II. LITERATURE REVIEWS

A. Basic knowledge on Alkaline phosphatase

Genetic and Expression

Alkaline phosphatase (E.C.3.1.3.1; ALP) has been continuously and extensively investigated for more than 70 years. ALP are encoded for by at least four different gene loci : tissue non-specific (TNALP), intestinal (I-ALP), placental (P-ALP), and germ-cell ALP (Figure 1) (Van Hoof and De Broe, 1994). The tissue non-specific alkaline phosphatase gene, expressed in osteoblast, hepatocytes, kidney, early placenta, and other cells, is located on chromosome 1p 34-p36.1 (Swallow *et al.*, 1986 ; Weiss *et al.*, 1988). In 1994 Miura *et al.* reported that molecular size of liver and bone ALPs protein molecule were identical, suggesting that the tissue non-specific ALP is probably encoded from a single genetic locus. In addition, evidence from selective modification by glycosidase indicates that differences between liver and bone ALP isoenzymes are located on their carbohydrate side chains (Moss and Whitaker, 1985).

Adult intestinal alkaline phosphatase has been mapped to chromosome 2q 34-q 37, where the loci that encode for alkaline phosphatase of the mature placenta and the closely similar (placental-like) germ cell alkaline phosphatase are located (Martin *et al.*,1987). These last three genes and their products have correspondingly similar sequences of base and amino acids. The similarities are particularly marked in the case of placenta and germ cell alkaline phosphatase, which differ by only 7-10 amino acid residues (98% homology), depending on sources. The sequence homology between the placental and intestinal isoenzymes is 86.5%, and between intestinal and TNALP is only to 56.6% (Moss, 1992).

The primary structures of human alkaline phosphatases contain a sequence of 36 amino acids conformationally close to the active center that is absent from the enzyme from *Escherichia coli.* Placental, germ cell, and intestinal alkaline phosphatase show stereospecific, uncompetitive inhibition by inhibitors and the action of inhibitors depend on residues located in this region. In particular, patterns of inhibition is played by the residue at position 429. This position is occupied by glutamine in placental ALP, serine in intestinal ALP, and histidine in the less inhibited TNALP. In germ cell alkaline phosphatase, residue 429 is glycine (Moss, 1992;

Hoylaerts and Millan, 1991). This is one of the differences between the primary structures of ALP isoenzymes.

The evolution of the ALP gene family has presumably involved the duplication of a primodial TNALP gene to create the TNALP gene and intermediate I-ALP gene, followed by additional duplications of the latter to create intestinal, placental, and germ cell ALP genes (Figure 1).

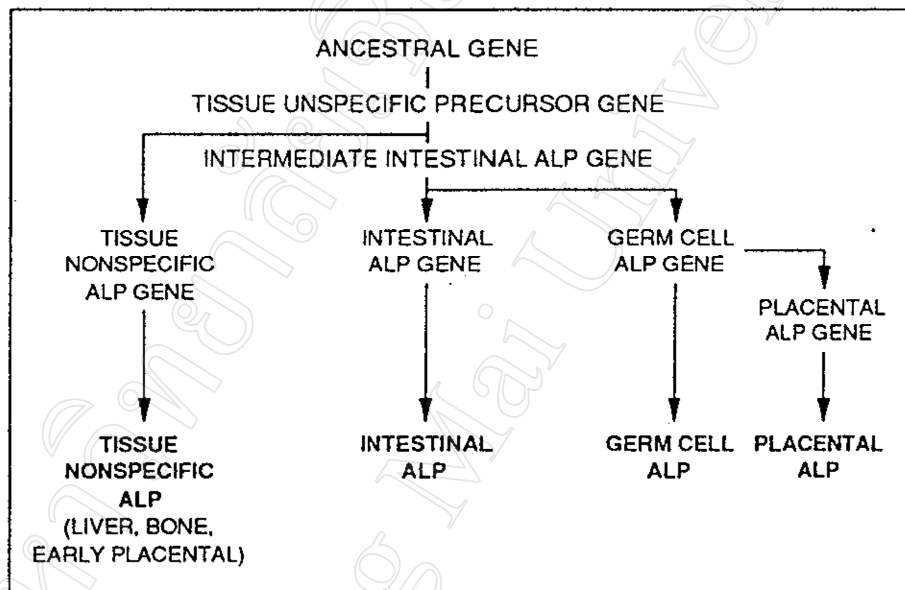

Figure 1 Evolution of the ALP gene family. (Van Hoof and De Broe, 1994)

Three-Dimensional Structure of ALP

In human serum as well as in many other species, ALP predominantly circulates as a hydrophilic homodimer. Crystallographic observations of the ALP molecule from *Escherichia coli* revealed the three-dimensional structure of dimeric ALP : a bat like figure with a metal

ion tripet in each active-site region. Zinc can bind to all of these sites, but binds particularly strongly to four sites per dimer, magnesium occupying two sites of the dimer (Figure 2) (Van Hoof and De Broe, 1994; Kim and Wyckoff, 1991).

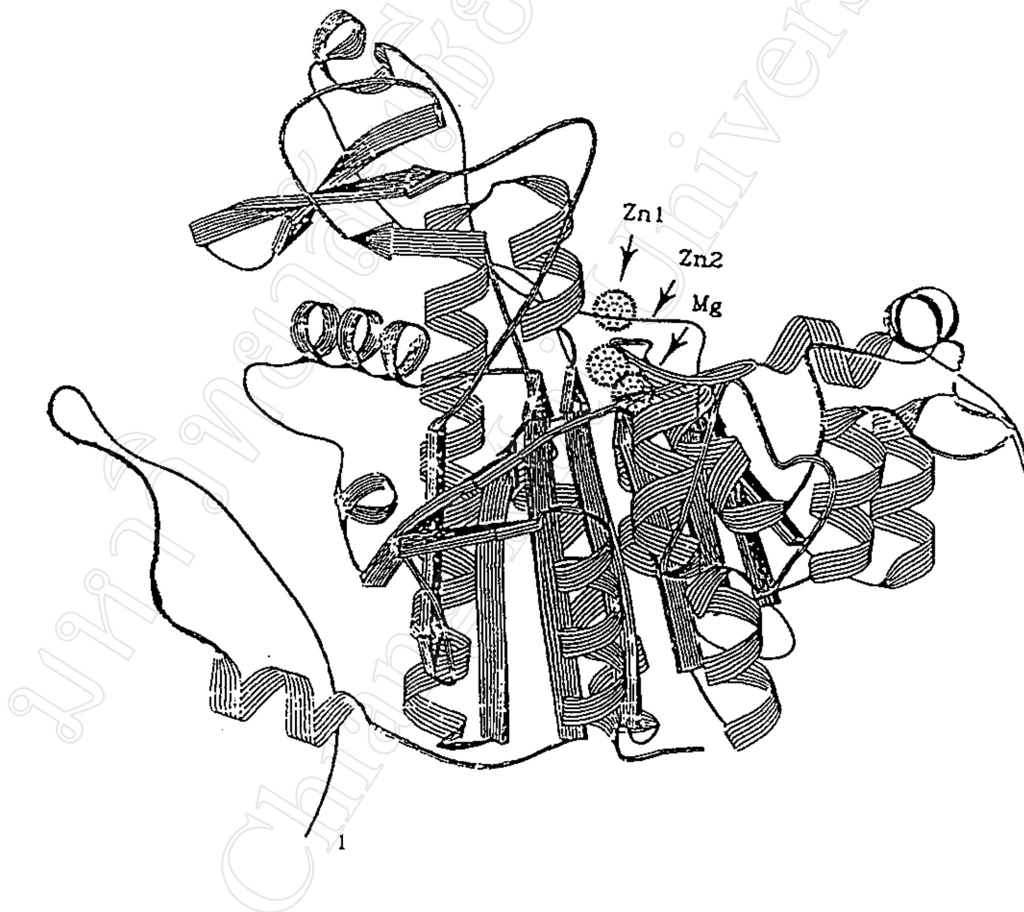

Figure 2 Ribbon drawing of a monomer of ALP. The three metals are shown as stippled spheres. The monomer consists of a ten-stranded central β-sheet flanked by 15 helicles and another three-stranded β-sheet, and a helix on the the top (Kim and Wyckoff, 1991).

Post-translational modifications of ALP

Different isoforms of ALP may result from post-translational modifications of the parent enzyme structure by either addition of chemical moieties or partial degradation. These changes can occur intracellularly, such as the addition of carbohydrate side chains, or even after the proteins are released from cells into plasma.

Modifications of the protein chain, such as the addition of sialic acid , can result in a large number of subtypes with different net charges and physical properties, thus allowing separation and identification of the isoenzyme activities. For example, a comparison of heat stability and catalytic properties of ALP from bone, liver, and kidney indicates that these isoforms result from different post-translational modifications of a single gene product of TNALP common to them all (Christenson *et al.*, 1996).

Membrane Anchoring of Alkaline phosphatase

Alkaline phosphatase is recognized as a large group of proteins attached to the outer surfaces of cells by a COOH-terminal glycan-phosphatidylinositol (GPI) anchor. The attachment of the GPI anchor to the enzyme protein during biosynthesis requires cleavage of a 29 residue, COOH-terminal peptide, exposing a new COOH-terminal residue, Asp^{484} to which the anchor is attached. Several mechanisms of release of GPI-anchored alkaline phosphatase molecules from the cell membrane can be envisaged, with the subsequent generation of different isoforms depending on the presence or absence of the GPI anchor (Figure 3).

Products of various specific phospholipases action are often only slightly different : e.g., the products of cleavage of the GPI anchor by phosphatidylinositol-specific phospholipase C or D (Figure 3) would differ only by the presence or absence of a terminal phosphate group in the released enzyme molecules. The presence of a phosphatidylinositol-specific phospholipase D in plasma probably accounts for the appearance of the non-aggregated, dimeric form of hepatocyte-derived alkaline phosphatase in that fluid (Raymond *et al.*,1991). However, it is not yet apparent whether release of alkaline phosphatase into plasma by phospholipase action is a rate-limiting step, or, if it is ,whether changes in phospholipase activity contribute to altered concentration of alkaline phosphatase in plasma in diseases. In view of these consideration,

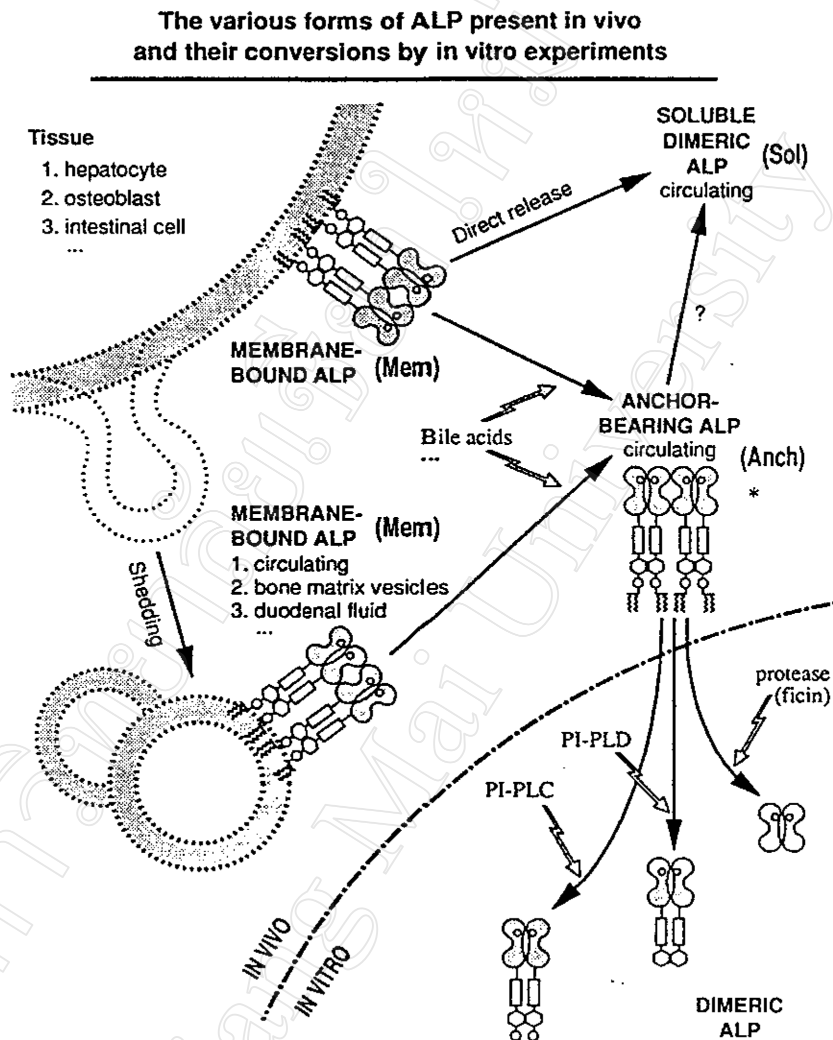

Figure 3 Schematic repesentation of the various form of ALP that are present *in vivo* and their conversions by *in vitro* experiments. This drawing represents only one of the several structures of anchor-bearing ALP* (Van Hoof and De Broe, 1994).

although still speculative, the anchoring mechanism of ALP might offer a clue to a physiological role of this enzyme.

B. Physiological role of ALP

ALPs are widely distributed in nature; they are present in all species, from bacteria to human. This is an indication that the enzyme is involved in fundamental biochemical processes. Millan (1992) suggests that possible clues to its functions were (1) The GPI attachment of ALP to the cytoplasmic membrane, (2) the uncompetitive inhibition properties to ALP, and (3) the extracellular matrix-binding domain of ALP.

ALP in the regulation of lipid transport. Day, Feher *et al.*(1992) have shown that the postpandial rise in serum I-ALP activity was significantly greater following a long-chain fatty acid meal than following a medium-chain fatty acid meal in healthy human under physiological conditions. In addition, a theory of absorption of I-ALP bound to lipid (Keiding, 1964) led to the speculation that I-ALP might be involved in lipid transport.

Transport of IgG molecules during pregnancy. According to Makiya *et al.*(1982), placental ALP fulfills the requirements needed to permit the transmission of functional antibodies to the fetus. The authors presented evidence that P-ALP has the potential to act as an Fc receptor, the presence of which is necessary to provide the fetus with maternal IgG as an acquired passive immunization during gestation.

ALP in bone formation. The following roles were postulated for bone ALP in the mineralization process :

1. Hydrolysis of organic phosphate esters resulting in high local Pi concentration, facilitating precipitation of calcium phosphate on bone.
2. Destruction of physiological crystal growth inhibitors such as inorganic pyrophosphate and ATP through its hydrolase activity.
3. Action as a Pi transporter.
4. Active transport of Ca^{2+} or Pi through its ATPase activity.

ALP in the Liver. Liver ALP is expressed at the sinusoidal and, to a lesser extent, biliary pole of the liver cell. Although liver ALP generally represents half or more than half of serum total ALP activity in healthy adults, no precise function can as yet be attributed to this isoform. The

way in which ALP is anchored to the liver cell membrane might offer a clue to its physiological role in this particular organ, but because gross deficiency of the liver isoenzyme in congenital hypophosphatasemia does not appear to give rise to any obvious clinical manifestations, it is doubtful that ALP fulfills a key function in the human liver (Van Hoof and De Broe, 1994).

C. Clinical significance

Although ALP isoenzyme is present practically in all tissues of the body, especially at or in the cell membranes, the form present in the sera of normal adult probably originates mainly in the liver, with up to half the total activity coming from the skeleton. Changes in the relative proportion and concentration of ALP isoenzymes and total ALP activity may occur as a result from a damage tissue of the original organ under pathological processes.

In disorder of bone, increased enzyme production results in elevated levels of bone ALP because of increased osteoblastic activity. The highest levels of serum ALP activity are encountered in Paget's disease as a result of the action of the osteoblastic cells as they try to rebuild bone that is being reabsorbed by the uncontrolled activity of osteoclasts (Moss and Henderson, 1994).

Increased serum ALP levels in hepatobiliary disease presumably results from injury to hepatocytes as well as from the accumulation of bile acids as a result of cholestasis. Although ALP of the liver origin can be increased in serum during any type of active liver disease, the serum level is especially sensitive to biliary tract obstruction, whether intrahepatic or extrahepatic, whether mild or severe, or whether localized in a small area of the liver or more extensive. As a general rule, the degree of ALP elevation reflects the severity of obstruction and the amount of biliary tissue involved.

An explanation for some of the alternative ALP isoenzymes detectable in serum, especially in hepatobiliary disease, is provided by the presence of inositol-specific phospholipase D activity in serum and its apparent in bile. In hepatobiliary disease, a hydrophilic, non-aggregating ALP is released from hepatocytes into serum by the action of phospholipase D. Since this phospholipase D is absent in bile, a higher-molecular-mass aggregate of ALP is released from hepatocytes forming a so-called fast-liver fraction. This fast-liver fraction is regarded as valuable

evidence of obstructive liver disease, particularly in the extrahepatic circulation (Raymond *et al.*, 1991).

In conclusion the elevation of ALP in plasma could be attributed by the following etiologies :

1. Liver and biliary tract origin

Extrahepatic bile duct obstruction
Intrahepatic biliary obstruction
Liver cell acute injury
Liver passive congestion
Drug-induced liver cell dysfunction
Space-occupying lesions
Primary biliary cirrhosis
Sepsis

2. Bone origin (osteoblast hyperactivity)

Physiologic (rapid) bone growth (childhood and adolescent)
Metastatic tumor with osteoblastic reaction
Fracture healing
Paget's disease of bone

3. Placental origin

Pre-eclampsia

4. Other

Thyrotoxicosis
Primary hyperparathyroidism

D. Clinical useful of serum ALP measurement

Measurement of serum alkaline phosphatase is useful in differentiating hepatobiliary disease from osteogenic disease. Alkaline phosphatase activity greatly increases (10 times) as a result of membrane-localized enzyme synthesis after extrahepatobiliary obstruction such as cholelithiasis or gallstones. Intrahepatic biliary obstruction is also accompanied by an increased serum ALP activity, but the degree of increase is smaller (two to three times). When doubt exists as to the origin of the increase ALP values, several alternatives could be used to identify. One possibility is the use of another enzyme that provides similar information to ALP in liver disease but is more specific for liver origin.

Enzymes that have been widely used for this purpose are 5'-nucleotidase (5'-NT), and gamma-glutamyltransferase (GGT).

Identifying the organic origin of serum ALP isoenzyme provides useful markers of the altered condition of the respective organ. Most often ALP isoenzyme is requested in order to determine whether bone or liver is the source of an elevated level of total serum ALP activity. Specific ALP isoenzyme measurements are definitely better than total ALP measurements for detecting the diseases.

ALP isoenzymes associated with tumor are referred to as tumor markers. It is found that total ALP levels of tumor cell are greater than the normal limit. Human tumors are found to produce increased concentration of the placental, intestinal, and germ cell isoenzymes of ALP. The increased expression of variants of P-ALP, as well as the " Regan" and "Nagao" isoenzyme is associated with germ cell tumors. High-molecular mass ALP are expressed in hepatocellular carcinoma or a metastatic tumor (Nishio *et al.*, 1986).

E. Multiple forms of ALP

Alkaline phosphatase enzyme is composed of a number of different isoenzymes as follow (Van Hoof and De Broe , 1994) :

1. Liver isoenzyme

Liver ALP (L-ALP) mainly circulates in human serum as a soluble homodimer of two Mr 80,000 subunits. It is expressed at the sinusoidal and, to a lesser extent, biliary pole of the liver cell. Chakrabartty and Stinson (1985) showed by *in vitro* experiments that liver ALP was released from the plasma membrane by PI-specific phospholipase C action and had a dimeric molecular mass. Liver ALP is the isoenzyme most frequently elevated when total ALP levels are elevated. The liver ALP increases in the blood early in liver disease before most liver function tests show abnormalities. The wide group of conditions leading to increase in liver ALP included acute hepatitis, cirrhosis, fatty liver, drug induced liver disease, obstruction of biliary flow by carcinoma at the head of the pancreas, primary biliary cirrhosis, and metastatic carcinoma of the liver.

2. Fast-liver isoenzyme

Fast-liver ALP (FL-ALP) is a high molecular mass liver fraction (greater than 2 MDa) and accompanied by a so-called high- molecular mass liver ALP isoform (high-Mr ALP). This isoform has also been called fast-liver ALP, koinozyme, or bile ALP. It consists of liver ALP anchored to fragments of the liver cell membrane, showing various sized vesicles by electron microscopy (De Broe *et al.*, 1985) or from the aggregation in bile or plasma of liver ALP with lipid and protein (Price and Sammons, 1974). The biliary ALP is present in the serum of patients with cholestatic liver disease and in patients with hepatic metastatic of tumor (Nishio *et al.*, 1986). In addition, it has also been isolated in patients with viral hepatitis, alcoholic cirrhosis, and other liver disease.

3. Bone isoenzyme

Bone ALP (B-ALP) circulates in its soluble dimeric form. Bone ALP is present on the plasma membrane fragments of the osteoblasts , had an apparent Mr of 445 KDa. This isoenzyme is normally elevated in growing children and adults over the age of fifty. The highest total ALP values have been attributed to an increased bone isoenzyme level due to Paget's disease. An abnormally high bone isoenzyme level may also be indicative of bone cancer, osteomalacia. A decreased bone ALP in children may be attributed to cretinism or to hypophosphatasia.

4. Intestinal isoenzyme

Intestinal ALP (I-ALP) is expressed in intestinal mucosa and is abundant in the brush borders of epithelial cells. Recently, expression of this enzyme has also been observed in kidney localized in the distal (S_3) segment of the proximal tubule (Verpooten *et al.*, 1989).I-ALP is normally seen in the serum of subjects who have B or O blood types, especially after a fatty meal. Pathologically, the band may be present in perforation of the bowel, ulcerative diseases of the intestine and faintly in liver cirrhosis as well as intestinal perforation.

5. Placental isoenzyme

Placental ALP (P-ALP) is heat-stable enzyme present at high levels in the placenta. A trace amount of this enzyme can be detected in normal sera. P-ALP appears in the serum of pregnant women between the sixteenth and twentieth weeks of pregnancy and disappears within 3 to 6

days after delivery. It is also present in relatively small amounts in lung and cervix (Christenson *et al.*, 1996).

6. Renal isoenzyme

Renal isoenzyme originates from kidney tissues. The isoenzyme represents a disease state of the kidneys or rejection of kidney transplant.

7. Regan isoenzyme

Regan isoenzyme is the placental gene re-expressed by cancer cells. The enzyme could be isolated from the sera of patients with neoplasm. Because of the similarities to placental ALP , it has been referred to as carcinoplacental isoenzyme. Regan has been isolated from patients with lung cancer, breast cancer, and carcinoma of colon.

8. Nagao isoenzyme

Nagao isoenzyme is a variant of Regan isoenzyme. It has been isolated in metastatic carcinoma to the plural surfaces and in adenocarcinoma of the pancreas or bile duct.

F. Measurement of ALP activity and ALP isoenzymes

1. Measurment of total ALP Activity

Total ALP activity remains one of the most commonly measured enzyme activities in the clinical laboratory, and different substrates have been proposed for its determination (Crofton, 1982). Of these, para-nitrophenylphosphate (PNPP) is probably the most widely used. It is important to note that some isoenzymes have different affinities, depending on the substrate used, for example, I-ALP shows a lower affinity for PNPP compared with the other isoenzymes (Bretaudiere, 1977). The buffer also plays an important role : diethanolamine (DEA) activates ALP activity (Van Belle, 1976) but I-ALP is activated to a lesser extent than bone and liver ALP (Wallinder, 1989 ; Van Hoof *et al* ., 1990) therefore, depending on the amount of I-ALP present in the sample, total ALP activity will be more or less underestimated when using DEA as phosphoacceptor.

2. Methods of ALP isoenzyme separation

ALPs from different human tissues are not identical in all properties, although they are similar in many respects. Criteria that have been used to differentiate the isoenzymes and other multiple forms of ALP include difference in relative rate of reaction with various substrates, different in response to the presence of selected inhibitors, variation in stability to denaturation by heat or urea, differences in electrophoretic mobility, and in immunochemical characteristics. Methods of ALP isoenzyme separation are concluded as follow : (Moss *et al.*, 1994; Christenson *et al.*, 1996)

2.1 Heat inactivation

Placental ALP is its pronounced stability to heat at 65°C whereas other ALP isoenzymes can also be differentiated on the basis of their stability at temperatures lower than 65°C. At 56°C or 52°C , liver ALP is more stable than the bone ALP.

2.2 Chemical inhibition

Specific chemical inhibitors have also been applied to the characterization of ALP isoenzymes in serum. L-Phenylalanine markedly inhibits intestinal, placental, and Regan isoenzymes when present at a concentration of 5 mmol/L but has less effect on the isoenzymes of bone or liver. Levamisole preferentially inhibits bone and liver ALP and is effective at much lower concentration.

2.3 Immunological tecniques

Another approach to aid in discrimination between the ALP isoenzymes in serum includes the use of antisera to specific tissue phosphatases. Monospecific antisera to placental and intestinal ALP have been prepared, but antisera to either liver and bone ALP cross-react completely. Immunological methods provide the best measurements of placental or intestinal ALP.

2.4 Lectin precipitation

The effect of lectin from wheat germ agglutinin (WGA) on the ALP isoenzymes, with the bone isoenzyme being preferentially bound (Behr and Barnert, 1986), provides the quantitative measurement of ALP isoenzymes originating in liver and bone. Lectin precipitation method gives poor resolution of liver and bone isoenzymes eventhough it is a quick and simple method.

2.5 Chromatographic technique

Conventional gel chromatography can be used to separate ALP isoenzymes. It has only limited use in routine laboratory, as it does not allow differentiation between bone and liver ALP (e.g., DEAE Cellulose) (Karman *et al.*, 1984). High performance liquid chromatography (HPLC) has been described as a useful tool for routinely separating ALP. Four or six major peaks are separated. Apparently, no data are available on the performance of this method with low ALP activities (Schoenau *et al.*, 1986).

2.6 Electrophoretic techniques

Electrophoresis is a commonly used technique for the qualitative analysis of ALP isoenzymes in serum. Various supporting media, including agarose, cellulose acetate membrane, and polyacrylamide gel have been used with varying degrees of success. Conventional electrophoretic method separates ALP isoenzymes to the extent that the technique allows a visual estimate of their relative properties and quantity by densitometric scanning. However, the liver and bone zones usually overlap to some extent, it cannot be exploited to distinguish between these two tissues as the possible source of increased phosphatase levels.

Moreover, some techniques may be too cumbersome for routine use such as isoelectric focusing or polyacrylamide gel electrophoresis. Nowadays, however, good and reproducible results are obtained with commercially available agarose electrophoretic system. A combination of neuraminidase treated sample or incorporated WGA into gel or buffer before electrophoresis is needed to ensure reliable discrimination of liver and bone isoenzymes (Crofton , 1992 ; Jung *et al.*, 1989)

ALP isoenzymes in serum displays considerable heterogeneity, with respect to net molecular charge and also to some degree with respect to molecular size. The differences in charges originate at the level of the structural gene and others as the results of post-translation modifications (glycosylation). Very little is known about the glycosylation patterns of such ALP isoenzymes. By serial lectin affinity chromatography, Koyama *et al.* (1987) demonstrated heterogeneity of the sugar chain structure of normal and tumor-produced ALPs extracted from various tissues. Kuwana *et al.* (1991) reported differences in sugar chain structure of liver and bone ALPs, fractionated from serum samples, from that of Koyama *et al.* (1987) in the corresponding tissues. In addition, it has been elucidated that neuraminidase from two different bacterial sources, used different forms of substrates (Corfield *et al.*, 1981), can separate liver and bone ALPs and thus indicating differences in sialic acid orientation. Recently, Miura *et al.* (1994) evaluated the differences between the sugar moieties of liver and bone isoenzymes. The results showed that differentiating liver and bone ALPs may depend on the way sialic acid and the O-linked sugar chain are linked to the molecules.

The heterogeneity of two major forms of intestinal alkaline phosphatase was also reported (Komoda *et al.*, 1981). One component in small intestine was shown to be resistant to neuraminidase treatment while the other which is predominant in the colon could be converted by neuraminidase (Griffiths *et al.*, 1985). Kidney and bone ALP are proposed to be similar in glycosylation and cannot be clearly discriminated. The placental alkaline phosphatase detected in serum of pregnant woman shows the heterogeneity with respect to neuraminidase treatment and contain both N- and O-glycosylation attached to the isoenzyme molecules (Nosjean *et al.*, 1997).