V. DISCUSSION

Clinical chemistry tests are useful in evaluation of liver function and generally used to identify liver disease. According to the result, most of the common liver function tests in sera of patients with liver disease were significantly higher than those of the healthy subjects. It was found that the biochemical changes in sera of patients with differential diagnosis of liver disease were in accordance with the laboratory features of various forms of liver disease (Sherwin and Sobennes, 1996 ; Tietz, 1990). For example, hepatobiliary disease, such as hepatitis, was characterized by transaminases which increase greater than 5 times the upper limit of normal, with the ALT levels usually predominantly increased above the AST values. Alkaline phosphatase, as a tumor marker, was risen approximately 10 times above the upper limit of normal associated with the increases of serum bilirubin concentrations in sera of patients with hepatoma. In cholestasis, a marked increase in the ALP level with only a mild increase of transaminase above the upper limit of normal are the characteristic of this disease. Thus, referred to the results of liver function test, the difference is seen between the healthy subjects and patients with liver disease as well as among the patient groups.

The ALP activities measured by a modified method in 96-well microtiter plates,with a microplate reader (Model EL 340) were correlated well with the standard method measured with the Shimudzu double-beam UV 160 A spectrophotometer ($r = 0.991$). ALP activities determined by the microplate method must be multiplied by a factor of 1.5 for comparison with the measurements by the double-beam UV 160 A. The values determined in the applied experiments in a microplate reader such as determination of remaining ALP activity after lectin precipitation or heat inactivation were appreciable specifically to this modified procedure. Although the volume of determination was very small, the CV percentage of intra - and inter - assay confirmed the reliability of ALP activities measured in this instrument.

Initial characterization of ALP isoenzymes in serum samples used in this study was performed by the measurements of the ALP isoenzymes which sensitive to chemical inhibitors and heat (Physicochemical properties). ALP activities in sera of patients with liver disease were markedly inhibited by levamisole but slightly inhibited by L-Phenylalanine. The commercial placental and intestinal ALP isoenzymes exhibited the reverse results with these two inhibitors. Since levamisole has been shown

to be the potent inhibitor of the ALP from bone, liver and kidney (Van Belle, 1976) and L-Phenylalanine as an organ specific, stereospecific inhibitor of intestinal and placental ALP (Fishman *et al.*, 1963), therefore, the susceptibility of ALP to levamisole inhibition implied that the ALP isoenzymes containing in the serum samples of normal and patients were related to the tissue-non specific ALP isoenzymes. Heat treatment results of some serum samples clearly demonstrated the identity of these isoenzymes.

Tissue non-specific ALP isoenzymes are circulated in sera under both normal and pathological conditions. Forms of which normally presented are particular of liver or bone type. The kidney ALP isoenzymes is rarely detected even is sera of kidney disease patients (Miura *et al.*, 1988; Pfleiderer *et al.*, 1980). In this experiment, lectin precipitation of bone ALP was used to evaluate the composition of bone fraction in sera of patients with liver disease as compared to the normal controls. The lectin used was wheat - germ lectin which was reported to react with N-acetylglucosamine and N-acetylneuraminic acid (Lis and Sharon, 1973) on the carbohydrate side chains of the ALP molecules and precipitating the bone fraction from the other isoenzymes. WGA precipitation method has been suggested as a quick and simple alternative method for the quantitation of bone ALP (Klein *et al.*, 1989 ; Behr and Barnert, 1986). As shown in the result, the values of the % precipitating ALP activities did not correlate with the bone ALP values calculated by using the formula. This result was agreed with the previous studies that WGA could not completely separate bone from liver isoenzyme activity (Griffiths and Black, 1987 ; Farley *et al.*, 1993) or variable amounts of fast liver ALP co-precipitated with bone ALP (Rosalki and Foo, 1989). Farley *et al.* (1994) suggested that a two- site immunoradiometric assay and heat inactivation assay for quantitative measurement of bone ALP activity were superior to the WGA precipitation assay. However, in the recent study, Rauch *et al.*(1997) reported that the immunoradiometric assay and enzyme-linked immunosorbent assay did not have a detectable advantage over lectin affinity electrophoresis in the determination of bone ALP in children.

The electrophoretic method is widely used for the quantitative analysis of ALP isoenzymes in serum. The technique is suitable for separation of ALP isoenzymes in a large numbers of sera. This study showed that electrophoretic patterns of ALP isoenzymes on a CA membrane could not be used to distinguish the pathological conditions of the liver disease. The overlapping liver-bone bands were found in most of

all samples from healthy and patient subjects. The intestinal ALP band was presented in some sera of the healthy subjects with blood group O and B and occasionally seen in sera of patients with cholestasis, hepatoma, cholangiocarcinoma and hepatitis (no record data of blood group). Determination of ALP isoenzymes by CA electrophoresis provided ease of handling but the disadvantages of this method are a lack of sensitivity, poor resolution of the liver and bone isoenzymes (Siede and Sieffert, 1977).

Serum samples of normal and various diseases were shown to contain various ratio of liver and bone ALP isoenzymes (Schiele *et al.*, 1983). The method of separation of ALP isoenzyme components in serum by DEAE-Cellulose anion exchange chromatography (Karmen *et al.*, 1984) was unable to resolved bone ALP isoenzyme from serum (or from liver fraction). In this study, using a modified method of Mercer *et al.* (1994), 3 fractions of ALP isoenzymes were separated on DEAE Sepharose column. Fraction of the separated isoenzyme was obtained from all samples of normal and patient sera. The difference in their respectively heat stability between F1- liver isoenzyme and F2-expected bone isoenzyme included slightly different in electrophoresis mobilities on an agarose gel verified the identity of this isoenzyme. There was a good agreement of identification of liver ALP isoenzyme (F1) fraction between C- Neu treated samples before chromatography and C- Neu treated samples after chromatography following by agarose gel electrophoresis. Separation of ALP fractions on DEAE Sepharose column was more sensitive than that of CA electrophoretic method because the fast liver isoenzyme were resolved even in low activity (from results and Mercer *et al.*, 1994).

In this study, the distribution of fraction 4-fast liver isoenzymes along with the fraction 1-liver isoenzyme and fraction 2-bone isoenzyme were occasionally seen in normal serum and almost in sera of various groups of liver disease. This was also reported by some workers (Karmen *et al.*, 1984 ; Van Hoof *et al.*, 1990) that the fast liver ALPs were presents in the activity as low as 8 U/L in normal adult sera aged from 20-60 years. This activity was remained the same throughout life. In the isoenzyme separation, no carry over of one type of ALP isoenzyme fraction into the others. These data provided the reliability for evaluations of sugar chain moieties on the separated ALP isoenzyme molecules in sera of normal and liver disease patients.

Liver, bone and fast liver ALPs were demonstrated to possess an identical protein core (Miura *et al.*, 1994). The difference in their affinity of lectin precipitation and electrophoretic mobilities were attributed to their sugar chain heterogeneity (Rosalki and Foo, 1984 ; Kuwana *et al.*, 1991 ; Moss and Whitaker, 1985). Several kinds of lectins were used to differentiate the compositions of sugar moieties in the carbohydrate chains of the ALP glycoprotein (Cummings and Kornfeld, 1982 ; Miura *et al.*, 1989). WGA , Con A and PSA were reported to have affinity binding or selective precipitating with the ALP isoenzyme molecules containing GlcNAc, α- mannose and fucose respectively (Cummings and Kornfeld, 1982 ; Kuwana *et al.*, 1991)

In this experiment, the identities of sugar chain moieties of liver ALP isoenzymes in sera of various forms of liver disease patients were compared with each other and with the adult normal sera. The concentrations of lectins used in precipitation of ALP fractions were selected from the work of Farley *et al.* (1993). It indicated that the dose dependent of lectin on separating of liver and bone isoenzymes were maximum at 3.0 g/L WGA and 6 g/L Con A in a final precipitation solution. For PSA, a 1 g/L was used to differentiate bone isoenzyme in the various bone ALP standard preparations. At that described levels, it is seen that WGA precipitated liver ALP isoenzymes (F1) from sera of liver disease patients at different rates. High quantities of liver ALP isoenzyme precipitation were found in patient sera with cholestasis and cirrhosis. In other groups of patients, the lower quantities of the isoenzyme precipitation were demonstrated to be agreed with the level in the normal controls. Precipitation of bone ALP isoenzymes (F2) from all serum samples (including from serum of normal subjects) except from sera of patients with hepatitis and hepatoma were rather constant at the average rate at 50% remaining activity. These observations implied that, in the quantitative separation of liver-bone isoenzymes in sera by the method proposed by Behr and Barnet (1986), the paradoxical results of bone fractions obtained from WGA precipitation might not only cause by co-precipitating of fast liver ALP isoenzyme but also from the liver isoenzyme fractions and thus resulted in underestimating of liver isoenzyme fraction and overestimating of bone ALP isoenzyme fraction in an individual serum sample.

Precipitation of the isoenzymes by Con A from liver ALP fractions from sera of liver disease patients were found to be significantly higher than from sera of normal control. These could be suggested that

glycosylation on the liver ALP proteins may change according to the disturbance of liver cells during the development of liver disease. There were no different between normal and disease in precipitating by Con A from bone fractions (F2) except for hepatitis. Con A precipitated fast liver isoenzyme fractions more or less the same as the liver isoenzyme fractions separated from the same patient sample.

In conclusion, as compared to normal and bone isoenzyme fractions the degree of glycosylation by α-mannose on the carbohydrate chains of the liver and fast liver isoenzyme fractions may be alterably higher than in disease. The alteration may caused by the variation in Golgi body sugar chain processing.

Apart from WGA, PSA has been elucidated to have affinity binding to bone isoenzyme. The sugar moieties which are specific for affinity binding are fucose residues. In this study, the precipitation of bone isoenzyme (F2) fractions from samples of normal and patients were considerably less than those obtained from liver (F1) and fast liver (F4) ALP isoenzyme fractions. These results were somewhat differed from those of Kuyama *et al.* (1987) who subjecting the partially purified ALP from pooled serum of healthy children to the serial lectin affinity chromatography and the bone isoenzyme fraction was found to bind highly, reproducibly to the pea lectin-agarose column. However from the investigation of Farley *et al.* (1993), the binding and precipitating of PSA with bone isoenzymes were not obtained from several putative bone standard except for the Pagetic serum. From the studies of age and sex distribution of alkaline phosphatase isoenzymes in sera of normal children and adults, Van Hoof *et al.* (1990) found that in sera of normal children, two bone isoforms were presented. One bone variant disappear after age 17 for girls and after age 20 for boys. These may be suggested that the sugar moieties on the carbohydrate chains of the bone isoenzyme may merely be altered during the developing of bone disease but in the liver disease, the bone isoenzyme would not be modified and circulated normally in the circulation. The disagreement of results obtained from this study and between the literature may be focus on the variations of bone isoforms appeared in sera of normal children, adults and diseases.

In this study of sialic acid linkages, although it was uncertain that CMP-NANA concentration and α 2,6- sialyltransferase (ST) activity were optimal for the sialylation reaction. The sialo-form liver ALPs obtained from α 2,6- sialyltransferase treatments of C-Neu digested liver ALP fractions were found to be succeeded at one level. The patterns of

sialylation as investigated on the agarose gel were similar between normal and patients with liver disease except for hepatitis and cholestasis. The mobilities of liver α 2,6- sialylated ALPs were migrated more toward the anode than that of asialo ALPs but not equalled to the undigested controls. It has already been reported that, in mammalian cells, the sialic acid linkages at the terminal of glycoproteins are α 2,3- and α 2,6- types (Schachter *et al*, 1983). C-Neu is demonstrated to use both α 2,3 - and α 2,6- sialic acid linked substrates but V-Neu specifically used α 2,3- sialic acid linked substrate. In addition, C-Neu is preferred to use more α 2,6- sialic acid linkage than that of the other. Therefore, the different electrophoretic mobility of undigested liver ALP fraction and the α 2,6- ST treated fractions was likely due partly to the α 2,3- sialic acid linked composition of the carbohydrate chains on the liver ALP molecules. One evidence from this experiment supported this interpretation was that the mobility of the asialo-liver ALP fraction prepared from V-Neu (α 2,3- digestion) digested sample from hepatoma was unchanged after treating with α 2,6-ST. However, sialylation by α 2,3- ST of the liver ALP fraction from hepatoma digested with V-Neu was expected to explain this investigation but unfortunately the α 2,3- sialylation could not be performed because the α 2,3- ST was commercially unavailable. Failure to convert asialo liver-ALP fractions from hepatitis and cholestasis (bile duct obstruction) to sialo-forms after α 2,6- ST treatment was suggested to be caused by the variations of α 2,3- and α 2,6- sialic acid linked on sugar moieties which differed from the other liver-ALP fractions. As seen from the result, C-Neu and V-Neu digested patterns of these two isoenzyme fractions were different from those obtained from normal and the other forms of liver disease (which could be sialylated with α 2,6- ST).

In the study of sialic acid linkage of fast liver (F4) ALP fraction, although the sialylation reactions were performed only on two of fractionated samples from hepatoma and bile duct obstruction, the sialo-forms obtained from α 2,6- treatment recommended some possible sialylated manner of sialic acid at the end terminal of carbohydrate chains of this isoenzyme. The similarity of C-Neu and V-Neu digested patterns and the mobility of sialo-forms fast liver ALPs migrated on an agarose gel explained more about the fast liver ALP isoform as compared with the liver isoform. If the sialo-forms of C-Neu digested fast liver ALPs from all of patients sera were all of α 2,6- type and since the sugar moieties of the carbohydrate chains does not play a role in determination the net charge of the isoenzyme molecules, therefore the faster electrophoretic mobility of

this fast liver ALP fraction would depend mostly on the membrane bound baring component of this ALP molecule. This postulation was supported by a study of Mattiazzo and Romasamy (1993) that the inclusion of Triton X-100 in the WGA precipitation solution prevented the binding of lectin to the membrane-bound fractions and released ALP from the membrane and the formation of micells. The ALP that dissociated from the complex behave exactly as the liver ALP isoenzyme on the agarose gel electrophoresis.

In spite of the bone ALP fractions always present in all serum samples either of normal or diseases. The study of sialic acid linkage has not been demonstrated yet in this study. For reason, as reported by Miura *et al.* (1994), the bone ALP isoenzyme has different manner of linking sialic acid to the sugar chain moiety from that of the liver ALP isoenzyme. Base on its electrophoretic charge, asialo- bone ALP could not be converted to sialo-form by the α 2,3-ST treatment. The absence of sialylation of this ALP fraction was thought to be due to steric hindrance of α 2,3- ST.

As discussed above that there were heterogeneities of sialic acid linkages on the ALP isoenzyme molecules in sera of liver disease patients. By making use of neuraminidase in digesting of sialic acid from the ALP isoenzymes, the bone and liver ALP isoenzymes were separated on the cellulose acetate membrane. V-Neu at the same activity as C-Neu could not separate liver and bone isoenzymes in serum samples. The dependent of ALP isoenzyme separation on neuraminidase activity from different sources was reported by Jung *et al.* (1989). C-Neu and V-Neu activities between 2.3 - 145 U/L and 1.45 - 8.7 U/L, of the incubation mixture respectively, were optimal to separate liver and bone isoenzymes containing in an equal activity ratio in a serum sample. The higher or lower activities as used in this study, i.e. 0.36 U/L activity of V-Neu could not separate ALP isoenzyme in a serum sample by CA eletrophoretic technique. With C-Neu digested sample, the overlapping of fast liver with bone isoenzyme was the disadvantage of this technique. However in 1998, Siangprorh suggested that the overlapping pattern could be corrected by running the same serum sample, with and without neuraminidase treatment. Subtraction of activities between band on the two lanes of isoenzyme separation after scanning by densitometer helped reporting the activities of each isoenzyme composed in a serum sample.