam UV-Visible, spectrophotometer** (Shimudzu UV 160 A)

Total ALP activity was determined with a double- beam UV-visible spectrophotometer in the program CPS kinetic at wavelength 404 nm at 37° C. A 2.7 mL of AMP buffer and 200 µL of p- nitrophenyl phosphate substrate were placed in a cuvet . After mixing gently, the cuvet was placed into the cell compartment to warm at 37° C for 3-5 minutes. After that,100 µL of sample was added into the mixture and mixed thoroughly. The activity of the enzyme was measured at 404 nm, every 30 seconds for 4 cycles. The rate of change of absorbances against time or ΔA/min and total ALP activity was calculated, using data obtained from a linear portion of the reaction progress-curve. One unit of activity was defined as the quantity of enzyme catalyzing the hydrolysis of 1 µmole of substrate per minute under pH 10.3 at 37° C.

Calculations

The activity of enzyme was calculated from the following formula :

$$\text{ALP Activity U/L} = \frac{\Delta A/\text{min} \times 10^3 \times V \times 10^3}{\varepsilon \times S \times b}$$

Where $\Delta A/\text{min}$ = Absorbance change per minute at 404 nm.

10^3 = Conversion of mL to L

V = Total volume of reaction ; 3 mL

10^3 = Conversion of millimole to micromole

ε = Molar absorptivity of p-nitrophenol at 404 nm. 18 250 L. mol^{-1} .cm^{-1}

S = Sample volume in mL ; 0.1 mL

b = light path in cm; 1 cm

$$U/L = \frac{\Delta A/\text{min} \times 10^3 \times 3.0 \times 10^3}{1.825 \times 10^4 \times 1 \times 0.1}$$

$$= \Delta A/\text{min} \times 1{,}645$$

2.2 Total ALP activity measured with the microplate reader (Model EL 340)

ALP activity was determined at 37° C in a 96- well microtiter plate, in a final volume of 300 µL per well. Volumes of AMP buffer, PNPP substrate and sample were reduced ten timed less than standard method described above. Increase in absorbance at 405 nm of p-nitrophenol production were measured with microplate reader. All assay were performed in triplicate. ALP activities were reported as U/L using 22.7 L. mol^{-1} cm. as the millimolar absorptivity of p- nitrophenol at 405 nm for calculation.

The activity of enzyme measured in microplate reader was calculated from the following formula:

$$\text{ALP Activity (U/L)} = \frac{\Delta A/\text{min} \times 0.3 \times 10^3}{22.7 \times 0.01 \times 1}$$

$$= \Delta A/\text{min} \times 1{,}321$$

2.3 Quality control of total ALP activity determination

Optimal condition variation (OCV) or intra-run assay of ALP determination was performed in 20 identical aliquots of pooled human serum control assayed in Shimudzu UV 160 A spectrophotometer or microplate reader EL 340. Routine condition variation (RCV) or inter-run assay was determined using the same serum control as the OCV assay. The RCV had to be done simultaneously with samples in each experiment of ALP determination in both instruments, Precision tested was evaluated from percentage of coefficient of variation (%CV) using the following formula (Kringle, 1994)

$$\% CV = \frac{SD \times 100}{\text{Mean value}}$$

[3.] Methods for determinations of physicochemical properties of ALP isoenzymes in serum

Human serum ordinarily contains a variable mixture of ALP isoenzyme from liver, bone, intestine and placenta (during pregnancy). In liver disease, biliary ALP isoenzyme or the other abnormal isoenzymes may also be presented. To elucidate that ALP isoenzymes containing in serum samples from patients diagnosed as liver disease are from liver origin, the physicochemical properties of the isoenzymes in sera were studied.

3.1 Studies of the chemical inhibitors of the ALP isoenzymes in serum

L-Phenylalanine and levamisole inactivate different ALP isoenzymes at different rates. L-Phenylalanine markedly inhibit intestinal , placental and Regan isoenzymes but has less effect on the isoenzymes of bone or liver. Levamisole preferentially inhibits bone and liver ALP isoenzymes. The inhibition most probably results from the formation of a poorly dissociable enzyme inhibitor - substrate complex.

The chemical inhibition procedure was adapted from various studies (Farley *et al.*, 1993, Van Belle, 1976, Griffiths *et al.*, 1992). Serum samples were divided into three 100 µL aliquots and placed in each cuvet which appropriately labeled for control , LP (sample with L-Phenylalanine

inhibitors) and LV (sample with levamisole inhibition). After 2.6 mL of AMP buffer was added, 100 μL of distilled water, 100 μL of 500 mmol/L L-Phenylalanine or 100 μL of 5 mmol/L levamisole was mixed into each reaction cuvet. All reaction mixtures were mixed thoroughly and incubated at 37° C for 10 minutes before starting the enzymatic reaction by adding 200 μL of PNPP substrate. Activities of ALP sensitive to chemical inhibitors were measured with double-beam UV 160 A and the remaining activity of the enzyme was calculated as a percentage of the respective control.

3.2 Heat inactivation of ALP isoenzymes in serum

Individual ALP isoenzymes are rendered catalytically inactive at different temperatures. The most remarkable property of placenta ALP isoenzyme is its pronounced stability to heat at a temperature of 65° C for 30 min but the liver or bone ALP isoenzyme are less stable than the placental ALP isoenzyme.

The procedure for heat inactivation assay was modified from Farley *et al.*(1993) ; Moss and Henderson (1994). It was carried out by incubating 100 μL of serum in a small polypropylene test tube sealed with parafilm. The test tubes was placed into thermostatically controlled water bath that was already stabilized at 65° C so that the surface of the serum in the tube was below the level of the water. After exactly 10 minutes, the serum tube was removed rapidly and then cooled in ice water. The alkaline phosphatase activities of the heated and unheated samples were determined by double-beam UV 160 A and the heat-insensitive ALP activity was calculated as a percentage of activity in the unheated control that had been kept in ice water.

Part II. Methods for preliminary screening of types of ALP isoenzymes composed in serum samples

[1.] WGA precipitation of bone ALP isoenzyme

Precipitation of the isoenzymes in serum with wheat germ agglutinin (WGA) reveals the amount of bone ALP isoenzyme presented in serum (Behr and Barnert, 1986). In this experiment WGA precipitation was performed in a mixture of 50 μL of serum in polypropylene microcentrifuge tube with a snap - tight lid and 50 μL of an aqueous

solution of WGA (3 g/L in acetate buffer, pH 4.5). After standing for 30 minutes at room temperature, the mixture was centrifuged for 5 min at 1000 x g. ALP activity was determined in neat serum ,the supernatant and parallel control containing 50 μL of serum and 50 μL of acetate buffer (without WGA) by Shimudzu UV 160 A method. Bone ALP isoenzyme precipitating was calculated as a percent of the respective control or using the following formula (Day, Saward *et al.*, 1992) :

$$\text{Bone ALP (U/L)} = (1.118 \times \text{total ALP}) - (2.35 \times \text{supernatant ALP})$$

[2.] Screening of ALP isoenzymes in serum by cellulose acetate electrophoresis

For qualitative assessment of the ALP isoenzymes in healthy subject and patient sera. A 1 μL of serum sample was electrophoresed at room temperature at 180 volts for 30 min on Titan III Iso-Vis cellulose acetate plate (Helena labs, Beaumont, TX, USA) that had been presoked in Tris-barbital-sodium barbital buffer, pH 8.8 for 20 minutes. ALP activity was visualized by incubation for 30 min at 37°C with a chromogenic enzyme substrate 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt in alkaline phosphatase indolyl blue reagent (kit). The membrane was fixed for 20 min in 5% v/v acetic acid, then, washed with distilled water, and finally dried at 40° C for 30 minutes. After inspection to ensure satisfactory separation and staining, the ALP isoenzymes were quantified by densitometric scanning at 610 nm using the Helena EDC densitometer. The isoenzyme bands were identified by comparing with the commercial liver-intestinal ALP isoenzyme control from Helena laboratories.

Part III. Methods for characterization of sugar chains on the fractionated ALP isoenzymes molecules

This experiment was performed to investigate changes in glycosylation of ALP isoenzymes which might be occurred in liver disease. The modification of carbohydrate side chains of ALP protein was studied in terms of type of sugar compositions and manner of sialic acid linkage.

[1.] Fractionation of ALP isoenzymes by anion exchange chromatography

The principle of this method is based on the fact that each individual alkaline phosphatase isoenzyme has different quantity of negative charges on the molecule. Thus liver, bone and biliary alkaline phosphatase isoenzyme were separated by their different preferences on an anion-exchange column. The isoenzyme proteins are eluted from the resin by gradient concentrations of eluted buffers at different rates.

Fractionation of ALP in serum on DEAE Sepharose were performed in sera of 3 healthy subjects and 10 patients with liver disease (two samples from each different group of liver disease). The procedure was as that modified from Mercer *et al.*(1994) and Karmen *et al.*(1984).

In experiment, a 75 µL of serum was applied to the top of 6X6 mm. column filled to a height of 1.8 cm. with DEAE Sepharose 6B-100. The ALP isoenzymes were separated from the column by step elutions with 2 mL of 100 mmol NaCl (buffer A), 6 mL of 150 mmol NaCl (buffer B) and 3 mL of 300 mmol NaCl (buffer C) per liter of 5 mmol Tris-HCL buffer pH 8.0 at 25 °C. For identification of peak fraction, each 0.5 mL of eluate was collected. The absorbances at 280 nm of each fraction was measured and plotted. For characterization of sugar chain moieties, 2 mL of buffer A eluted was collected as fraction 1 (F1). The first 2 ml of a second buffer (buffer B) elution was collected as fraction 2 (F2) and the other 4 ml, as fraction 3 (F3). The last fraction eluted with 3 ml of buffer C was collected as fraction 4 (F4).

All fractions obtained from column chromatography were dried in the Lioalpha-10 (Telstar, Spain) and kept at 4°C for further studies.

All steps for anion exchange (DEAE Sepharose 6B-100) chromatography were summarized as shown in Figure 4.

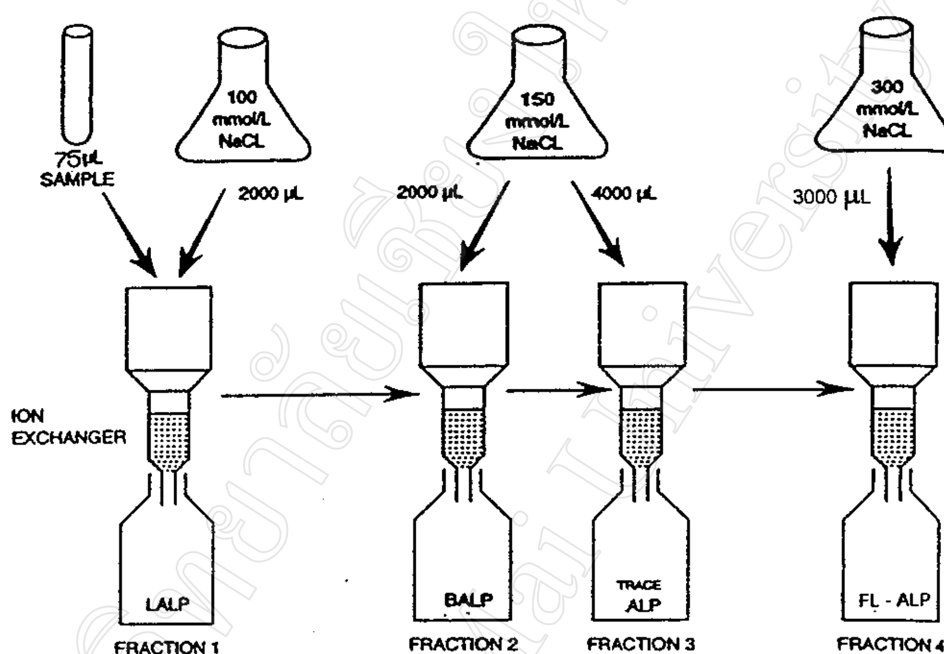

Figure 4. Ion- exchange column chromatographic separation of ALP isoenzymes.

[2.] Determination of protein in ALP isoenzyme fractions

Protein concentrations of fractionated ALP isoenzymes were determined by characteristic absorption of UV light at 280 nm and the formula used to calculate protein concentrations in each isoenzymes fraction was as followed :

Protein (g/L) = 1.55 X abs. at 280 nm - 0.77 X abs. at 260 nm

[3.] Identification of fractionated ALP isoenzymes

3.1 Identification of protein peaks of the separated ALP isoenzyme fractions

Serum sample showing various protein peaks eluted from DEAE Sepharose column chromatography were proved to contain glycoprotein ALP isoenzyme identical to the commercial control liver -bone ALP isoenzymes separated by the same conditions as the unknown. The experiment was performed in sera of normal and patient with hepatoma comparing with Gel ALK Phos Iso Control (Helena Laboratories, Beaumont, TX, USA). Sera or control, volume of 50 µL, was mixed with 20 µL of 1 U/ml neuraminidase solution (from *C. perfringens* ; C-Neu) and incubated at 37°C for 30 min. At the end of the incubation period, the mixtures were transferred to ice water. Chromatography of neuraminidase treated sera and control were carried out, by method described in part III-[1] , comparing with its neuraminidase untreated sample. The fraction of 0.5 ml eluate was collected, absorbances at 280 nm were measured in a Beckman Model 25 UV-Visible spectrophotometer. The absorbances were plotted for peak identification. Fractions from each protein peak were pooled, measured for ALP activity and protein concentration before lyophilization. After dissolving, they were identified on an agarose gel electrophoresis.

3.2 Electrophoresis on agarose gel (Miura *et al*, 1994 ; Gonchoroff *et al*., 1989)

Agarose gel electrophoresis of fractionated ALP isoenzymes was carried out on both the Titan gel alkaline phosphatase (HR) (Helena labs, Beaumont, TX, USA) and 0.8% agarose gel prepared in this laboratory. ALP isoenzymes were separated on the basis of their molecular sizes and electrophoretic mobility. Each lyophilized fraction was dissolved with 150 µL of distilled water before electrophoresed. Five microliters of different dissolved fractions were subsequently applied on the template slits on agarose gel and waited for 10 minutes after the last sample had been applied for absorption. After the template was carefully removed, an electrophoresis was carried out at 4° C for 70 min at 90 volts. As soon as the gel was removed from the chamber, it was immediately stained for 40 min at 45° C with the Titan gel alkaline phosphatase reagent (kit)

containing 5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitroblue tetrazolium. After incubation, the gel was washed in 10% v/v acetic acid for 5 min, then, removed and rinsed with distilled water for 30 seconds. The gel was dried approximately 20 minutes at 50-60° C. Band of each fractionated ALP isoenzyme was identified by comparing with liver-bone ALP commercial control and serum controls for the fast liver and intestinal isoenzyme.

3.3 Identification of Fraction 1 and Fraction 2 ALP isoenzymes by heat inactivation (Farley *et al.*, 1993, Rosalki and Foo, 1984)

Fraction 1 and 2 in the eluates were identified and quantified by differential ALP isoenzymes sensitive to heat inactivation. The lyophilized fractions were dissolved with 150 µL of distilled water . After that, 30 µL triplicate aliquots of each fraction in polypropylene tubes sealed with parafilm were heated at 52° C for 15, 30 and 60 minutes accordingly. Samples were then cooled in ice water and assay for ALP remaining activity in a microplate reader. The untreated samples were simultaneously measured for total ALP activity and used as the control without heat treatment. Heat-insensitive ALP activity was calculated as the percentage of activity of the unheated control.

[4.] Lectin precipitation of sugar moieties of the fractionated ALP isoenzymes (Farley *et al.*, 1993)

Several lectins have been used to characterize the internal sugars in carbohydrate site chains of ALP isoenzymes. The lectins used in this experiment were 12 g/L wheat germ agglutinin (WGA), 24 g/L concanavalin A (Con A) and 4 g/L *Pisum sativum* agglutinin (PSA). Each lectin was prepared as an aqueous solution in distilled water. The lyophilized fractions 1, 2 and 4 were dissolved in 350 µL of distilled water before used. Each fractions was divided into four 75 µL aliquots and placed in four polypropylene test tubes. One aliquot was then added with 25 µL of distilled water, and the other aliquots were added with 25 µL of different aqueous lectin solutions respectively. They were thoroughly mixed and incubated at 37° C for 30 minutes. After that, the mixtures were centrifuged at 1000 x g for 5 minutes The supernatant was assayed in triplicate for remaining activities in a microplate reader. Lectin-insensitive

ALP activity was determined as the difference between the lectin-treated samples and the distilled water-treated control.

[5.] Study of sialic acid linked to the sugar chain moieties

5.1 Preliminary studies of asialo-ALP isoenzyme preparation in serum

5.1.1 Preparation of asialo-ALP isoenzymes in serum

ALP isoenzymes in pooled human serum (total ALP = 259 U/L) were digested with neuraminidase to eliminate the charge of sialic acid (Miura *et al.*, 1994). Desialylation of ALP was achieved by mixing duplicate 50 μL aliquots of pooled human serum and 40 μL of 1 unit /mL neuraminidase (C-Neu) in polypropylene test tubes. Following that, the mixtures were incubated at room temperature for 3 or 18 hours (overnight). After the incubation time, the asialo-ALP mixtures were then cooled in ice water and kept for cellulose acetate electrophoresis.

5.1.2 Heat inactivation of neuraminidase activity in the asialo-ALP isoenzymes (Mendla and Cantz, 1984)

To inactivate of neuraminidase activity in the asialo-ALP isoenzyme preparation in serum. One 20 μL aliquot of the 3 h neuraminidase-treated sample were heated in water bath at 52° C for 60 minutes and then cooled in ice water. A second 20 μL aliquot was kept in ice water as an unheated control. For 18 h neuraminidase-treated serum, 20 μL triplicate aliquots of sample were also subjected to heat at 52° C but for various lengths of time (15, 30 and 60 minutes). The tubes containing the remaining of 18 h neuraminidase-treated enzyme were immediately kept in ice water as well as an unheated control. Aliquots of neuraminidase treated with or without heat inactivation of serum samples were evaluated for neuraminidase (sialidase) activity and effect of heat treatment of neuraminidase on ALP isoenzymes in a microplate reader.

5.1.3 Measurement of total sialic acid (TSA) in serum

Measurement of total sialic acid (TSA) in serum was carried out by micromethod periodate-resorcinol assay (Modified by Surangkul, 1998). In principle, N-acetylneuraminic acid (NANA) was oxidized to 7-

carbon aldehydes with low concentration of periodic acid. Then resorcinol and copper ions react with 7-carbon aldehydes to form a colored compound, which could be measured at 620 nm.

The procedure was performed by adding 40 µL of samples or pure standard sialic acid (2.5-10 µg/well) solution into the wells of 96-well microtiter plate. Then, 50 µL of 1.3 mM periodic acid was added to each well and mixed by shaking the plate for 5 min on a microplate shaker at room temperature. After that, the plate was placed in an ice box for 60 min and then 100 µL of 0.6 g/dL of resorsinol reagent was added and mixed by shaking for 5 minutes. Later on, the plate was covered with a microtiter plate cover and heated for 60 minutes at 80° C. At the end of the incubation time, the plate was removed and placed on a shaker for about 2 minutes and let it cooled down to room temperature. Then 100 µL of 95% tert-butyl alcohol was added to each well and the mixture was mixed thoroughly once again for 2 minutes. The absorbance at 620 nm of the reaction in microwell was measured immediately by a programmable microplate reader. A standard curve for TSA assay was constructed and the level of TSA in samples were calculated using a linear curve fit.

For quality control of sialic acid measurement, The precision of TSA assay was tested by percentage coefficient of variation (%CV). The coefficient of variation for both intra- and inter assay were calculated according to the formula described in part I - [2] , 2.3.

5.2 Sialylation of ALP isoenzyme fractions

5.2.1 Optimization of neuraminidase (sialidase) activity on ALP isoenzyme fractions

Optimal condition for fractionated ALP digestion, prepared for sialylation, depended on sources and activities of the neuraminidase used. Neuraminidase of different sources and activities from *Vibrio cholerae* (V-Neu) and *Clostridium perfringens* (C-Neu) were applied to the ALP isoenzyme fractions.

To determine the optimal activity, two units/mL of stock C-Neu was diluted (v/v) to 41.6, 62.5 and 125 mU/L with distilled water and used. Triplicate aliquots of 25 µL of aqueous fraction 1 was added with 10 µL of various concentrations of neuraminidase prepared as above and mixed thoroughly. After incubation at room temperature for 3 h, asialo-ALP were then cooled in ice water for further examination.

To select sources of neuraminidase used for desialylation, the 125 mU/L concentration of both neuraminidases were compared for the digesting effects on fraction 1 and 2 samples. Then the asialo-ALP obtained from various concentration and types of neuraminidase applications on ALPs fractions were examined on an agarose gel electrophoresis as described in part III - [3] , 3.2.

5.2.2 Preparation of asialo-ALP fraction

Neuraminidase digestion of the ALP isoenzyme fractions were adapted from 5.1.1 and method described by Miura *et al.* (1994). Fraction 1 separated from healthy subjects and patients sera (two samples from each group of discriminated liver disease) and fraction 4 from patient sera with hepatoma and cholestasis were subjected to desialylation. The lyophilyzed fractions were dissolved with 150 μL of distilled water and 25 μL of aqueous fractions were then added with 10 μL of 125 mU/L neuraminidase (C-Neu) in polypropylene test tubes. The mixture was mixed thoroughly and left to stand for 3 h at room temperature before cooling in ice water.

5.2.3 Sialylation of asialo-ALP isoenzymes by α 2,6 - sialyltransferase (α 2,6-ST)

This assay measures a transfer of NeuNAc from CMP- NeuNAc substrate by α 2,6-sialyltransferase (α 2,6-ST) to the acceptor asialo-form ALP prepared by neuraminidase treatment. Sialylation of ALP was performed by the method described by Miura *et al*. (1994), Schachter *et al.*(1983).

Briefly, A 10 μL of asialo-ALP preparation was placed in a polypropylene tube and the following reagents were added respectively; 5 μL of 60 mmol/L CMP- NeuNAc, 10 μL of 2% bovine serum albumin, 10 μL phosphate buffer (0.5 M, pH 6.8) and 5 μL of 1.5 U/L α 2,6-sialyltransferase. The mixture was then mixed thoroughly with autopipette and incubated at 37° C for 2 h. Exactly, at the end of incubation period, the mixture was removed and then cooled in ice water. The α 2,6-ST treated ALP was later examined by an agarose gel electrophoresis. The band of sialylated ALP isoenzyme was compared with its respective asialo ALP and non-treated neuraminidase control isoenzyme fraction.

Part IV. Application of carbohydrate heterogeneity of ALP molecules for separation of serum ALP isoenzymes by cellulose acetate electrophoresis

According to the findings that sialylation on the ALP isoenzyme molecules in serum were heterogeneous. Therefore selective digestion of sialic acid from the ALP molecules may help in separating ALP isoenzymes in an electrophoretic field. In this study, neuraminidase of two different sources, C-Neu or V-Neu in the concentration (or activity) of 0.36 U/L of a final incubation mixture was used to treat serum samples from various diagnosis of the liver disease patients. The method applied from the use of C-Neu or V-Neu to desialylate of ALP isoenzyme fraction. The activity of neuraminidase used was increased proportionally with the activity of ALP in serum. Thus, 50 µL of serum sample containing ALP activity not exceeded than 400 U/L, was mixed with 20 µL of 1.25 U/mL neuraminidase solution in a polypropylene tube. The mixture was left at room temperature for 3 h before performing electrophoresis on cellulose acetate membrane by the method described in Materials and Methods part [2].

Part V. Statistical analysis

All data are shown as the average of triplicate (mean ± SD). Correlation coefficients were determined by linear regression. Comparisons were made by analysis of student unpaired t-test, using the statistical functions in Microsoft excel 7.0 program on a Microsoft windows for personal computer.

IV. RESULTS

Part I. Evaluation of serum specimens for pathological conditions

[1.] Routine laboratory data in healthy subjects and patients with liver disease

Results of liver function test in sera of healthy subjects and patients were summarized in Table 1. In patient group, the mean values and ranges in most of all tests except protein and cholesterol exhibited abnormal patterns. The activities of ALP averaged 10 times higher than healthy subjects and ranged from 113 to 2,518 U/L with a mean of 666.49 U/L. Total ALP, AST, ALT, TBIL and DBIL activities were significantly ($p<0.0001$) increased in sera of patients and the average protein and albumin levels were lower as compared with the healthy subjects.

Table 2 showed profiles of liver function test in sera of patients with different pathological conditions of liver disease. In patients with hepatitis, serum AST and ALT activities were markedly elevated and the ALT activities being predominant over the AST activities. Changes in both forms of bilirubin concentrations in serum samples were also observed.

In cirrhosis patients, the albumin levels in sera were decreased, accompanied by the characteristic increase in the globulin levels. The increase of ALP activity was slightly as compared with the normal (Table 1). AST activity was higher than ALT and slightly increased in total and direct bilirubin were demonstrated in sera of this group of patient. Significant increases of alkaline phosphatase, total and direct bilirubin and cholesterol levels were shown in sera of hepatoma patients. The patterns of albumin and total protein values were the same as that found in sera of patients with cirrhosis.

In patients with cholestasis (caused by gallstones), the ALP values were higher than in the cholangiocarcinoma group, whereas in both groups there was a similar increase in serum bilirubin levels.

[2.] Analytical precision of ALP activity determination

The analytical precision determined by analyzing ALP activities in pooled serum in both Shimudzu UV 160 A and microplate reader were shown in Table 3. In the determination of OCV (intra - assay), the precision of analytical method determined in Shimudzu UV 160 A were varied with the activities of ALP in pooled serum controls. The increasing of mean activity of the enzyme reduced the percentage of coefficient of variation (%CV). The %CV obtained by analyzing an abnormally low level of ALP in pooled serum in a microplate reader was higher than that obtained from Shimudzu UV 160 A, (compared with the low level control serum) but this variance was still accepted. In routine analyses (inter - assay) of control pooled sera that had been used previously for evaluation of the OCVs in both instruments, it was found that the RCV mean values were nearly the same as OCV. The %CV under most of routine conditions were greater than that found under optimal conditions and the ratio of RCV to OCV was less than two for both ALP analytical methods in both instruments. Figure 5 showed an example of the distribution of analytical ALP values performing in a microplate reader.

A comparison between the microplate method for ALP activity determination with the standard kinetic assay in Shimudzu UV 160 A was found to be correlated with a correlation coefficient of 0.991. There was a good correlation between the two methods but with small constant bias of the results. ALP activities determined by the microplate method must be multiplied by a factor of 1.5 for comparison with the measurements by Shimudzu UV 160 A and a Merk-MEGA automate II analyzer.

[3.] Physicochemical properties of ALP isoenzymes in serum

Sensitivity to chemical inhibitors and heat

Characterization of ALP isoenzymes in healthy and patient sera were tested by chemical inhibition and heat inactivation. The results for sensitivity to chemical inhibitors were illustrated in Figure 6. The ALP isoenzymes in most healthy and patient sera were very sensitive to levamisole but insensitive to L-Phenylalanine except placental and intestinal ALP isoenzyme controls. However, ALP isoenzymes in sera of three patients and only one healthy subject were insensitive to levamisole, these samples were then subjected to heat inactivation. They were found

to sensitive to heat inactivation at 65°C. The percentage of remaining ALP activity in the samples were decreased to less than 10% of total ALP activity. These data indicated that the ALP isoenzymes in healthy and patient sera in this study expressed the characteristic of tissue-non specific ALP particularly as the liver or bone ALP isoenzymes.

Part II. Preliminary screening of types of ALP isoenzymes composed in serum samples

[1.] Quantification of bone ALP in healthy and patient sera by WGA precipitation technique.

For screening of approximated quantity of bone ALP isoenzyme in serum, WGA precipitation was determined in patients and healthy sera. Table 4 summarized the ranges and mean values of % precipitating ALP activity and bone ALP values after precipitation. WGA precipitated approximately 21% in the whole group of patients having increased total ALP activity compared with 24.8% of healthy subjects who had normal ALP values. Mean bone ALP activities calculated using the formula gave paradoxically results compared with the precipitating ALP activities. In ten healthy subjects and nine patients had a calculated bone ALP activity of less than 0 U/L. In patients with cirrhosis, the precipitating ALP activity was 21.7% whereas the bone ALP decreased to 1.8%. The precipitating ALP activity in sera of healthy subjects were 24.8 % whereas they had bone ALP activity only 10.7%. However, these data suggested that there were the bone components in sera of healthy subjects and various group of patients with liver disease. No correlation of precipitating bone ALP activities with those calculated by using the formula described in Materials and Methods section.

[2.] Patterns of ALP isoenzymes in serum using cellulose acetate electrophoresis.

Serum samples of healthy subjects and each differentiated group of patients with liver disease showed different ALP isoenzymes on cellulose acetate membrane. Electrophoretic patterns in sera of all patients with cholestasis and hepatoma showed two bands of ALP isoenzyme. One of the bands moved faster towards the anode than the major liver band,

indicating its greater negative charge, and is referred to as the fast liver band. In addition, the intestinal band was found in two cases of patients with cholestasis and one case of patient with hepatoma .

In sera of patients with cirrhosis, hepatitis and cholangiocarcinoma, the diffuse band of liver - bone ALP isoenzymes and the intestinal bands were presented in two cases of patients with cholangiocarcinoma and one case of patient with hepatitis and cirrhosis.

In sera of healthy subjects, only one of the overlapping diffuse liver - bone ALP isoenzyme band was presented. Two subjects with blood groups O and B showed an additional band of the intestinal ALP isoenzyme (data not shown).

Table 1. Liver function test findings in sera of healthy subjects and total patients with liver disease.

Test [a]	Reference ranges	Healthy subjects (N = 36)	Patients (liver disease, N = 47)
		Mean ± SD (range)	Mean ± SD (range)
TP, g/dL	6.0 - 8.5	7.73 ± 0.52 (6.8 - 8.7)	6.99 ± 0.89 (5.2 - 9.5)
Alb, g/dL	3.2 - 5.0	4.93 ± 0.36 (4.1 - 5.8)	3.1 ± 0.58 (1.8 - 4.3)
Glob, g/dL	2.8 - 3.5	2.81 ± 0.47 (2.1 - 4.0)	3.96 ± 0.69 (2.9 - 5.6)
ALP, U/L	23 - 98	68.69 ± 17.31 (34 - 98)	666.49 ± 558.65 [b] (113 - 2,518)
Chol, mg/dL	150 - 250	201.58 ± 37.67 (136 - 293)	198.45 ± 79.32 (45 - 491)
AST, U/L	3 - 35	20.3 ± 6.97 (11- 39)	179.66 ± 205.64 [b] (25 - 910)
ALT, U/L	7 - 33	16.97 ± 12.07 (5 - 59)	168.68 ± 230.2 [b] (11 - 1,239)
TBIL, mg/dL	0.2 - 1.0	0.50 ± 0.22 (0.18 - 1.05)	13.15 ± 11.37 [b] (0.26 - 41.52)
DBIL, mg/dL	0 - 0.2	0.17 ± 0.08 (0.07 - 0.44)	8.41 ± 7.41 [b] (0.16 - 25.15)

[a] Mean ± SD (range) is shown for all tests.

[b] Very highly significant difference ($p<0.0001$) V.S. healthy subjects.

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Table 2. Comparison of liver function tests in sera of patients with differential diagnosis of liver disease.

Disease	N[a]	TP (g/dL)	Alb (g/dL)	Glob (g/dL)	ALP (U/L)	Chol (mg/dL)	AST (U/L)	ALT (U/L)	TBIL (mg/dL)	DBIL (mg/dL)
Hepatitis	10	7.39[b] (0.44)	3.28 (0.53)	4.11 (0.38)	785.6 (823.5)	196.3 (48.7)	332.1 (304.1)	391.8 (382.3)	11.43 (10.92)	7.51 (7.11)
Cirrhosis	7	7.07 (1.31)	2.89 (0.42)	4.18 (1.14)	153.6 (58.7)	158.4 (34.3)	105.1 (78.5)	136.0 (158.6)	5.68 (3.17)	3.00 (3.00)
Hepatoma	10	6.84 (0.81)	2.94 (0.48)	3.90 (0.49)	1136.5 (424.7)	301 (82.8)	197.4 (156.1)	144.2 (120.4)	20.59 (11.83)	13.89 (8.22)
CHCA	10	7.03 (1.11)	2.92 (0.75)	4.11 (0.65)	375.5 (113.1)	150.5 (55.7)	104 (66.5)	84.6 (115.7)	14.78 (11.54)	9.15 (6.73)
Cholestasis	10	6.53 (0.67)	2.91 (0.46)	3.62 (0.73)	727.4 (396.6)	174 (53.5)	141.3 (225.8)	90 (83.6)	11.0 (12.0)	6.86 (7.42)

[a] Number of patients.
[b] Mean (SD) was shown for all tests.

Table 3. Analytical performances of the measurements of ALP activities in two different instruments.

Measurement / instruments	Level of pooled serum control	OCV (Intra - assay)			RCV (Inter - assay)		
		N	Mean ± SD	%CV	N	Mean ± SD	%CV
Shimudzu UV 160 A spectro-photometer *	high	20	166.0 ± 3.54	2.13	20	167.0 ± 5.14	3.07
	low 1	20	92.64 ± 1.99	2.14	20	93.79 ± 3.34	3.56
	low 2	20	20.79 ± 0.88	4.23	20	21.91 ± 1.48	6.75
Microplate reader **	low 3	20	26.68 ± 1.85	6.93	20	27.67 ± 2.88	10.4

* Method used for determination of total ALP activity in serum samples.

** Method used for determination of ALP isoenzyme activity in fractionated serum samples.

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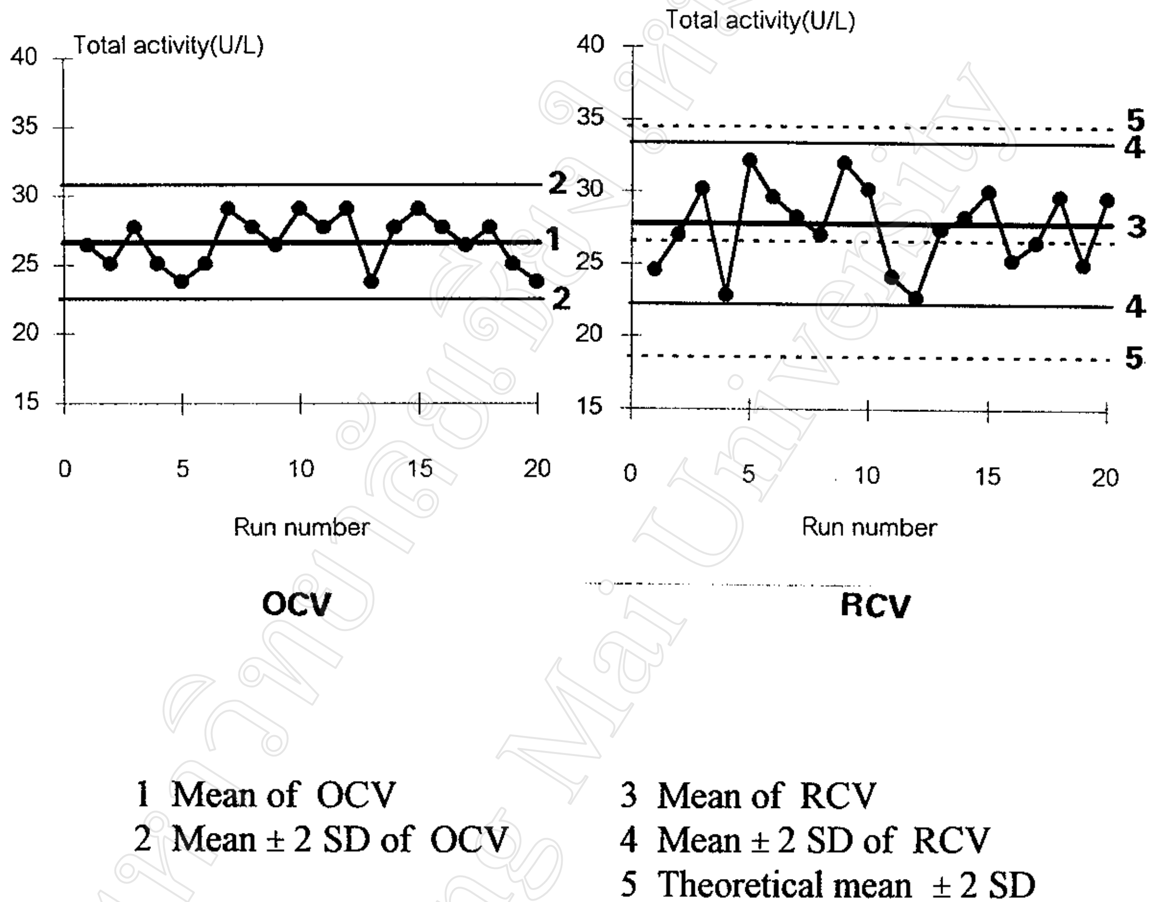


1 Mean of OCV
2 Mean ± 2 SD of OCV

3 Mean of RCV
4 Mean ± 2 SD of RCV
5 Theoretical mean ± 2 SD

Figure 5. The quality control chart showing intra- and inter- assay precision of ALP values in pooled serum control performed in a microplate reader.

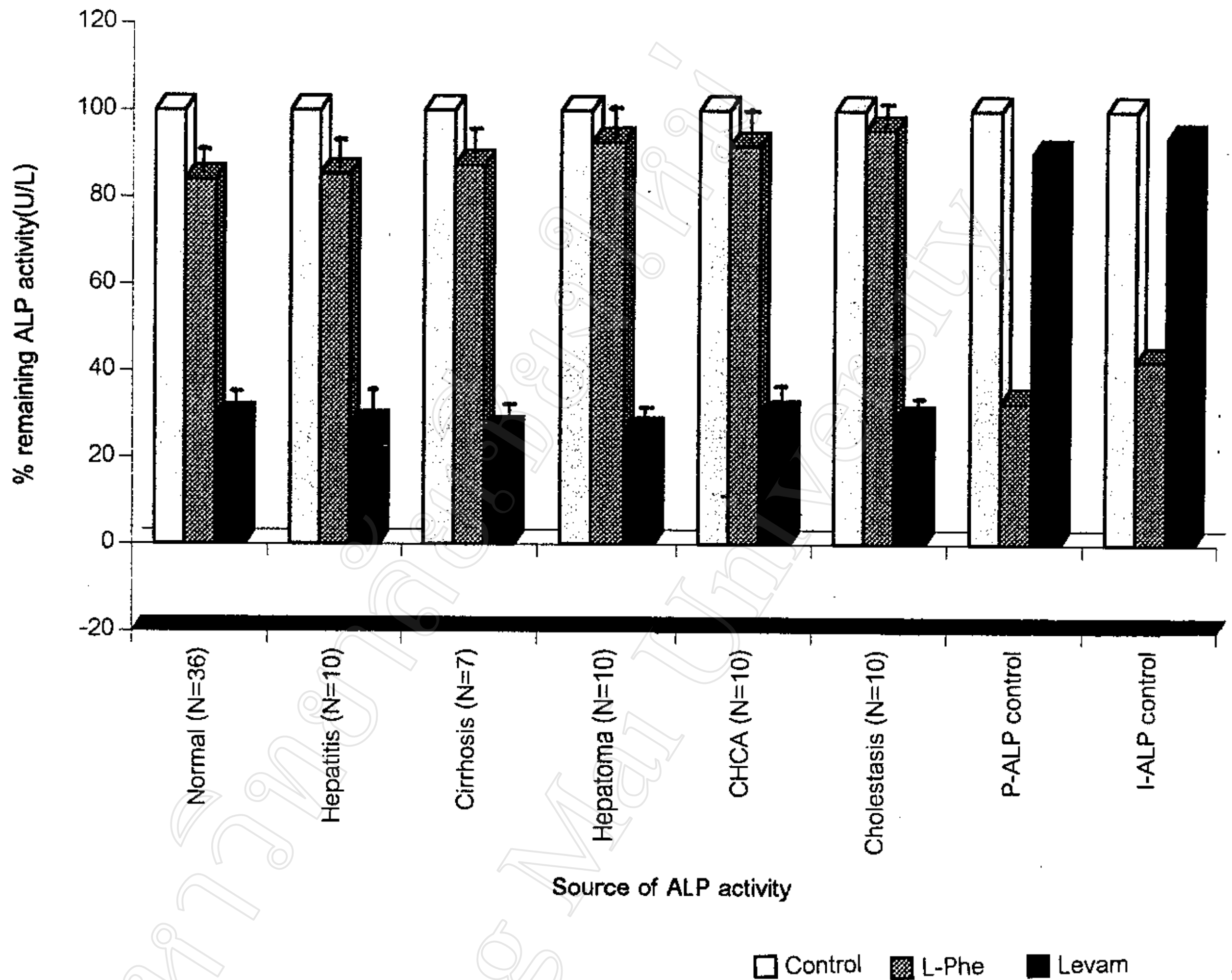


Figure 6. Sensitivities of ALP activities to the chemical inhibitors. Percent of remaining ALP activity when assayed in the presence of the indicated inhibitors (Phe = L-Phenylalanine, Levam = levamisole). Data shown are averages of duplicates and expressed as a percentage of the activity of the untreated control.

Table 4. Quantitative measurement of bone ALP isoenzyme in sera of patients and healthy subjects.

Source of ALP activity	N [a]	WGA precipitation Mean (range)			
		Basal ALP activity, U/L [b]	% precipitating ALP activity	Bone ALP activity(U/L) [c]	%Total bone ALP activity(U/L)
Healthy	36	42.8 (23 - 68)	24.8 (6.5 - 48.4)	4.9 (-25.5 - 40.8)	10.7 (0.03 - 65.8)
Hepatitis	10	347.2 (60- 1,012)	20.6 (3.8 - 58.3)	132.4 (-17 - 783)	36.6 (2.3 - 77.3)
Cirrhosis	7	86.8 (64 - 130)	21.7 (4.9 - 46.7)	1.6 (-61.7 - 68)	1.8 (0.01 - 75.5)
Hepatoma	10	496.3 (224- 832)	15.5 (2.7 - 49.2)	226.8 (-159 - 708)	44.8 (7.8 - 92.9)
CHCA	10	180 (102 - 248)	16.9 (1.7 - 37.9)	52.8 (-11 - 200)	31.9 (3.4 - 96.4)
Cholestasis	10	315.7 (16.8-672)	24.5 (7.7 - 44.8)	186.4 (16 - 504.7)	55.4 (10.2 - 99.7)

[a] Number of patients or healthy subjects.
[b] In untreated samples (without WGA)
[c] Bone ALP was calculated using the formula as described in Materials and Methods section.

Part III. Characterization of sugar chain on the fractionated ALP isoenzyme molecules.

[1.] Fractionation of ALP isoenzymes by anion exchange chromatography.

ALP isoenzymes were isolated to four fractions(fraction 1 - 4) in the column eluates. The elution profiles were obtained after eluting 75 μL of serum samples with 8 separate addition of buffer A and B for fraction 1 and 2, followed by 8 and 6 separate additions of buffer B and C for fraction 3 and 4, respectively. The absorbance peak at 280 nm of the eluated collected as 22 consecutive individual 0.5 ml fractions were illustrated in Figure 7. From the elution profiles, The highest peak was observed in fraction 1 for all samples. No protein peak was found in fraction 3 eluate and the peak of fraction 4 was found in only patients groups. Furthermore, the highest peak of fraction 4 were found in patients with cholestasis and hepatoma.

[2.] Identification of fractionated ALP isoenzymes.

The fractionated ALP isoenzymes containing in protein eluates obtained from chromatographic method was identified by separating the untreated against the neuraminidase - treated sample on DEAE Sepharose anion exchange chromatography.

In Figure 8 the elution profiles of ALP isoenzyme separated from sera of healthy subject and patient with hepatoma were compared with the profile of a commercial liver-bone ALP isoenzyme control. As shown from Figure 8 that the peak height of each protein fractions of the neuraminidase untreated healthy and patients sample were at the same as eluting positions of the liver-bone control (fraction 2 for F1, fraction7, 8 for F2 and 18, 19 for F4). Alternatively, after treating with neuraminidase (C-Neu), it was shown that not only the isoenzyme containing proteins separated from samples and control, were eluted slower from the column but the absorbances at 280 nm of all the eluted peaks were also reduced, particularly, significantly observed in F2 of hepatoma sample. After each fraction was concentrated and subjected to agarose gel electrophoresis, all of protein fractions were proved to contain ALP isoenzymes (Figure 9).

The purity of separated ALP isoenzyme in the fractionated eluate fraction was also evaluated on agarose gel electrophoresis. Fraction 1 behaved like the liver ALP isoenzyme and fraction 2 and 4 resembled to bone and fast liver type respectively (Figure 10 compared with the commercial control or serum control). As shown, no carry over of fraction 1 and fraction 2 - ALPs into fraction 4- ALP (lane 1) and no carry over of fraction 4- ALP into fraction 1 and fraction 2- ALPs (lane 2 and 3) respectively. As compared with lane 5, the ALP fraction (F4)in lane 1 was identified as fast liver ALP whereas the ALP fractions in lane 2 (F2) and 3 (F1) which compared with lane 8 (commercial control) were identified as bone or liver ALPs. To discriminate the ALP isoenzymes in lane 2 (F2) and 3 (F1), the rate of heat treatment of ALP isoenzyme in fraction 2 was examined comparatively with ALP isoenzyme in fraction 1 separated from the same serum sample. Fraction 1 and fraction 2 were inactivated by heat at 52 °C at different rates (Figure 11). The % remaining ALP activity in fraction 1 and 2 fractionated from healthy and patient sera were differed approximately 22% and 13 % respectively at 30 min of heat treatment and the differences were equal to 25 % for both samples at 60 min of heat inactivation. From data, it is indicated that ALP isoenzyme in fraction 2 was more sensitive to heat than of fraction 1. In healthy and patients samples, the ALP activities in fraction 1, after heating for 60 minutes at 52 °C, reduced by 71.2% and 56.2% whereas in fraction 2 activities of the enzyme were reduced by 92.1% and 79% respectively. Therefore fraction 1 and 2 were distinguished by heat as liver and bone ALP isoenzymes.

From these results, it could be concluded that Fraction 1, 2, and 4 contained liver, bone and fast liver isoenzymes respectively. The eluates collected as fraction 3 were discarded for all of samples fractionated by using this anion exchange chromatographic columns.

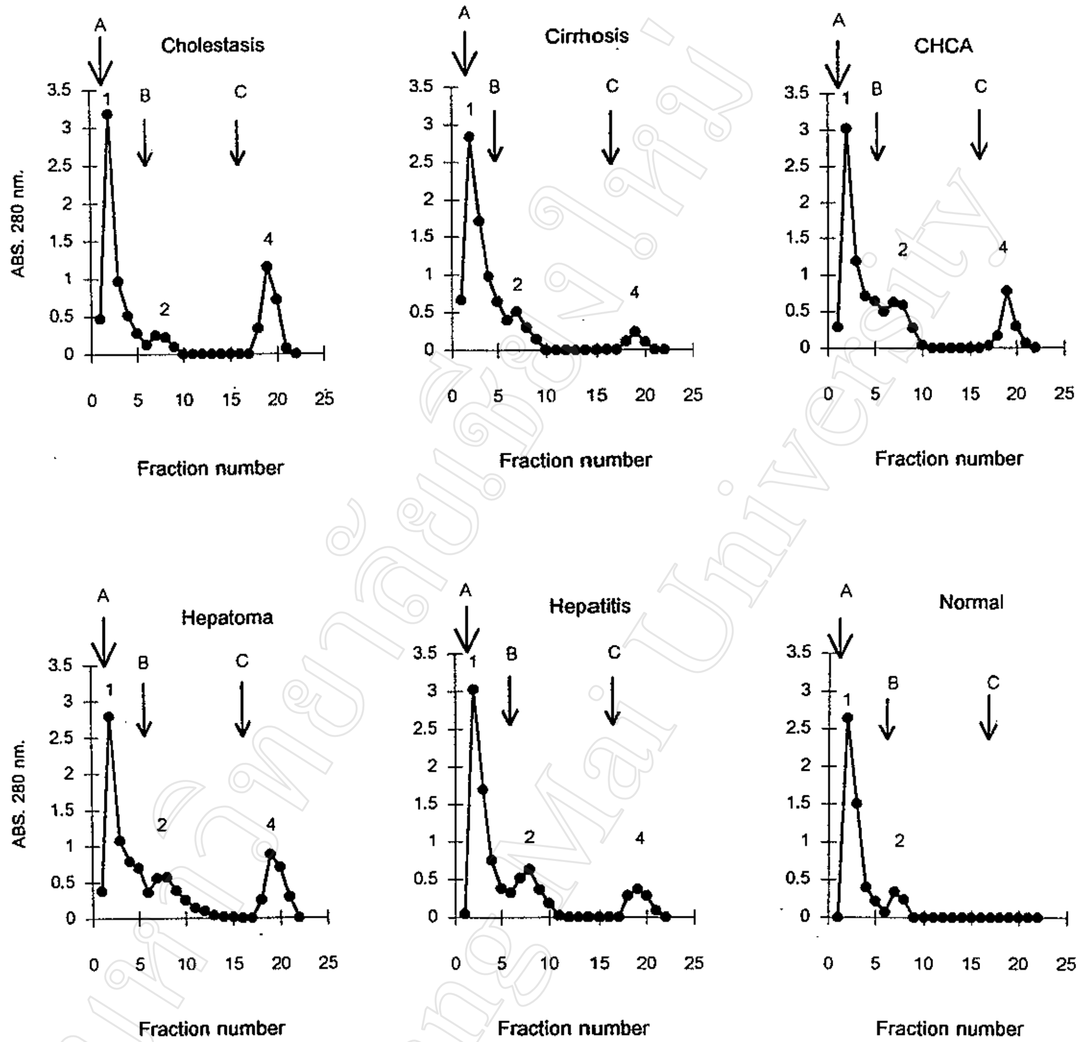
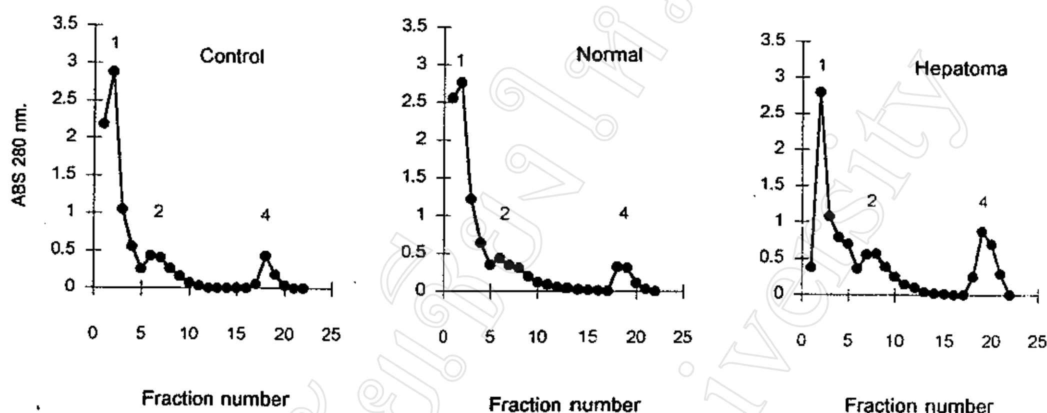


Figure 7. Elution profiles of ALP separated from sera of liver disease patients and healthy control by DEAE Sepharose anion exchange chromatography.

A,B and C were 100, 150 and 300 mmol NaCl in 5 mmol/L Tris buffer pH 8.0. The eluates of 0.5 mL fraction were collected and 1,2 and 4 were pooled eluates of fraction 1,2 and 4 respectively (see text).

Samples without C-Neu treatment



Samples with C-Neu treatment

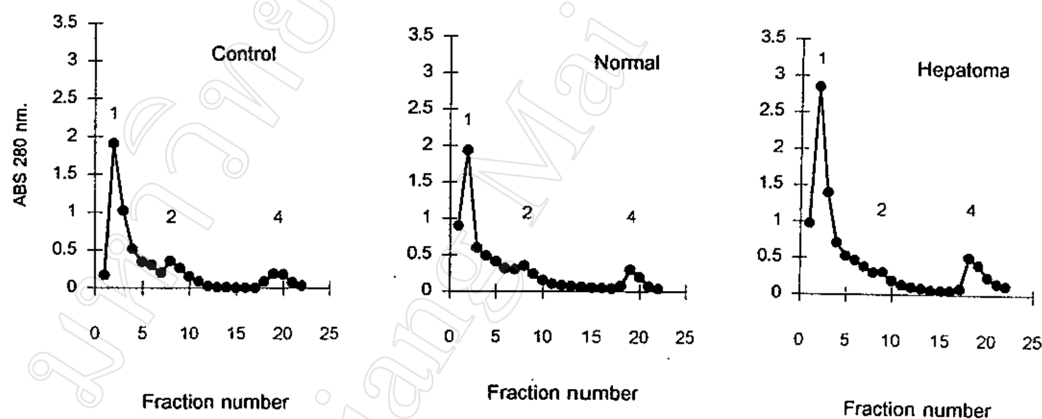


Figure 8. Elution profile of ALP separated from control, normal and hepatoma sera by DEAE Sepharose chromatography using the same condition as Figure 7. Upper and lower elution profiles were from samples without and with C-Neu treatment before chromatography respectively.

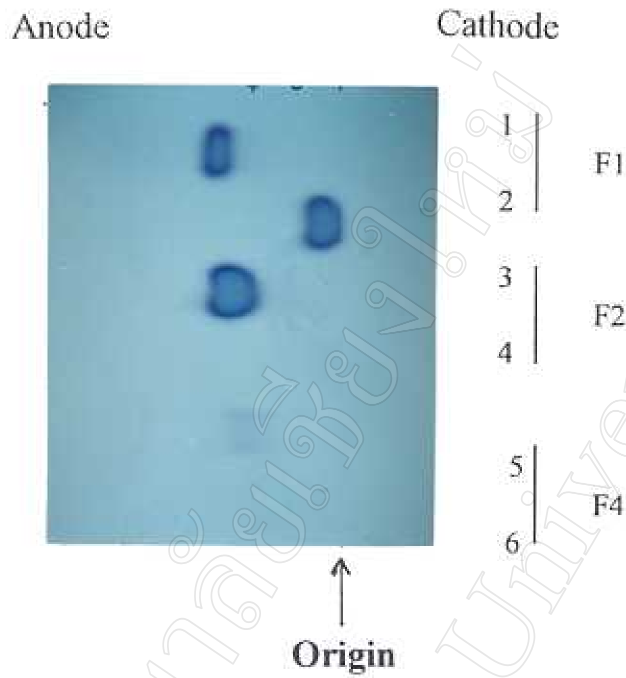


Figure 9. Agarose gel electrophoresis of ALP isoenzymes fractionated from hepatoma serum, demonstrating the patterns of migration of the fractionated ALPs as compared with the respective protein peaks in Figure 8.

Each pair of lanes represents serum untreated (odd Nos) and pretreated (even Nos) with neuraminidase before fractionating by DEAE Sepharose column chromatography.

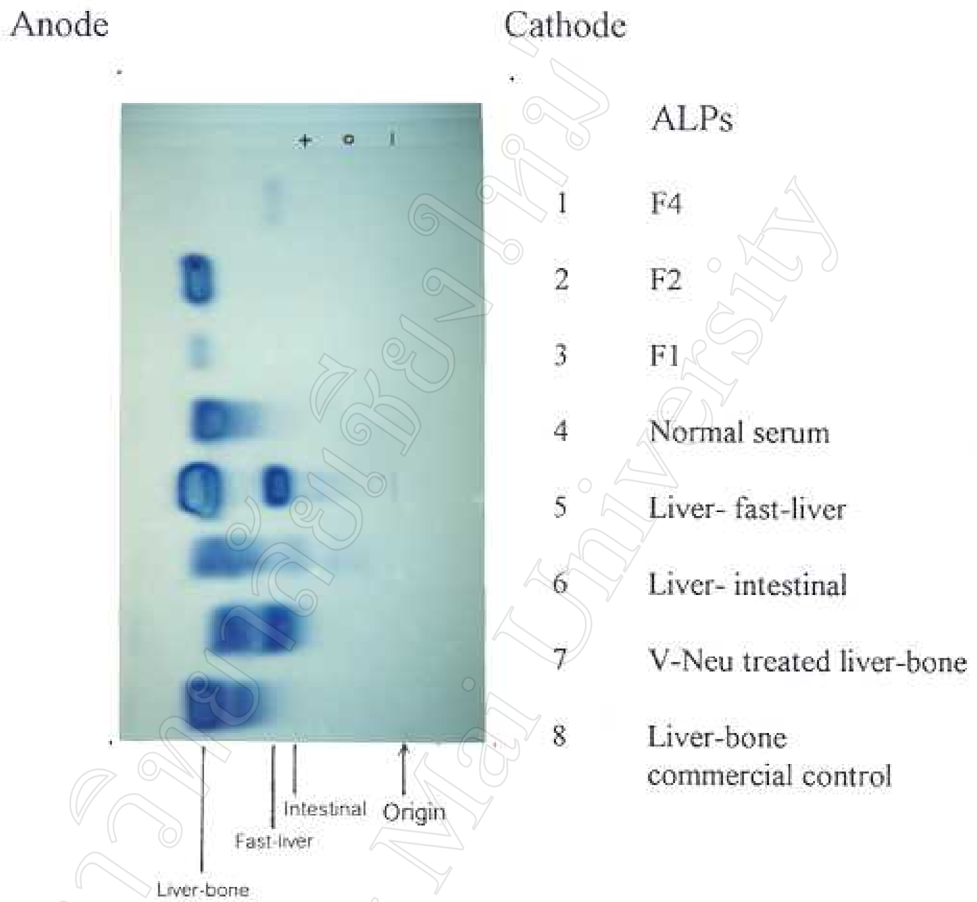


Figure 10. Agarose gel electrophoresis of column- fractionated ALP isoenzymes (lane 1-3) compared with various control sera (lane 4-6) and a commercial control from Helena laboratories, USA (lane 8 and 7 was V-Neu treated control in lane 8).

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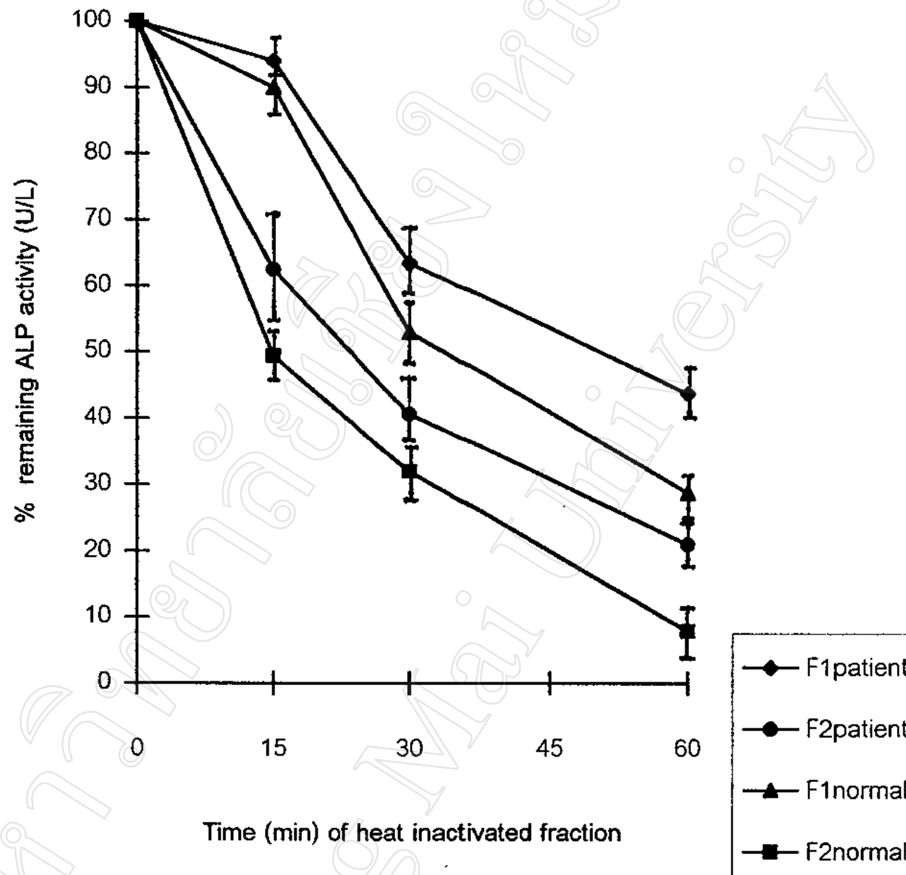

Figure 11. Heat inactivation of fraction 1 and 2 ALP isoenzyme activities. Percentage of remaining ALP activity is shown as a function of time (for 15,30 and 60 min) at 52°C. ALP activity was measured in triplicate aliquots and expressed as a percentage of the activity of the unheated control.

[3.] Lectin precipitation of sugar moieties of the fractionated ALP isoenzymes

Fraction 1 (liver ALP isoenzyme) precipitation

Precipitation with lectins of fractionated ALP isoenzymes revealed differences between healthy subjects and various groups of patients with liver disease. As summarized in Table 5, basal ALP activities of patient groups were higher than those of the healthy controls and the activity in patients with cholestasis was significantly higher than other group of patients ($p<0.05$ vs. healthy subjects). At 3 g/L of WGA precipitation of fraction 1 showed statistical results difference ($p<0.05$) for cholestasis and cirrhosis as compared with healthy subjects and it could not be used to distinguish among patients with cholangiocarcinoma, hepatoma, hepatitis, and healthy controls.

ALP isoenzymes in fraction 1 of patient groups were more sensitive to precipitation by 6 g/L of Con A than healthy controls. In patients with cholestasis, cirrhosis and cholangiocarcinoma, precipitation with Con A was significantly increased to approximately 85% as compared with 60% of the healthy controls ($p<0.05$). In addition, Con A precipitated from 72% to 76% of fraction 1 from the group of hepatoma and hepatitis patients which were still higher when compared with the controls.

Precipitation with PSA of fraction 1 could not distinguish between the patient groups from the healthy controls. At 1 g/L of PSA precipitated approximately 60% in patients with cholestasis, cirrhosis and cholangiocarcinoma. The fraction 1 of hepatoma group was more sensitive to precipitation with PSA than healthy and some of patient subjects, whereas the hepatitis group was less sensitive to the PSA precipitation.

Fraction 2 (bone ALP isoenzyme) precipitation

As shown in Table 6, All patients with hepatoma and hepatitis had more basal ALP activity than healthy subjects and the ALP activity in hepatitis group was significantly higher than controls ($p<0.01$). WGA precipitation efficiencies were found to be significantly increased in the hepatoma and hepatitis patients as compared with the healthy controls ($p<0.05$), and the other groups of patients with liver disease.

For Con A precipitation, fraction 2 of the hepatitis group was more sensitive to Con A precipitation than those of the other patient groups as compared with the healthy controls. Con A precipitated fraction 2 from 42 % to 60% in healthy subjects and in the patients with cholestasis, cirrhosis, cholangiocarcinoma and hepatoma. Precipitation efficiencies with PSA could not be used to distinguish the patient groups from the healthy subjects.

Fraction 4 (fast liver ALP isoenzyme) precipitation

Most of patient groups had variable basal ALP activity for fraction 4 (Table 7). WGA and PSA precipitated about 60% of the fraction 4 from sera of various groups of patients with liver disease. On the other hand, fraction 4 of patient groups were more sensitive to precipitation with Con A than any other lectins used to differentiating this isoenzyme molecules. Con A precipitated from 78 % to 80 % of fraction 4 from cholestasis and cirrhosis, whereas precipitation efficiencies were lower to 64% and 67% in the other groups of patients.(Statistically no differences)

Taken together, these results concluded that precipitation efficiencies of WGA and Con A were varied on sugar moieties of ALP isoenzyme in fraction 1 and fraction 2 separated from patient and healthy sera. WGA precipitation varied from 50-80% of sugar chains composed on the liver and bone ALP isoenzyme molecules. Patterns of precipitation were heterogeneous among the various conditions of liver disease and normal. Con A precipitation of liver and fast liver ALP isoenzymes in sera of patients with liver disease were significantly higher than healthy subjects ($p<0.05$). No significant differences between patients and healthy groups on precipitating with Con A from the bone ALP isoenzyme except for patients with hepatitis. PSA precipitation affected on liver ALP of patients with hepatoma and on the fast liver isoenzyme in one case of cirrhosis.

Table 5. Effect of lectins on precipitation of fraction 1, liver ALP isoenzyme, separated from sera of patients and healthy subjects.

Sources of ALP activity	Basal F1 ALP activity, U/L[a]	Percent remaining ALP activity after ppt. With[b]		
		WGA	Con A	PSA
Cholestasis 1	17.2[c]	23.1[c]	15.4[c]	46.2
Cholestasis 2	18.5[c]	21.4[c]	14.3[c]	42.9
Cirrhosis 1	14.5	27.3[c]	13.6[c]	54.6
Cirrhosis 2	9.3	25.6[c]	14.3[c]	42.9
CHCA 1	7.9	58.3	16.7[c]	50.0
CHCA 2	11.9	50.0	14.4[c]	44.4
Hepatoma 1	11.9	50.0	27.8	33.3
Hepatoma 2	14.5	48.2	22.7	27.3
Hepatitis 1	14.5	45.5	22.7	72.7
Hepatitis 2	18.5	39.3	23.6	64.3
Healthy subject 1	6.6	44.0	40.0	50.1
Healthy subject 2	6.5	39.9	31.8	44.5
Healthy subject 3	6.6	34.9	41.1	47.6

[a] In untreated controls (without lectin).

[b] Percent of basal ALP activity remaining after precipitation with lectin.

[c] $p < 0.05$ VS. healthy control.

Table 6. Effect of lectins on precipitation of fraction 2, bone ALP isoenzyme, separated from sera of patients and healthy subjects.

Sources of ALP activity	Basal F2 ALP activity, U/L[a]	Percent remaining ALP activity after ppt. With[b]		
		WGA	Con A	PSA
Cholestasis 1	4.6	42.9	57.2	85.7
Cholestasis 2	4.1	48.4	58.1	80.6
Cirrhosis 1	7.9	58.3	50.0	66.7
Cirrhosis 2	4.1	64.5	51.6	67.7
CHCA 1	7.9	50.0	41.7	66.7
CHCA 2	7.4	53.6	35.7	55.4
Hepatoma 1	17.2	38.5[d]	53.9	84.6
Hepatoma 2	11.9	36.0[d]	55.6	74.0
Hepatitis 1	17.2[c]	15.4[d]	30.7	76.9
Hepatitis 2	15.9[c]	20.0[d]	23.3	70.0
Healthy subject 1	9.9	53.3	40.0	93.3
Healthy subject 2	8.9	55.0	45.0	80.0
Healthy subject 3	8.1	54.5	45.5	81.8

[a] In untreated controls (without lectin).
[b] Percent of basal ALP activity remaining after precipitation with lectin.
[c] $p < 0.01$ VS. healthy control.
[d] $p < 0.05$ VS. healthy control and the other groups of patient.

Table 7. Effect of lectins on precipitation of fraction 4, fast liver ALP isoenzyme, separated from sera of patients and healthy subjects.

Sources of ALP activity	Basal F4 ALP activity, U/L[a]	Percent remaining ALP activity after ppt. With[b]		
		WGA	Con A	PSA
Cholestasis 1	6.6	40.0	20.0	60.0
Cholestasis 2	6.6	40.2	20.7	41.8
Cirrhosis 1	11.9	44.4	22.2	46.5
Cirrhosis 2	6.6	40.0	20.0	36.0
CHCA 1	5.9	66.7	33.3	55.5
CHCA 2	7.9	50.0	33.3	41.7
Hepatoma 1	14.5	50.0	36.4	54.5
Hepatoma 2	9.3	47.1	32.7	42.8
Hepatitis 1	11.9	55.6	33.3	44.4
Hepatitis 2	7.9	51.7	33.3	45.0

[a] In untreated controls (without lectin).
[b] Percent of basal ALP activity remaining after precipitation with lectin.

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[4.] Study of sialic acid linked to the sugar chain moieties

4.1 Evaluation of preliminary studies

Preliminary studies of desialylation of ALP isoenzymes were carried out in serum samples. To estimate a time suitable for digesting sialic acid from the ALP isoenzyme in serum, the neuraminidase treated samples were incubated at 3 or 18 h at room temperature. After cellulose acetate electrophoresis, the patterns of isoenzymes incubated at different lengths of time were compared. These were shown in Figure 12 that the mobility of neruraminidase treated ALP isoenzyme for 3 h (lane 7) was slightly different from treating for 18 h (lane 3). To prevent sialylated reaction of α 2,6-sialyltransferase (α 2,6- ST) from interfering activity of neuraminidase, heat treatments on neuraminidase activity in sera after digesting the ALP isoenzyme at two different lengths of time were carried on. Results showed in figure 12 that heat inactivation of neuraminidase used to desialylation of ALP isoenzyme also affected on stability of ALP by decreasing the intensity of color staining in the same serum sample (lane 4,5,6 ; 18 h C-Neu treated samples inactivated by heat at 52°C for 15,30 and 60 min respectively and lane 8 ; 3 h C-Neu treated sample inactivated by heat at 52°C for 60 min).As in Figure 13 ,the % remaining ALP activity plotted against the amounts of sialic acid liberated from isoenzyme molecules (digested activities of neuraminidase were evaluated by the amounts of sialic acid liberation) were reduced as a function of inactivation time. Neuraminidase activity was inactivated for about 10 % and 15 % in serum sample digested with neuraminidase for 3 and 18 h respectively. Along with these, ALP activities were also reduced by 30 % and 50 % for 3 h and 18 h treated samples respectively.

For further studies, the ALP digestion with neuraminidase for 3 h at room temperature, without heat inactivation of neuraminidase activity, was the selected conditions in desialylation of the fractionated ALP isoenzymes.

The analytical precision of TSA assay was assessed by analyzing of sialic acid in pooled human serum. The coefficient of variation for both intra- and inter assay were 6.87 % and 10.5 %, respectively.

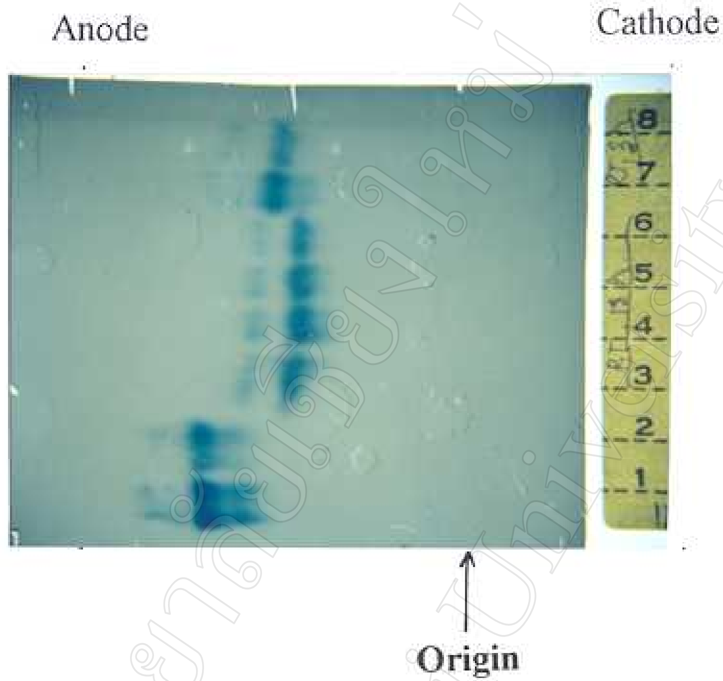

Figure 12. Effect of heat on ALP isoenzymes incubated with neuraminidase at different lengths of time, demonstrating by cellulose acetate electrophoresis.

Lane(1) neat serum, lane (2) untreated control serum, lane(3-6) 18 h C-Neu digested serum; lane (3) unheated control and lane (4-6) heated at 52° C for 15,30, and 60 min respectively. Lane (7-8) 3 h C-Neu digested serum; lane (7) unheated and lane (8) heat treated at 52° C for 60 min of 3 h.

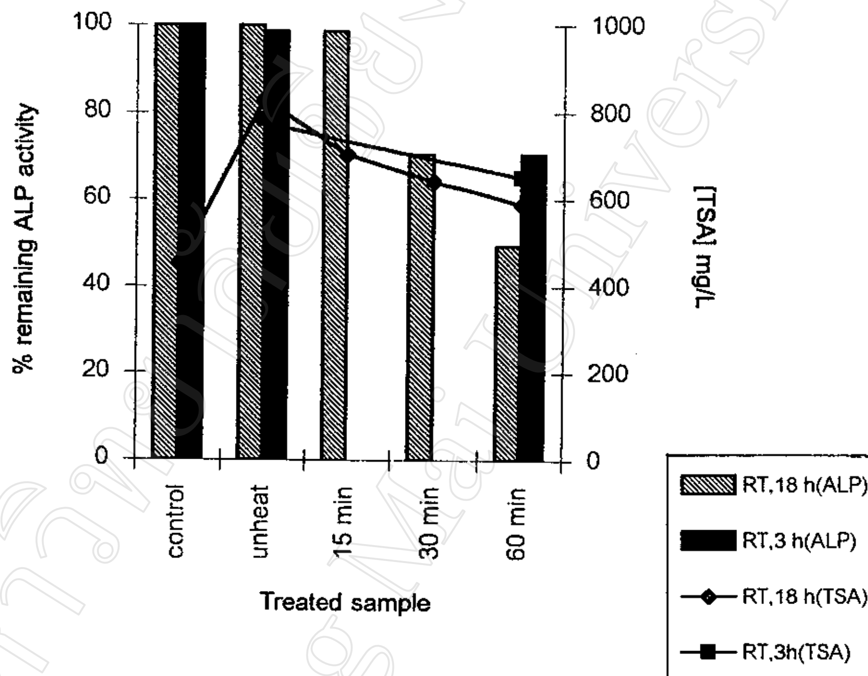

Figure 13. Heat inactivation of neuraminidase activity (measured by the releasing of TSA product), which affected on ALP stability in desialylated serum.
Control = C-Neu undigested serum, unheat = C-Neu digested serum without heat treatment and the rests were C-Neu digested serum with heat treatment at different lengths of time.

4.2 Sialylation of ALP isoenzyme fraction

4.2.1 Optimization of neuraminidase concentration on the digestion of ALP isoenzyme fractions

Result from previous experiment introduced the optimal condition used to desialylation of ALP isoenzymes in serum. Because of the fractionated ALP isoenzymes had less activities than those in serum samples, therefore, the optimization of neuraminidase concentration was needed. The various concentrations and types of neuraminidase used to digest partly purified ALP isoenzymes were incubated with fraction 1 of all fractionated ALP samples and fraction 2 of hepatitis. The asialo- ALP isoenzymes obtained from desialylations were examined by an agarose gel electrophoresis. As shown in Figure 14, the selected concentration and type of neuraminidase was 125 mU/L C- Neu. This concentration was used to prepare asialo-ALP isoenzymes in all subsequent experiments.

4.2.2 The digesting effect of different sources of neuraminidase on ALP isoenzyme fractions

Figure 15 showed the effect of C - Neu and V - Neu treatment on liver ALP fraction. In comparative migrations of asialo- ALP bands on agarose gel electrophoresis, the mobility of neuraminidase-treated liver ALP with C-Neu and V-Neu in healthy subject and patients with cirrhosis and cholangiocarcinoma were moved equally toward the anode. In hepatoma and cholestasis, the mobility of liver ALP treated with C-Neu was markedly slower than that of V - Neu. In contrast, the mobility of C - Neu treated liver ALPs from patient with hepatitis was faster than that as compared with V - Neu treated sample.

4.2.3 Electrophoretic patterns of α 2,6-sialyltransferase treated asialo liver ALPs

The electrophoretic patterns of the asialo liver ALPs (prepared by the selected conditions as in 4.2.1) treated with α 2,6- sialyltransferase (α 2,6- ST were shown in Figure 16 (a-c). The sialylated liver ALPs were found to move more toward the anode than those asialo- ALPs in healthy subject and patients with cirrhosis, hepatoma and cholangiocarcinoma (Figure 16 a). In addition, the mobility of sialylated liver ALPs in patients

with hepatitis and cholestasis were unchanged by treatment with α 2,6- ST (Figure 16 b). In case of fast liver ALP fraction (fraction 4), after treatment with α 2,6-ST, the mobility of sialylated fast-liver ALP in patients with cholestasis and hepatoma were moved more toward the anode than their corresponding asialo- ALP (Figure 16 c, lane 6 and 8 respectively).

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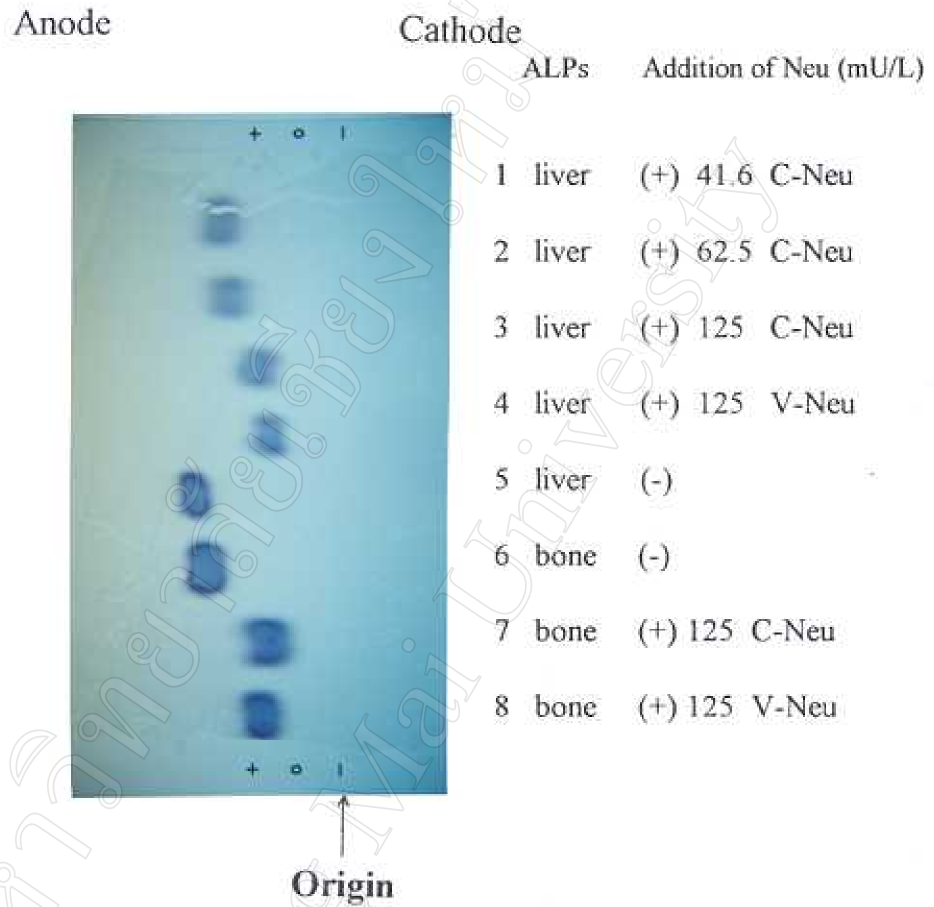

Figure 14. Effect of various concentrations of C-Neu and comparison effect of C-Neu and V-neu at the concentration 125 mU/L on the fractionated ALP isoenzymes, identified by an agarose gel electrophoresis without (-) and with (+) neuraminidase (C-Neu).

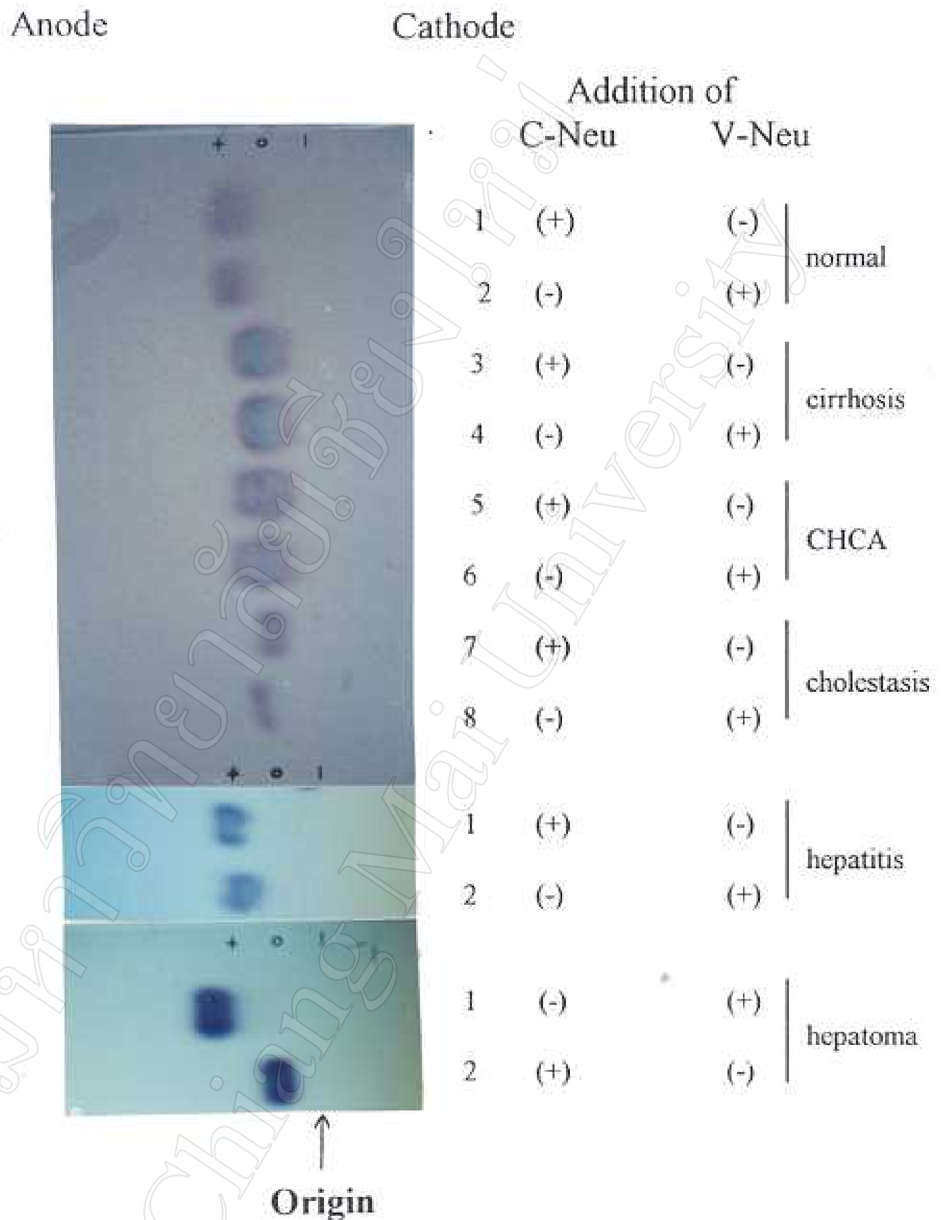

Figure 15. Agarose gel electrophoretic patterns of C-Neu and V-Neu desialylation of fractionated liver ALP isoenzymes from healthy subject and patients with cirrhosis, CHCA, cholestasis, hepatitis and hepatoma (separate run) without (-) and with (+) neuraminidase (C- and V-Neu).

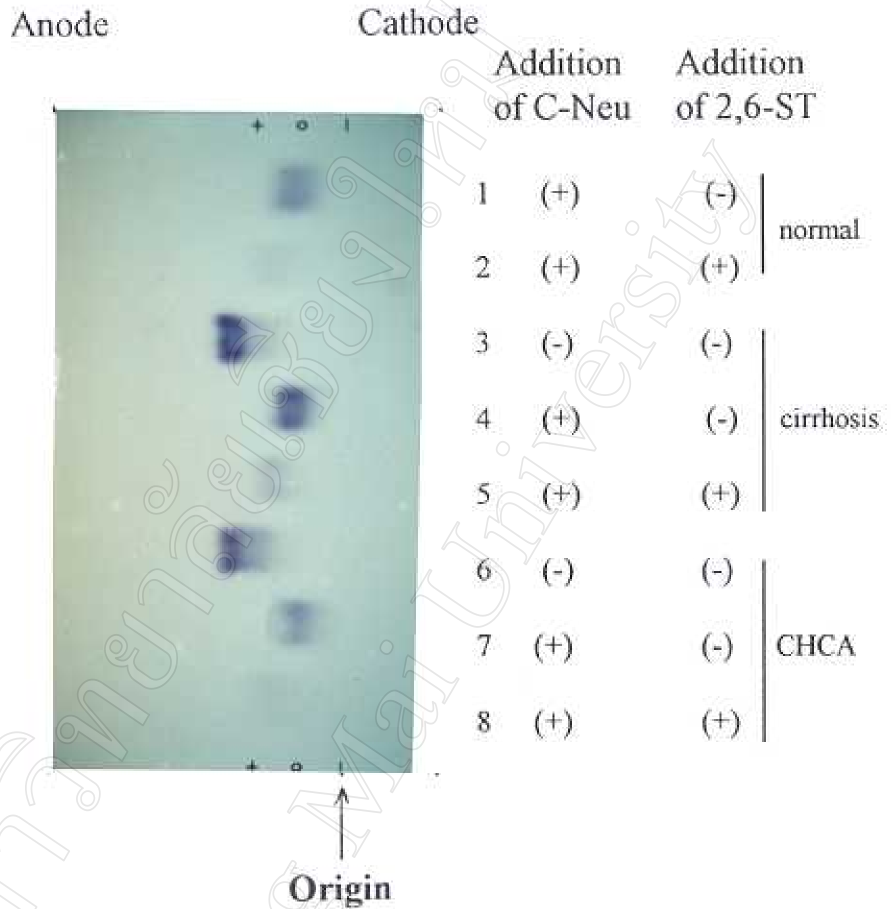

Figure 16 a. Agarose gel electrophoretic patterns of liver ALP isoenzyme treated with α 2,6- ST in healthy subject as compared with cirrhosis, CHCA patients without (-) and with (+) neuraminidase (C-Neu) or α 2,6- ST.

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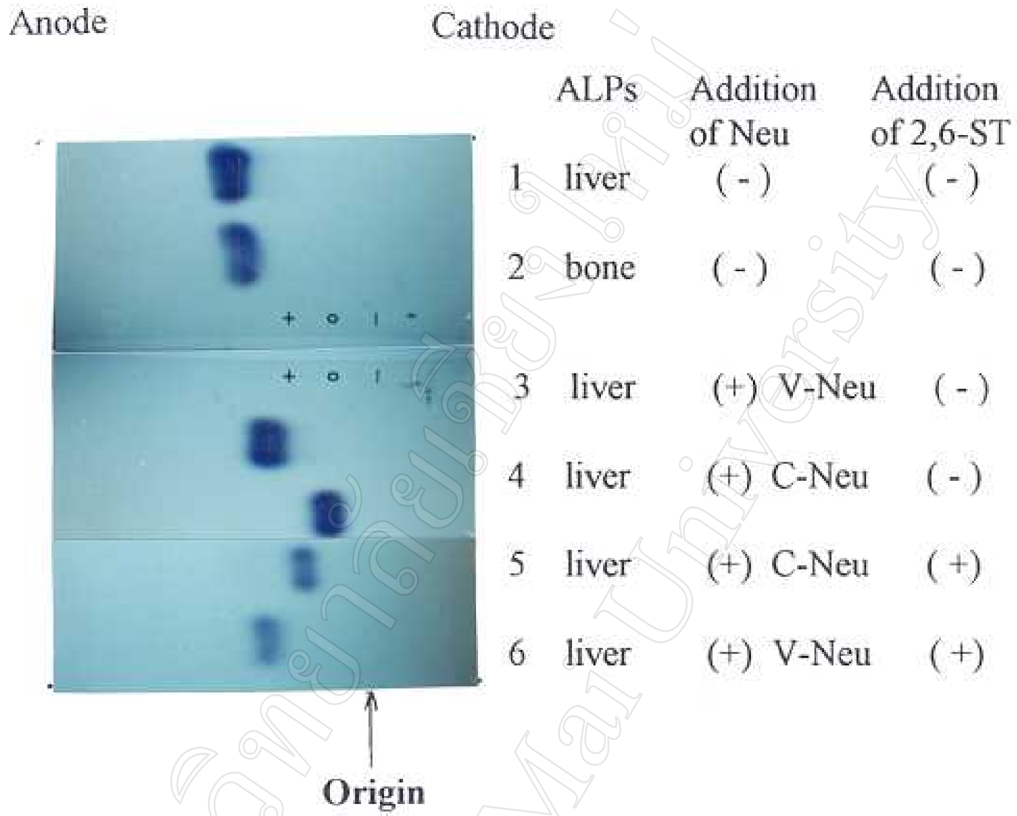

Figure 16 b. Agarose gel electrophoretic patterns of liver ALP isoenzyme treated with α 2,6- ST in patients with hepatoma (separate run) without (-) and with (+) neuraminidase (C-Neu) or α 2,6- ST.

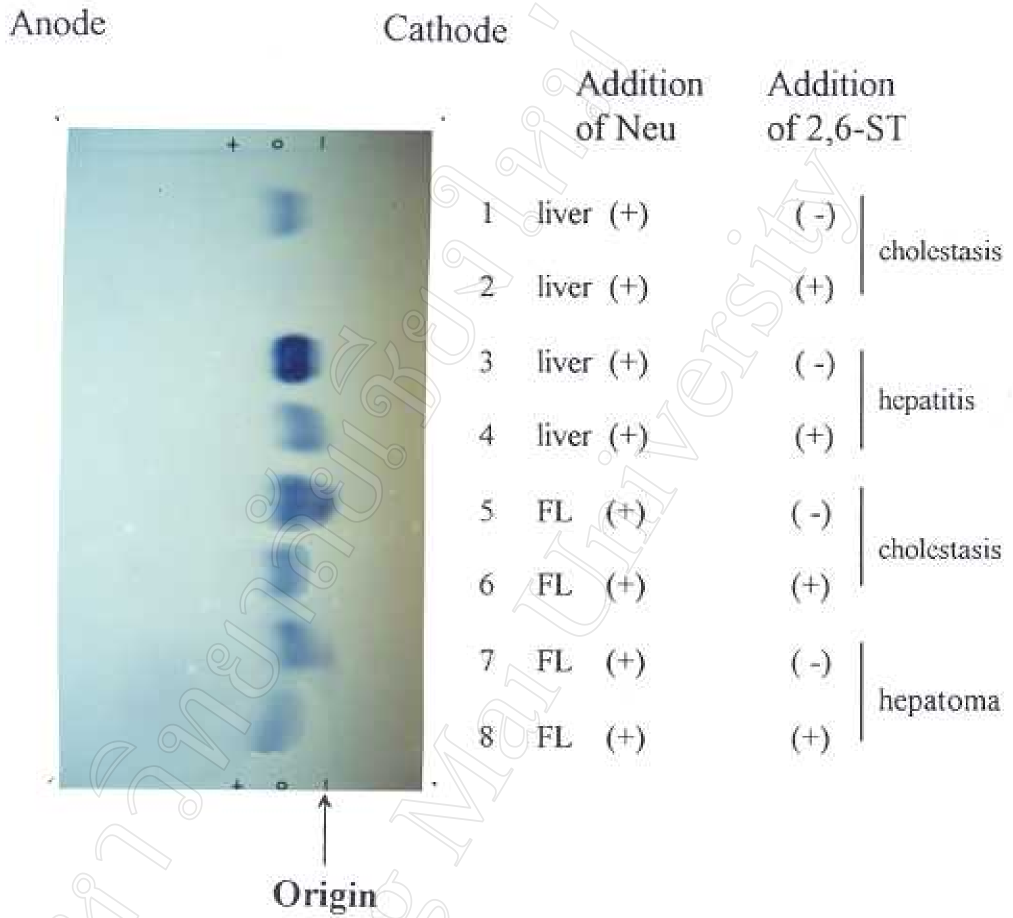

Figure 16 c. Agarose gel electrophoretic patterns of liver ALP isoenzyme treated with α 2,6- ST in patients with cholestasis, hepatitis, and hepatoma without (-) and with (+) neuraminidase (C-Neu) or α 2,6- ST. (FL : fast-liver ALP).

Part IV. Application of sialic acid linked- properties of the ALP molecules on the separation of ALP isoenzymes in serum by CA electrophoresis

In an attempt to apply the knowledge of sialic acid linkages on different ALP isoenzyme molecules reported in this study to the laboratory work use for discriminating these ALP isoenzymes in sera of patients with liver disease. Electrophoresis separation of ALP isoenzymes on cellulose acetate membrane was performed. Result in figure 17 showed the patterns of ALP isoenzyme separation scanned by the Electrophoresis Data Center (EDC). It was found that V-Neu at the same activity with C-Neu could not separated ALP isoenzymes in serum samples by CA electrophoresis (data not shown). C-Neu pretreated samples before CA electrophoresis showed very good separation of bone and liver ALP isoenzymes for serum samples from hepatitis, cirrhosis patients and commercial liver- intestinal control. The mobility of liver ALP isoenzyme of pretreated serum was retarded and migrated nearly the cathode (compared with the control). In serum from hepatoma and cholestatic patients, there were the overlapping of the bands that moved toward the anode. As compared with the same serum sample but non- treated condition, this hepatoma sample contained fast liver ALP isoenzyme. The overlapping bands after pretreating with neuraminidase was suspected to be the fast liver and bone isoenzymes.

Anode

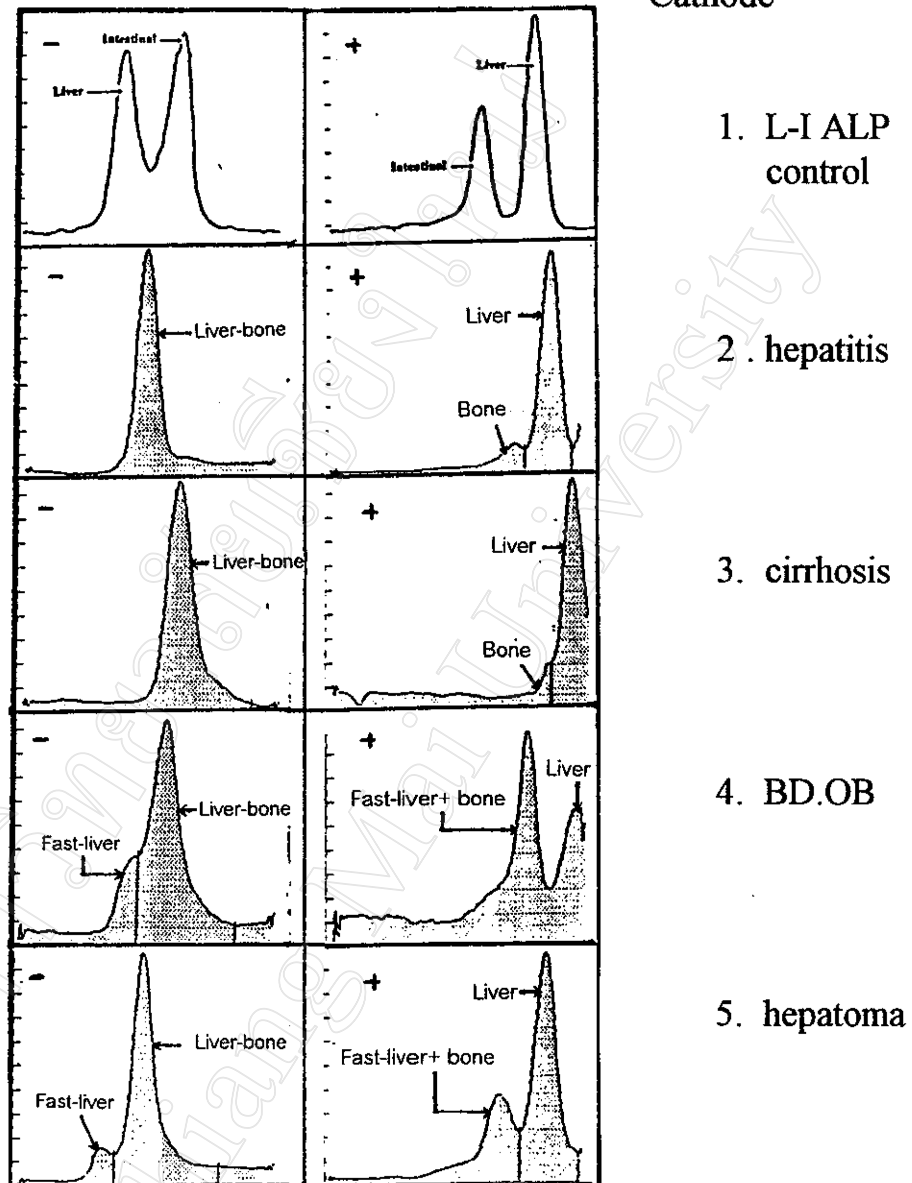

Figure 17. Densitometric scanning of ALP isoenzymes in serum of liver disease patients by CA electrophoresis. (1) liver-intestinal ALP commercial control (Helena laboratories, USA), (2) hepatitis, (3) cirrhosis, (4) bile duct obstruction, and (5) hepatoma untreated (-)and treated (+) with C-Neu.