# SERUM-β-N-ACETYLHEXOSAMINIDASE IN NORMAL AND PATIENTS WITH OBSTRUCTIVE JAUNDICE


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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
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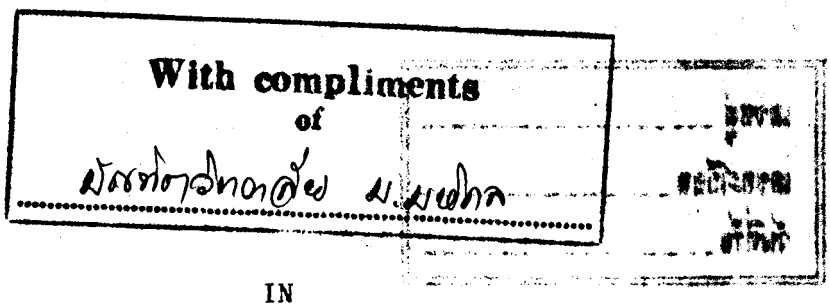


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Kanit Vichitphan

ชื่อวิทยานิพนธ์ β-N-Acetylhexosaminidase ในซีรั่มของคนปกติ และผู้ป่วยที่เป็นโรคทางเดินน้ำดีอุดตัน

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## บทคัดย่อ

ได้ทำการศึกษาวิเคราะห์ระดับเอ็นซัยม์ β-N-acetylhexosaminidase (HEX) ในซีรั่มโดยวิธี heat inactivation โดยใช้เครื่องวัดการเรืองแสง (spectrofluorometer) การวิเคราะห์เอ็นซัยม์นี้สามารถแยกเอ็นซัยม์ได้เป็น 2 ไอโซซัยม์ คือ HEXA (ไม่ทนความร้อน) และ HEXB (ทนความร้อนได้ดี) การศึกษาในครั้งนี้ได้วิเคราะห์หาระดับเอ็นซัยม์ดังกล่าวจากซีรั่มรวมทั้งสิ้น 276 ราย ซึ่งประกอบด้วย คนปกติที่มีสุขภาพแข็งแรงสมบูรณ์ 120 ราย ผู้ป่วยโรคอื่นที่ไม่ใช่โรคตับ 49 ราย และผู้ป่วยโรคทางเดินน้ำดีอุดตัน 107 ราย พบว่าค่าเฉลี่ย (mean $\pm$ SD) ของ total HEX เท่ากับ 18.5 $\pm$ 3.5 U/min, 20.8 $\pm$ 5.6 U/min และ 38.9 $\pm$ 14.6 U/min ตามลำดับ ส่วนของระดับ HEXB เท่ากับ 6.3 $\pm$ 1.9 U/min, 9.0 $\pm$ 3.8 U/min และ 18.9 $\pm$ 9.6 U/min ตามลำดับ HEXA เท่ากับ 12.1 $\pm$ 2.0 U/min, 11.8 $\pm$ 3.7 U/min และ 20.1 $\pm$ 6.5 U/min ตามลำดับ เมื่อนำทั้ง 3 กลุ่มมาเปรียบเทียบกันพบว่า ผู้ป่วยโรคทางเดินน้ำดีอุดตันมีปริมาณของ total HEX, HEXB และ HEXA สูงกว่าในคนปกติและ ผู้ป่วยโรคอื่นอย่างมีนัยสำคัญทางสถิติ ($P < 0.001$) จากผลการวินิจฉัยชิ้นเนื้อได้แยกผู้ป่วยออกเป็น 2 กลุ่ม คือ ผู้ป่วยโรคทางเดินน้ำดีอุดตันจากสาเหตุที่ไม่ใช่มะเร็ง 10 ราย และผู้ป่วยโรคทางเดินน้ำดีอุดตันจากมะเร็ง 25 ราย เมื่อนำมาเปรียบเทียบกันพบว่าปริมาณของ total HEX, HEXB และ HEXA สูงขึ้นในผู้ป่วยโรคทาง

เดินน้ำดีอุดตันจากก้อนมะเร็ง แต่ไม่มีนัยสำคัญทางสถิติที่ $P < 0.05$ สาเหตุที่ทำให้ปริมาณของเอ็นซัยม์สูงขึ้นอาจจะเนื่องจาก ความบกพร่องของระบบกำจัดเอ็นซัยม์ HEX ของตับ และอาจจะเนื่องจากเซลมะเร็งเองมีการส่งเอ็นซัยม์ HEX ออกมามากกว่าเซลธรรมดา

เมื่อทำการหาความสัมพันธ์ระหว่าง total HEX, HEXB และ HEXA กับกรดน้ำดีรวมในซีรั่มที่อดอาหารของผู้ป่วยทางเดินน้ำดีอุดตัน 17 ราย พบว่าเฉพาะ total HEX และ HEXA เท่านั้นที่มีความสัมพันธ์กับกรดน้ำดีรวมอย่างมีนัยสำคัญทางสถิติ ($P < 0.05$) โดยมีสัมประสิทธิ์แห่งสหสัมพันธ์(r) เท่ากับ 0.511 และ 0.644 ตามลำดับ ความสัมพันธ์นี้สนับสนุนความคิดที่ว่าปริมาณน้ำดีที่เพิ่มขึ้นในระบบไหลเวียน จะไปทำลายผนังเซลของคุฟเฟอร์เซล ทำให้การกำจัด HEX ลดลงมีผลทำให้ปริมาณเอ็นซัยม์ในซีรั่มสูงขึ้น

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## ABSTRACT

β-N-Acetylhexosaminidase in human serum were studied by heat inactivation using spectrofluorometric method. This enzyme consist of two major forms; HEXA (heat labile) and HEXB (heat stable). Two hundred and seventy six serum samples from normal subjects (n = 120), control subjects (n = 49) and obstructive jaundice patients (n= 107) were analysed. The values (mean ± SD) of total HEX were 18.5 ± 3.5 U/min , 20.8 ± 5.6 U/min and 38.9 ± 14.6 U/min respectively; HEXB were 6.3 ± 1.9 U/min, 9.0 ± 3.8 U/min and 18.9 ± 9.6 U/min respectively; and HEXA were 12.1 ± 2.0 U/min, 11.8 ± 3.7 U/min and 20.1 ± 6.5 U/min respectively. The total HEX, HEXB and HEXA in obstructive jaundice patients compared to normal and control subjects were statistically significant increasing ($P < 0.001$). In according to tissue diagnosis, the benign (n=10) and malignant (n=25) biliary obstruction were determined. The results showed the increasing of total HEX, HEXB and HEXA in malignant biliary obstruction but no statistically signi-

ficant increasing at $P < 0.05$ . Increased level of HEX may defect in clearance HEX of the liver and over releasing from malignant cells.

Seventeen fasting sera of obstructive jaundice patients were studied the correlation between total HEX, HEXB and HEXA with total bile acids. The total HEX and HEXA were correlated to total bile acid ( $P < 0.05$) with correlation coefficient (r) of 0.511 and 0.644 respectively. These are consistent with the idea that elevated bile acid in circulation can damage the Kupffer cell membranes, impeding the clearance of the HEX from the circulation resulting in elevated serum levels.

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| | |
|---|---|
| 3α-HSD | 3α-Hydroxysteroid dehydrogenase |
| 4-MUGlcNAc | 4-Methylumbelliferyl-β-D-N-acetylglucosamine |
| β-NAD | β-Nicotinamide adenine dinucleotide |
| DEAE | Diethylaminoethyl |
| DM | Diabetes Mellitus |
| GlcNAc | N-Acetylglucosamine |
| HEX | β-N-Acetylhexosaminidase |
| HEXA | β-N-Acetylhexosaminidase A isozyme |
| HEXB | β-N-Acetylhexosaminidase B isozyme |
| HEXC | β-N-Acetylhexosaminidase C isozyme |
| HEXI | β-N-Acetylhexosaminidase I isozyme |
| HEXM | β-N-Acetylhexosaminidase M isozyme |
| HEXP | β-N-Acetylhexosaminidase P isozyme |
| HEXS | β-N-Acetylhexosaminidase S isozyme |
| HEX $A^{*}$ | β-N-Acetylhexosaminidase A isozyme in serum |
| HEX $B^{*}$ | β-N-Acetylhexosaminidase B isozyme in serum |
| HPLC | High performance liquid chromatography |
| IEF | Isoelectric focussing electrophoresis |
| Mr | Molecular weight |
| NMR | Nuclear magnetic resonance |
| pH | -log (Hydrogen ion concentration) |
| pI | Isoelectric point |
| SDS-PAGE | Sodium dodecyl sulphate - Polyacrylamine gel electrophoresis |

# CHAPTER I

# INTRODUCTION

## 1. β-N-ACETYLHEXOSAMINIDASE

### 1.1. BACKGROUND

The lysosomal hydrolase β-N-acetylhexosaminidase (2-Acetamido-2-deoxy-β-D-hexoside: Acetamidodeoxyhexohydrolase, EC 3.2.1.52) or β-hexosaminidase(HEX)is a complex group of glycoprotein (1,2) with terminal mannose or N-acetylglucosamine (3) and has both N-acetylglucosaminidase and N-acetylgalactosaminidase activity with pH optima between 4 and 5 (4,5,6). This enzyme is primarily of lysosomal origin and is concerned with the degradation of glycosidic linkages of the non-reducing terminal β-D-N-acetylglucosamine and β-D-N-acetylgalactosamine residuals on natural substrates as glycolipids, glycoproteins and glycoaminoglycans (7,8). It exists in various isozyme forms in human serum, urine, leucocytes, macrophages, body fluids and tissues (9). The isozymes can be seperated into two major forms (A and B) and several minor forms (i.e.$I_1$, $I_2$, S and P) on the basis of different charge properties (10,11). Furthermore, the HEX isozymes are composed of two different polypeptide chains, denoted α and β (12), that having been mapped to chromosome 15 and 5 respectively (7). For example, isozyme S contains only α-chain, isozymes B and P contain only β-chain while isozyme A is composed of both α-and β-chain (13,14,15). It has been controversy about the number of polypeptide chain forming HEX. In according to the molecular weight study in many laboratories have reported the structure as tetramer and hexamer(12,16,17). The relation amounts of different

isozymes vary between different sources and are altered in certain physiological and pathological condition (18). For instance, an increase of the serum isozyme P is seen in pregnancy and liver disease (19). Renal injury is associated with an increase of total HEX activity in urine and increased relative amount of the isozyme B (20,21). Total HEX is also increased in different form of cancer (22,23). An elevation of isozyme $I_2$ activity is seen in lymphocyte of patient with acute lymphocytic leukemia (24,25). Then, the usage of soluble, synthetic, chromogenic and fluorogenic substrates for the measurement of HEX hydrolyzing activity proved to be very helpful (26). Especially, the method predominantly used for preparative isozyme separation is ion exchange chromatography. The anionic exchanger DEAE coupled to various supports is the one most commonly usefulness (7).

## 1.2. ASSAY FOR β- HEXOSAMINIDASE

### 1.2.1. ARTIFICIAL SUBSTRATES

In case of quantitative assay the most common artificial substrate used to assay HEX is the N-acetylglucosamine derivative of the fluorescent compound 4-methylumbelliferone, 4-methylumbelliferyl-β-D-N-acetylglucosamine(4-MUGlcNAc) (7). There are variation to the conditions for this assay in tissues and in serum. However, all were developed the fluorescence of 4-methylumbelliferone after hydrolysis from the non-fluorescent 4-MUGlcNAc (27-30). The colorimetric substrate, p-nitrophenyl-β-D-N-acetylglucosamine is also widely used but is less sensitive and subject to

some interference from colored materials include tissue extracts or in body fluids (7). Hydrolysis is measured spectrophotometrically by several similar methods (31,32) that are based on earlier assays (33). Recently, two new colorimetric substrates have been described 2-methoxy-4-(2'-nitrovinyl)-phenyl-β-D-GlcNAc (34) and sodio-m-cresolfonaphthaleinyl-β-D-GlcNAc(35). Furthermore, HEX also hydrolyzes N-acetylgalactosamine derivatives of these substrates. Although the Km's are lower than those estimated with N-acetylglucosamine derivatives, the Vmax values are 3-9 fold lower (31,33,36,37). Optimal conditions for HEX assays have been studied extensively but vary with the nature of the enzyme source and should be checked before significant decision are made on the results of an assay (7). The pH optimum is usually between 4.1 and 4.5. Acetate buffers inhibit HEX, thus the assays are normally preformed in citrate-phosphate buffers (38,39). Because of the high sensitivity with the 4-methylumbelliferone substrates HEX are often performed in dilute solution, and under these condition, 0.1-0.3 % albumin should be added to prevent protein denaturation or absorption of the enzyme on the vessel walls. Many commercial preparation of albumin contain HEX activity, however, and should be assessed with appropriate blanks (36).

In quanlitative assays the HEX activity can be demonstrated in tissue sections, culture fibroblasts, or in bands of native (undernatured) enzyme seperated on electrophoretic system using a number of colorimetric reagents. Pugh and Walker (40,41) used α-naphthyl-β-D-GlcNAc and naphthol AS-LC-β-D-GlcNAc, while Hyashi (42) used naphthol AS-BI-β-D-GLcNAc coupled to diazonium salt, fast Garnet GBc, or hexazonium pararosanilin. Fluorescent detection can

also be used on electrophoretic media. Bands are identified after incubation with 4-MUGlcNAc (43).

### 1.2.2. NATURAL SUBSTRATES.

The isozyme of HEX can be distinguished to some degree by their natural substrate specficities, as well as by difference in their charge and stability. However, there are several problem in studying the hydrolysis in aqueous media. Not only are there several HEX isozymes but there are difficult to prepare, often involving lengthy purification and may require detergents or natural activators for optimal hydrolysis (7). The detergents sodium taurocholate or sodium taurodeoxycholate (44,45) and the natural activators as albumin and ovalalbumin (44,46) greatly increase natural substrate activity by both HEXA and HEXB. Moreover, there are difficulties with the stability and purity of the substrates as well as the isolated enzymes. Thus, the interpretation of data on natural substrate hydrolysis by HEX is difficult (7). There are several important natural substrates in vivo. Gangliosides especially $GM_2$ ganglioside is one of glycosphingolipid containing equimolar amounts of ceramide, glucose, galactose, N-acetylneuraminic acid and N-acetylgalactosamine. The β-linked hexosamine is at the nonreducing end of oligosaccharide chain, and the linkage can be cleaved by HEXA but not by HEXB in vivo. The terminal hexosamine in the related glycolipid $GA_2$ (Asialo $GM_2$), and in globoside $GL_4$, however, can be hydrolyzed by both major HEX isozymes (46). Glycoaminoglycan (GAG) are oligosaccharides composed of disaccharide units with varying degrees of sulfation. Most contain a uronic acid and hexosamine in the

disaccharide. If the hexosamine is β-linked and at the nonreducing end of the molecule, it is a potential substrate for HEX for example hyaluronic acid, keratan sulfate, dematan sulfate. Although Glycominoglycan are catabolized by HEX, these compounds are not stored nor excreted in excessive quantities in patients with defects in HEX activity (47). Glycoproteins are asparagine-linked oligosaccharides on glycoproteins contain GlcNAc residuals, serine-and threonine-linked oligosaccharides contain both GlcNAc and GalNAc residual (48). Glycoproteins are degraded in liver in the lysosome. HEX in lysosome can cleave terminal β-1,2, β-1,4 and β-1,6-linked GlcNAc residuals from oligosaccharide side chain of glycoprotein (49). Steroid: Tomasi et al (50) demonstrated that HEXA, B and S can hydrolyze GlcNAc residuals from dehydrocpiandrosterone-3-β-N-GlcNAc and testersterone-17-β-N-GlcNAc.

## 1.3. CHARACTERIZATION OF β- HEXOSAMINIDASE

There are several techniques in characterization of HEX isozymes depending on their properties but the general and popular methods are discussed. Firstly, ion exchange chromatography, a separation method in which component with different net charges are separated when a gradient of increasing ionic strength is used as the eluent. In this method the anionic exchanger DEAE coupled to various supports (eg. Cellulose, Sephadex, Sapharose 6B-Cl) is used to adsorb the HEX isozymes. The isozymes are then deabsorbed by the increasing of NaCl concentration gradient and continuously are eluted from the column. Secondly, isoelectric focusing electrophoresis (IEF), the sample components are separated in a pH-gradient

according to differences in isoelectric point (pI). Then, each of HEX isozymes can be separated by IEF in difference peak that depend on isoelectric point. According to this method the different in pI of isozymes, and fine structure are elucidated. Thirdly, gel filtration, the gel acts as a molecular sieve separating molecules with difference in molecular size and weight. Then, the molecular weight (Mr) of undenatured enzyme is determined by gel filtration. Forthly, SDS-PAGE, the sample components are separated based on their differences in net charge, size and shape. The sodium dodecyl sulfate (SDS) denatured protein at disulfide bond. The Mr of the fine structure, polypeptide chain, are elucidated by SDS-PAGE. In addition, the oligosaccharide structure of carbohydrate and amino acid sequence of polypeptide chains were determined by both of the reversephase HPLC and $^{1}H$ NMR spectroscopy (51,52). Finally, the heat inactivation method are designed to calculate the quantity of HEXA and HEXB activity as a percentage of total activity in fluid or tissues. A reliable differential assay method by heat inactivation is important because the easiest and most reproduceable procedure. A comparison is thus made between the HEX activity in heated and unheated sample. By convention the residues activity is commonly call HEXB, although in serum it is largely HEXI (7). However, the several method are used to characterize HEX isozymes that did not mention.

A number of researchers believed that lysosomal hydrolases are first synthesized at special population of rough microsome as bound enzyme protein is transported from rough microsome through smooth microsome, Golgi apparatus, to primary lysosome. As enzyme transportation there would be some addition of carbohydrate residuals

especially N-acetylneuraminic acid to the peptide backbone increasing the acidity and solubility of enzyme (53,54). Then, the precursor of high molecular weight and subsequently processed to mature forms, presumbly by protienase (55,56). Maturation of lysosomal enzymes occurs after their segregration from the secretory pathway and is accomplished, at least partly, within lysosomes. Like other lysosomal enzymes the HEX isozymes are glycoprotien and must be specifically target to their destination by cell (57). The molecular forms, the pathway, the modification, and the elimination of these enzyme remain to be established. Thus, the characterization of structure and function in cells, tissues, and body fluids are elucidated.

Although human placenta contain a lower proportion of lysosomal enzyme than kidney and liver, it general availability had made it the tissue of choice for large-scale purification. In according to ion-exchange chromatography, SDS-PAGE, IEF/SDS-PAGE and 2$^{nd}$ IEF/SDS-PAGE methods can show the characterization of human placenta HEX isozymes in Table 1. (7). While the conclusion of Geiger and Arnon (12) have indicated the subunit structure for HEXA was $\alpha_2\beta_2$ , HEXB was $\beta_2\beta_2$ and HEXS was $\alpha_2\alpha_2$. The Mr obtained for HEXA is 100,000, whereas HEXB appeared slightly larger, about 108,000. The number of disulfide bridges (inter-or-intra chain) are present in each β-subunit, whereas only one disulfide bridge connects the two α-chain in the $\alpha_2$ dimer. In addition, each α-chain contain one free sulhydryl group that is absent in the β-chain (Figure 1). The relationship of A to B isozyme appearent conversion of the rearrangment from α-subunit to new β-subunit that could be catalized by heating,

Table 1 Characterization of human placental hexosaminidase isozymes[a]

| HEX | Native pI | $K_m$ (mM) 4-MU-GlcNAc | Native $M_r$ ($\times 10^{-3}$) | Subunit $M_r$ ($\times 10^{-3}$) | Proposed polypeptide structure |
|---|---|---|---|---|---|
| S[b] | 4.2 | nd[f] | 120 | 2 x54 | 2α |
| A | 4.9 | 0.84 ± 0.10 | 120 | 54;56 | α(βaβb)[c] |
| $I_2$ | 5.3,5.6 | 0.73 ± 0.06 | 140 | 61;65 | "α"(βa"βb")[d] |
| D | nd | nd | 140 | 2 x 71 | 2δ |
| $I_1$ | nd | 0.85 ± 0.13 | 130 | 58;56 | preβ(βaβb)[e] |
| B | 6.9 | 0.71 ± 0.05 | 130 | 2 x 56 | 2(βaβb) |
| $B_A$[b] | 6.7 | 0.64 ± 0.06 | 130 | 2 x 56 | 2(βaβb) |

a : Published by Mahuran et al (7)

b : Produced by disruption and subunit rearrangement of HEXA.

c : Polypeptides within parentheses are covalently bound via a disulfide bridge(s) to form a single subunit.

d : Quotation marks indicate that the chain is structurally related but not identical.

e : Limited data are available to confirm this structure.

f : Not determination

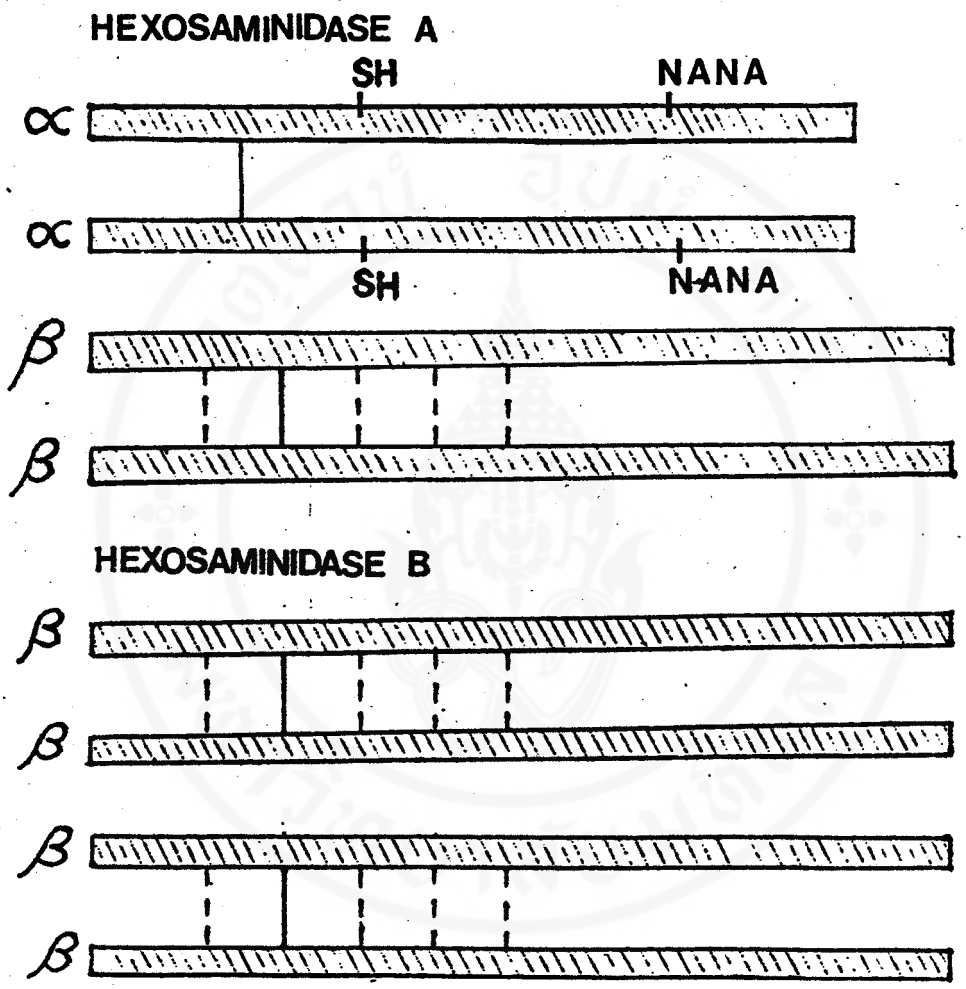


Figure 1 Suggested molecular structure of hexosaminidase A and B. Solid lines connecting two chains represent S-S bridge, broken lines represent either intra-or interchain S-S bridges. In the α-chains of hexosaminidase A, in addition to the interchain S-S bridges, one sulfhydryl group (SH) and one sialic acid residue (NANA) are present.

freeze-thawing, or treatment with mercurials such as merthiolate, a preservative used in some neuraminidase preparation (13,17,26,45). In the part of carbohydrate structure, Freeze et al(58)have reported the mannose residuals predominated, galactose was also found, indicating the presence of both a complex- (or hybrid) as well as high mannose type oligosaccharide. The kinetics of the HEX isozymes, using the nonlinear least square method and highly purified placenta HEX $B_A$, B, $I_1$, $I_2$ and A found no significant difference in Km for the 4-MUGlcNAc in 95 % confidence limit (Table 1). The Vmax values for HEXA and HEXB were about equal and twice those of HEX $B_A$, $I_1$ and $I_2$ (7).

Serum HEX activity has been assayed for many years in order to diagnose patients with Tay-sachs disease(TSD) or Sandhoff disease (SD) and to identify individuals who are heterozygotes for these gene. By DEAE-cellulose chromatography, Lowden(59) separated HEX of normal serum into 3 distinct peaks. HEXB is not bound to the ion exchanger but pass through in the void volumn. HEXI is loosly bound and eluted shortly after the NaCl gradient is applied at 0.02-0.03 M salt. HEXA is more tightly bound and requires a concentration of 0.13-0.15 M NaCl for elution (Figure 2). Morever, by IEF chromatography the HEX pattern are at least 7 peaks of enzyme activity. HEXB activity separated with a pI of approximately 6.9. It is followed by isozymes with pI values 6.7, 6.3, 6.1, 5.7, 5.4 and finally HEXA at 5.0 (Figure 3,4). The serum isozymes have been name HEXA, B,I etc., like the tissue enzymes, however, there are important difference between serum and tissues isozymes. Ninety percent of serum HEXA ($HEXA^s$) have difference from HEXA in tissues, for example, the molecular

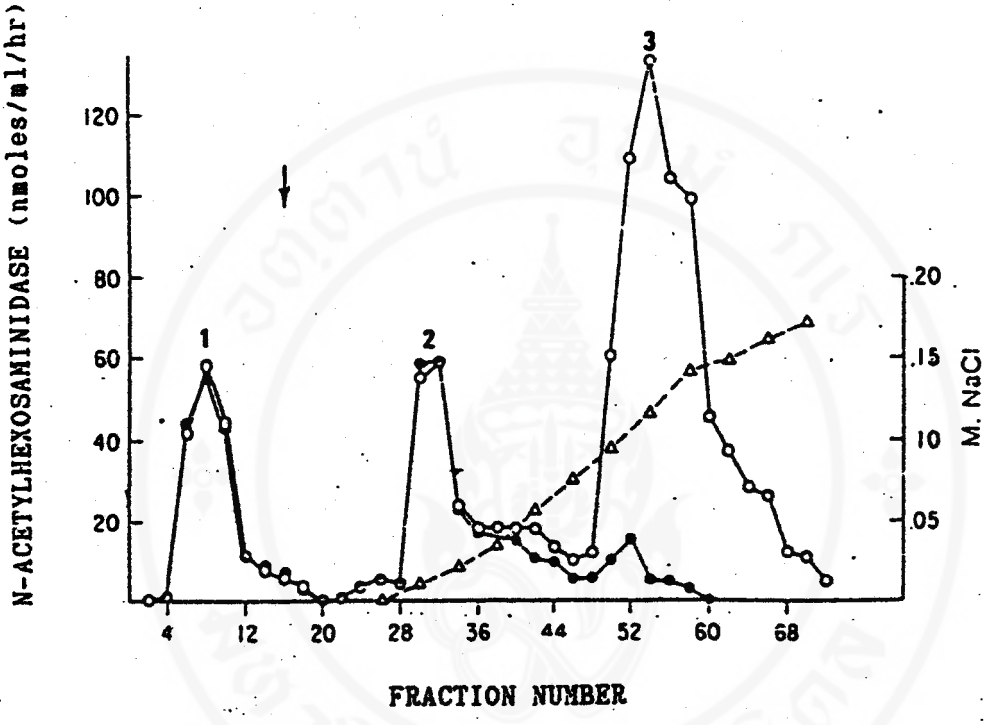


Figure 2 DEAE chromatography of normal serum hexosaminidases. Serum (0.5 ml) was dialysed and applied to a 2x3 cm column of DEAE cellulose. The column was eluted with a gradient of NaCl in 0.07 M sodium phosphate buffer. Fractions (1 ml) were assayed for total hexosaminidase (o_____o) and for heat-stable hexosaminidase (●___●) Conductivity measurements were used to determine the molarity of NaCl in the effluent (Δ_____Δ) . Arrow indicates start of gradient in eluting buffer.
1 = HEXB , 2 = HEXI , 3 = HEXA

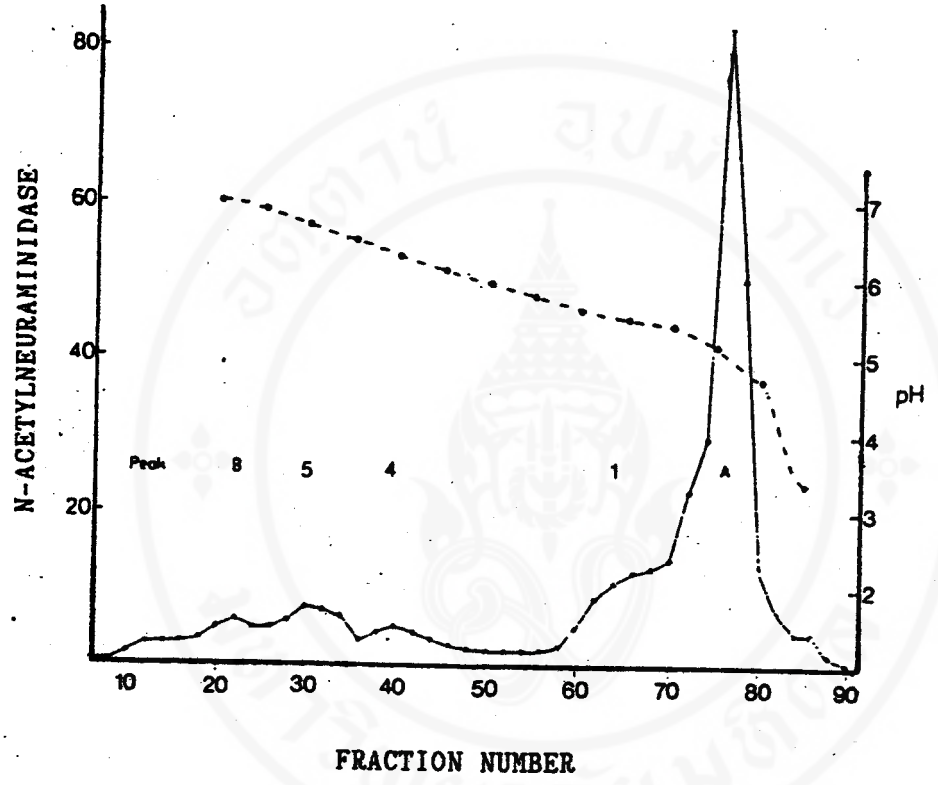


Figure 3 Isoelectric focussing of normal serum hexosaminidases. 0.5 ml serum applied to a mini column containing 27.5 ml of pH 4-8 ampholines at 2.5 ml pH 4-8 ampholines at 2.5% in a 0-50% sucrose gradient. Fractions of 5 drops were collected and assayed.

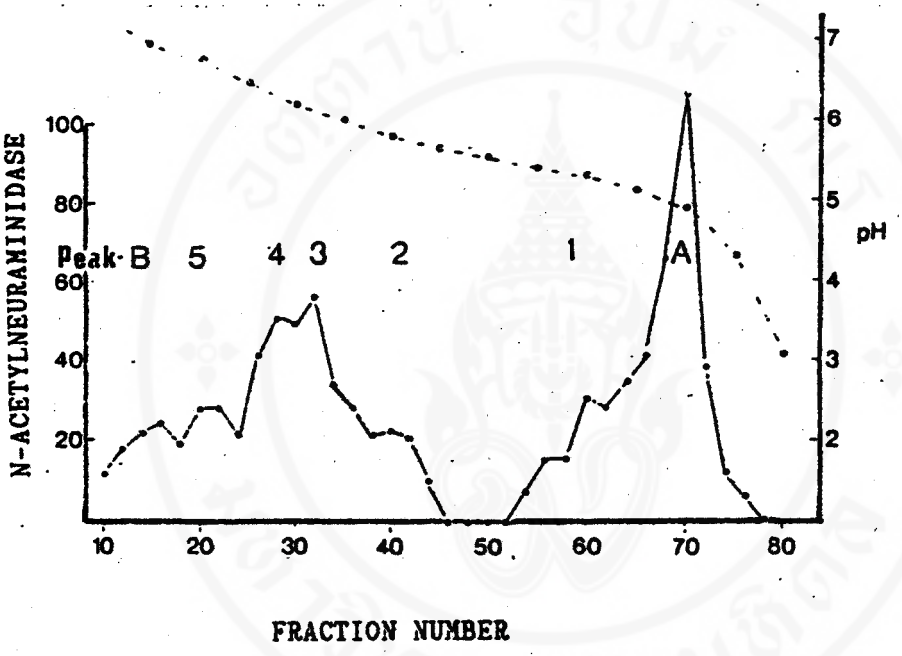


Figure 4 Isoelectric focussing pattern of serum hexosaminidases from an obligate Tay-Sachs heterozygote during pregnancy (15 weeks gestation). See Fig 3. for details.

weight slightly larger, more basic, and neuraminidase sensitive than tissues HEXA (60). On the other hand, a $HEXA^{c}$-like isozyme has been found to be the predominant form in tears as well as in synovial, seminal, and cerebrospinal fluids (61). Zuhlsdorf et al (9) separated serum HEX by SDS-PAGE. Using the Western blot technique with specific anti-HEXA serum, they concluded that 80 % of α-and β-chain in serum were in the precursor form and had pIs of 5.2-5.3. Moreover, the carbohydrates are predominantly endo H-resistant and contain some sialic acid group (62). In the part of serum HEXB appear to contain primarily of β-precursor with endo H-resistant. It is considerably more resistant to heat denaturation than $HEXA^{s}$ and had pIs of 7.6 - 7.7. A comparison between tissue and serum HEXB is more different because HEXB is only identified as isozyme is not bound to a pH 6 or 7. But it is interesting that in serum the amount of DEAE-unbound HEXB is reduced (3-15% of the total activity) as compared to tissue sources, with corresponding increase in the HEXI fraction (62,63). The HEXIs (intermidiates) are a heterogenous population of minor HEX isozymes whose pIs are between those of the two major isozymes, HEXA and HEXB (60). The HEX activity peaks are numbered starting at the beginning of the salt gradients thus HEXI, is slightly more acidic than HEXB but more basic than the rest of HEXIs (59). In serum the predominant forms are $I_1$ and $I_2$ , and they contain exclusively pre-β-chain, but in placenta $HEXI_2$ contains a polypeptide structurally related to α chain (7). The neuraminidase sensitivity suggest that the pre-β-chain are variable sialylated to produce the HEXIs (9). Comparison of serum HEXIs with the HEXIs described in placenta is quite different. The serum HEXIs are heat stable and are increased of pregnancy or diseases but in placenta HEXIs are not

heat stable, and the tissue HEXIs in general remain constant during pregnancy (7).

In conclusion serum enzymes are the product of secretion by fibroblasts, endothelial cells, smooth muscle cells, macrophages, lymphocytes, and granulocyted (9). Bearpark and Stirling (49) have shown that infused $HEXA^{S}$ was rapidly cleared from the circulation when sialic acid was removed. The oligosaccharide chain had a terminal galactose residual, presumably the galactose terminal glycoprotein were removed by the hepatocyte-dependent, receptor mediated plasma clearance pathway (64). Glycoproteins and various tissue-derived lysosomal hydrolases with terminal mannose or GlcNAc residuals are also specifically cleared from plasma following infusion, by a second receptor-system (65); the liver Kupffer cell are primarily responsible for this receptor-mediated uptake (66) which may represent simply a general pathway for the clearance and lysosomal degradation of circulating glycoproteins. These two glycoprotein clearance pathways would rapidly remove from the circulation any HEX isozymes that did not contain fully sialylated, complex-type oligosaccharide moities. The resulting serum HEX isozymes thus are the result of the concentration of the most sialylated HEX isozymes secreted by variuos cells (7).

## 1.4. β-HEXOSAMINIDASE AND DISEASES



The physiological functioning of the HEX is perhaps best clarified by the consequences of their absence. Moreover, the isozymes of HEX can be distinguished to some degree by their natural

substrate specific, as well as by differences in their charge and stability. All HEX isozymes catabolite, all the artificial substrates. In Sandhoff disease patients total HEX inability to degrade lipid hexosaminidases lead to storage of $GM_2$ ganglioside, asialo $GM_2$ ganglioside and globoside. Both HEXA and HEXB are absent from all tissues (resulting from defects in $\beta$ chain synthesis) (47). In classical Tay-sachs disease inability to degrade $GM_2$ ganglioside. The enzyme dificiency is not as complete, HEXA is absent (resulting from defects in $\alpha$ chain synthesis) and a normal or even elevated level of HEXB (28). Moreover there are exist several minor forms of HEX (HEXC, which has a higher pH optimum; HEX $I_1$, $I_2$ (P) which are found in serum, particularly of pregnant women; HEXM, found in male urine; and yet other form seen in fibroblasts from patients with I-cell disease) (67). These minor form could represent intermediates in the synthesis or degradation of HEXA and HEXB, or be unrelated. Although their biological function is unknown, the minor forms should not be ignored, if only because they may interfere with analytical determinations of HEXA and HEXB and with interpretation of the results (67). The alteration of HEX activity and proportion of HEX isozymes occur in various kind of diseases.

I-cell disease, the serum form I-cell patients has a tenfold increase in total HEX activity, a large amount of this increase in the HEXI region, with a smaller increase in HEXB. The HEXB also appeared to be more neuraminidasesensitive than normal serum HEXB when separated on Carboxymethyl-cellulose (62).

Diabetes Mellitus (DM), the total activity in serum and urine is also increased in newly diagnosed patients with DM; the high level can be reduced by insulin treatment (68). In pregnancy, the total HEX activity increase, predominantly of the HEXI. Using DEAE-cellulose chromatography can be clearly that in normal, the activity ratio of A/B are 4.3 $\pm$ 1.33. During pregnancy the ratio falled slightly to 3.01 $\pm$ 1.08 (59,65). While, rheumatoid arthritis, the HEX isozyme pattern in synovial fluid taken from patients showed the presence of tissue-like HEXA (mature HEXA) and HEXB. After remission, the peptide returned to one resembling normal serum (61).

Renal disease, the presence of glomerular damage in patients with Goodpasture disease could be detected by the increase in the $HEXA^{s}$ form in urine. In contrast, patients with nephrotic syndrome show increased amounts of the tissue-like HEXA and HEXB in urine, reflecting tubular epithelium damage (61). It has also been suggested that an increase in the total urinary HEX activity could be used as an early warning sign of renal transplant rejection or renal disease (69).

Various kinds of cancer also show an increment in total serum HEX activity, predominantly in the HEXI (61). Ellis et al (24) suggested that the ratio HEXI: $HEXA^{s}$ could be used in the differential diagnosis of leukemia in children. The total serum HEX level were significantly elevated in sera of subjects with female genital cancers, and in subject with ovarian cancer (70). Furthermore, the HEX of various tumor seems to offer an interesting problem for the

study of enzyme markers of tumors. Studies in HEXA and HEXB in human colon (71,72), breast (72), renal (73) and ovarian carcinoma (74) indicated that total HEX and HEXB activity increased. In different studied on human ovarian(74) and prostatic tumors(76), the activity of HEXB was reported to be with in normal ranges. In addition to these major types of isozymes, variant form of the isozymes in tumor tissues have been described. Recently, an atypical thermolabile form of human HEXB was observed in cell line of colonic carcinoma (77) as well as in tumors metastasized to liver (6). Finally, the elevation activity of HEX and atypical thermolabile form of HEXB was observed in human lung cancer. This finding indicate that the formation of mixed disulfide bonds in the tumor HEXB increases the net negative charge and results in the appearence of a heat labile form (78).

Numerous investigators have found altered activity levels and properties of specific glycoside in malignant tissues, since glycosidases are concerned with removing specific sugar residuals from glycoconjugated and since glycoconjugates are involved in cell-cell interactions, altered glycosidases could modify soluble on cell surface glycoconjugated and lead to decreased adhesiveness, increased invasiveness, and the matastatic spread of cancer (79,80). The isozyme compositions and/or altered isozyme properties exist in human primary cancerous and metastatic tumor tissues (6, 24, 61, 71, 73, 75, 76, 77, 81). A number of these studies have specifically reported altered electrophoretic and kinetic properties of HEX isozymes in human cancerous tissues. Kimball et al (77) reported on a variant HEX isozyme (designed F) in a human colonic carcinoma cell line of high metastatic potential which has a pI near that of HEXB

but is more thermolabile and has lower activity at acidic pH values when compared to normal HEXB. Chatterjee et al (75) have found that HEXB from ovarian adenocarcinoma is more labile to heat and acidic pH values than is HEXB from normal ovarian tissue. Narita et al (81) have very recently reports on the presence of an atypical HEXB in human lung adenocarcinoma which is very similar to the atypical HEXB$^{c}$ described above and the one previously characterized in human liver metastasis. These variant HEXB usually have slightly more acidic pI values, exhibit significantly decreased thermostability and have subtle differences in their pH optimum curved (decreased activity at acidic pH values) when compared to normal HEXB. The evidence suggesting that the atypical HEXB in human lung carcinoma is due to an enzyme protein containing a sulfhydyl modefication (possibly reacted with glutathione) (81).

The liver diseases, the various study about the HEX in liver diseases, together with malignancy in liver diseases (5,10,89,90,91). The increasing in total HEX activity and percentage of HEXB are shown in Table 2,3 and probably result from two causes. One, defect in clearance system: Earlier studies have shown that the clearance of HEX, a lysosomal enzyme, from blood is performed by non-parenchymal cells of the liver. From fractionation techniques, autoradiography and histochemical studied of rat liver tissue have revealed that the non-parenchymal cells are the principle cell engaged in the recognition of terminal mannose or GlcNAc residuals such as HEX (82,83) by the mannose / glucosamine receptor(84). There are several impairment in clearance of receptor. First, the non-parenchymal cell cannot display their optimum clearance properties since they have lost

contact with much of the portal blood (85). Second, the hemodynamic disturbance, virus as well as toxic agents, particularly alcohol, attributed the phenomenon to depression of the reticuloendothelial system (86). Finally, the increasing of bile acid in the circulation or in hepatocytes may produce change in membrane of cell (include Kupffer cells) by acting as detergents and disrupting membrane bound receptor function. Especially, the Kupffer-cell membrane is even more acceptable to detergent action of bile acid than the sinusoidal membrane (87). There are the results in decreasing clearance of HEX that are normal cleared by this mechanism. Two, overproduction and releasing, there are many tumors themself may be responsible for the elevated HEX level by increased production (5). Furthermore, in macrophages it has been possible with several agents, e.g. endotoxin or antigenic stimulation, to induce of intarcellular content and release of lysosomal hydrolases (88).

Table 2 Cancer patients and hexosaminidase enzyme by fluorometric detection (5).

| Group description(N) | HEX activity (U/min) Mean ± SD | Percent HEXB Mean ± SD |
|---|---|---|
| Normal (18) | 18.4 ± 5.0 | 29 ± 9 |
| Pathological (27) | 19.0 ± 8.0 NS* | 25 ± 8 NS* |
| Cancer (108) | 29.2 ± 21 <.05*,.02+ | 28 ± 9 NS*, NS+ |
| Cancer patient subgroups | | |
| - No metastasis (43) | 21.6 ± 12.2 NS*, NS+ | 28 ± 8 NS*, NS+ |
| - Liver metastasis (35) | 39.0 ± 28.6 <.01*,.001+ | 27 ± 10 NS*, NS+ |
| - Non-liver metastasis (24) | 26.5 ± 15.8 <.05*, .05+ | 28 ± 9 NS*, NS+ |
| - Liver and other metastasis(6) | 37.4 ± 12.7 <.001*,.001+ | 34 ± 10 NS*, <.05+ |

NS : no significant (P > 0.01)

* : P values compared to normal values.

\+ : P values compared to pathological values.

Table 3 Liver diseases and hexosaminidase enzyme by colorimetric detection.

| Liver disease patients | Refe-rence | NO | HEX activity (U/min) Mean ± SD |
|---|---|---|---|
| Normal control | 89 | 19 | 15.8 ± 3.5 |
| Cirrhosis (Alcoholic 20, Hepatisis 15,Cryptogenic) | 10 | 45 | 24.9 ± 8.9 |
| Primary biliary cirrhosis | 10 | 10 | 36.6 ± 9.5 |
| Alcoholic liver disease | 89 | 8 | 19.3 ± 9.5 |
| Acute hepatitis A | 90 | 45 | 28.9 ± 10.1 |
| Acute hepatitis B | 90 | 45 | 25.6 ± 12.7 |
| Chronic hepatitis B ($HB_sAg$ -positive) | 90 | 12 | 34.4 ± 26.6 |
| Chronic hepatitis B ($HB_sAg$ -negative) | 90 | 17 | 24.0 ± 9.5 |
| Viral hepatitis | 89 | 5 | 23.3 ± 7.4 |
| Cholestasis (Choledochol-ithiasis 5, other 5, Malignant biliary duct or pancrease 6) | 10 | 16 | 40.3 ± 10.6 |
| Benign extrahepatic obstr-uction (gallstones) | 89 | 10 | 23.10 ± 4.7 |
| | 91 | 14 | 30.24 ± 2.84 |

Table 3 (conti.)

| Liver disease patients | Refe-rence | NO | HEX activity (U/min) Mean ± SD |
|---|---|---|---|
| Prolonged intrahepatic cho-lestasis | 91 | 15 | 39.4 ± 2.44 |
| Malignant of extrahepatic obstruction (Carcinoma of head of pancrease 14, cholangioca-cinoma 1) | 91 | 15 | 45.49 ± 4.34 |
| Malignant of extrahepatic obstruction | 89 | 16 | 47.6 ± 14.7 |

## 2. OBSTRUCTIVE JAUNDICE

Jaundice is a condition in which the scleras, mucous membranes and skin become abnormally yellow as a result of an increased concentration of bilirubin in the blood, usually greater than 3 mg per dl. It can result from a varity of disorders from life-threatening disease of the liver and biliary tract to innocuous impairment of hepatic bilirubin transport. Plasma bilirubin concentration reflects a state of dynamic equilibrium between bilirubin production and hepatic bilirubin clearance, or some combination of the two (93). Simple classification of jaundice is into three predominant types. Pre-hepatic, there may be increased bilirubin load on the liver cell. Hepatic, there may be a disturbance in uptake, transport and conjugate of bilirubin within the liver cell. Finally cholestasis might be due to obstruction to the biliary tract before the bilirubin reaches the intestine. Then, the clinical classification of the jaundice by Warren and Kune are shown in Table 4 (92).

In most patients, jaundice reflects the presence of cholestasis. Cholestasis was originally coined by the morphologist to imply viable accumulation of bile in cells and biliary passages, usually associated with dilated bile canaliculi, the clinician defines as an exess of biliary substances in the blood, and in common usage implies interference with bile flow into the duodenum asscoiated with jaundice. However, the process sometimes involves only part of the liver and jaundice may be absent even through other clinical and biochemical features pointing the cholestasis are present (94). Cholestasis, thus, has been divided into the type

Table 4 Clinical classification of jaundice

| |
|---|
| 1. *Increased Bilirubin Load*<br>Excessive haemolysis<br>Congenital haemolytic anaemias<br>Acquired haemolytic anaemias<br>Haemolytic disease of the newborn<br>Incompatible blood transfusion<br>Haemolysis caused by drugs and infections<br>Primary "shunt" hyperbilirubinaemia |
| *2. Defective Bilirubin Transport and Conjugation*<br>Gilbert's syndrome<br>Grigler-Najjar Syndrome<br>Neonatal jaundice<br>Acute viral hepatitis } Frequently other factors<br>Chronic hapatitis } involved such as cholestasis<br>Hepatitis caused by drugs } and excessive<br>and infection } haemolysis |

Table 4 (conti.)

*3. Cholestasis*

Intrahepatic cholestasis

- Cholestatic form of acute viral hepatitis
- Acute alcoholic hepatitis
- Postnecrotic cirrhosis (sometimes)
- Dubin - Johnson and Roter syndromes
- Drug-induced cholestasis
  - Methyl testosterone
  - Phenothiazine drugs - chlorpromazine
    - prochlorperazine
    - trifluoperazine
  - Oral hypoglycaemics - chlorpropamide
    - glibenclamide
  - Erythromycin estolate
  - Imipramine
  - Methyl dopa
- Cholestatic jaundice of pregnancy
- Oral contraceptives
- Postoperative cholestatic jaundice
- Primary biliary cirrhosis
- Benign recurrent idiopathic cholestasis

Table 4 (conti.)

3. Cholestasis (conti.)

Mechanical bile obstruction - extrahepatic cholestasis

- Choledocholithiasis
- Malignant obstruction
  - Bile duct
  - Gallbladder
  - Pancreas
  - Papilla of Vater
  - Duodenum
  - Liver
- Benign strictures
  - Traumatic (postoperative)
- Stenosis of the papilla of Vater
- Primary sclerosing cholangitis
- Recurrent pyogenic cholangitis
- Pancreatic inflammation
  - Acute pancreatitis
  - Chronic pancreatitis
  - Pancreatic cysts
  - Pancreatic abscesses
- Parasitic infestation
  - Hydatids
  - Ascaris (roundworm), opisthorchis (liver fluke)
- Congentital biliary atresia

resulting from obstruction (intrahepatic or extrahepatic) and the type without obstruction. The extrahepatic biliary obstruction most common obstacles to the flow of bile in the extrahepatic biliary passages are stones, tumors, and strictures. Gallstones may lead to sufficient dilation of the proximal ducts that bile may flow around them. Moreover, a ball-value effect of the stone may cause temporary biliary obstruction. Benign and malignant intrinsic tumor of the bile ducts result in jaundice, as do extrinsic tumors that directly invade the duct wall and fix it. The parasite and myocotic condition, choledochal cyst, duodenal diverticular, or hepatic artery aneurysms may cause obstruction. The intrahepatic mechanical biliary is obstruction mechanical interference with bile flow in circumscribed portions of the intrahepatic ductal system, as from intrahepatic stones, leads to focal cholestasis. Carcinoma at the bifurcation of the common duct at the hilum of the liver with all the manifestations of an extrahepatic process. The tumor begins in the hepatic duct and may later involve the other. Suppurative cholangitis, usually ascending and often associated with abcess formation, was previously a common cause of jaundice (95).

Carcinoma of the extrahepatic bile ducts is more prevalent in men, and usually presents as gradually progressive obstructive jaundice. According to data collected by Schoenfied (96) biliary ductal carcinoma occur with about one-fifth the frequency of carcinomas of the gallbladder, and one-eight the frequency of pancreatic carcinomas (carcinomas of head of pancrease). The major sites of predilection are the bifurcation of the common hepatic duct, the middle of the common bile duct, and the ampulla of Vater, Bile

duct carcinomas are fairly frequently found in societies where infections with the liver fluke, *clonochis sinesis* and *opisthorchis viverrini*, is common. It is believed that the chronic obstruction by these agents leads to inflammation which, in turn, leads to dysplastic effects of possibly carcinogenic bile sterols (97). In case of obstruction from acute and chronic cholecystitis, the inflammatory diseases of the gallbladder, are almost invariably the result of previous cholelithiasis. Acalculus cholecystitis may complicate bacteraemia from elsewhere. Other causes of cholecystitis without gallstone include clostridia, polyarteritis, steroid treatment and trauma. The gallbladder is usually contracted with a thickened, sometimes calcified, wall but may be cystic. The mucosa is ulcerated and scarred (98).

## 3. OBJECTIVES

3.1. To study and compare the activities of serum HEX and isozymes in normal, control and patients (liver diseases with obstructive jaundice) subjects.

3.2. To compare serum HEX and isozymes in benign and malignant biliary obstruction.

3.3. To study the correlation between HEX and total bile acid in fasting serum of normal and patient subjects.

# CHAPTER II

# MATERIALS AND METHODS

## 1. MATERIALS

### 1.1 CHEMICAL REAGENTS

| NAME | SUPPLIER |
|---|---|
| 1. Bilirubin | MERK (Germany) |
| 2. Chloroform | MAY&BAKER (England) |
| 3. Cholic acid, sodium salt | SIGMA (U.S.A.) |
| 4. Citric acid, monohydrate | MERK (Germany) |
| 5. Diaphorase (17 units/mg solid); Ec. 1.8.1.4 from *Clostridium Kluyveri* | SIGMA (U.S.A.) |
| 6. Glycine (Essential ammonia free) | SIGMA (U.S.A.) |
| 7. Hydrochloric acid (37% W/W) | MERK (Germany) |
| 8. 3 α- Hydroxysteroid dehydrogenase (8.9 units/mg solid )Ec. 1.1.1.50. from *Pseudomonas testosteroni* | SIGMA (U.S.A.) |
| 9. Methyl alcohol | MAY&BAKER (England) |
| 10. 4-Methylumbelliferyl-N-acetyl-β-D-glucosaminide | SIGMA (U.S.A.) |
| 11. 4-Methylumbelliferone (Free acid) | SIGMA (U.S.A.) |
| 12. β-Nicotinamide adenine dinucleotide | SIGMA (U.S.A.) |
| 13. Resazurin, sodium salt | SIGMA (U.S.A.) |
| 14. Sodium carbonate, anhydrous | BDH (England) |

| NAME | SUPPLIER |
|---|---|
| 15. Sodium hydrogen phosphate, dibasic.12$H_2o$ | MERK (Germany) |
| 16. Sodium hydrogen phosphate, monobasic.$H_2o$ | CARLO ERBA (Italy) |
| 17. Sodium nitrite | BDH (England) |
| 18. Sulfanilic acid, monohydrate | MALLINCKRODT (U.S.A.) |
| 19. Tris (hydroxymethyl) aminomethane hydrochlorid | SIGMA (U.S.A.) |

## 1.2 INSTRUMENTS

| NAME | MANUFACTURER |
|---|---|
| 1. Spectrofluorophotometer model RF-5000 | Shimadzu corporation, Kyoto (Japan) |
| 2. Controller unit model DR- 15 | Shimadzu corporation, Kyoto (Japan) |
| 3. Color graphic display unit model GDU-10C | Shimadzu corporation, Kyoto (Japan) |
| 4. Spectrophotometer model UV-160 | Shimadzu corporation, Kyoto (Japan) |
| 5. The others : | |
| pH-meter model ion analyzer150 | Corning limited (England) |
| Analytical balance | Sartorius research (W. Germany) |
| Water bath | Blue M eletric company (U.S.A.) |
| Vortex mixer | Scientific industries (U.S.A.) |

NOTE : Units 1,2,3 are connected for fluorescence determination (Figure 5).

Figure 5 Layout of Photometer (1), Controller (2) and color graphic display unit (3)

## 2. METHODS

### 2.1 SUBJECTS

#### 2.1.1. Normal subjects

Two groups of normal serum sample were obtained.. The first is 95 fasting serum of healthy subjects from medical students, graduate students and technician of Siriraj Hospital (65 males and 30 females) ages ranging from 17 to 57 years. The second group is 25 serum of healthy blood donors from Blood Bank Unit of Ramathibodi Hospital (18 males and 7 females) ages ranging from 23 to 41 years.

#### 2.1.2. Control subjects.

Forty nine fasting serum sample of patients were obtained from Srinagarind Hospital, Khonkaen University (35 males and 14 females) ages ranging from 15 to 81 years. These patients were proved by clinical assessment and biochemical screening that they were absent from liver diseases. Especially, the value of bilirubin exist in normal range.

#### 2.1.3. Patient subjects.

Seventeen fasting serum and ninety serum of liver disease patients were obtained from Siriraj Hospital and Srinagarind Hospital (72 males and 35 females). All of them were proved being obstructive jaundice by clinical diagnosis and biochemical screening test. Especially, the biochemical metabolism of bilirubin showed abnormality.



### 2.2 REGENTS:

#### 2.2.1. Reagent for β-N-Acethylhexosaminidase

##### 2.2.1.1. Citrate - phosphate buffer, 0.04 M, pH 4.4

Dissolve 0.84 gm of citric acid monohydrate and 1.432 gm of $Na_2HPO_4.12H_2o$ in 100 ml distilled water, as solution A and B respectively. Adjusted solution B pH to 4.4 with solution A

2.2.1.2. Glycine - Carbonate buffer, 0.17 M, pH9.9

Dissolve 6.38 gm of glycine and 9.01 gm of sodium carbonate anhydrous in 500 ml distilled water, as solution A and B respectively. Adjusted solution B pH to 9.9 with solution A.

2.2.1.3. 4 -Methylumbelliferyl-N-acetyl-β-D-glucosaminide solution, 1.0 mM.

Dissolve 0.00379 gm of 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide in 10 ml of citrate-phosphate buffer, 0.04 M, pH 4.4

2.2.1.4. 4-Methylumbelliferone stock standard solution 2 mM.

Dissolve 0.00176 gm of 4-methylumbelliferone in 5 ml of glycine-carbonate buffer, 0.17 M, pH 9.9. This solution always keeps at 4°c.

2.2.2. Reagent for total bile acid.

2.2.2.1. Phosphate buffer, 0.065 M, pH 7.4

Dissolve 0.9 gm of $NaH_2PO_4.H_2O$ and 2.33 gm of $Na_2HPO_4.12H_2O$ in 100 ml of distilled water, as solution A and B respectively. Adjusted solution B pH to 7.4 with solution A.



2.2.2.2. Tris - HCl buffer, 0.05 M, pH 9.0

Dissolve 3.03 gm of tris (hydroxymethyl) amino methane hydrochloride in 500 ml of distilled water, so adjusted pH to 9.0 with 6 N hydrochloric acid.

2.2.2.3. 3α-hydroxysteroid dehydrogenase (3α-HSD) stock solution, 0.048 U/ml

Dissolve 0.0001 gm of 3α-hydroxysteroid dehydrogenase in 2 ml of cool distilled water. Then aliquot 0.5 ml of stock solution in plastic tube, stored at -20° c and used before one month.

2.2.2.4. 3α-HSD working solution, 0.0048 U/ml.

Dissolve 0.5 ml of 3α-HSD stock solution in 4.5 ml of cold distilled water.

2.2.2.5. β-Nicotinamide adenine (β-NAD) dinucleotide solution, 0.012 gm/ml

Dissolve 0.12 gm of β-NAD in 10 ml of cold phosphate buffer, 0.065 M, pH 7.4.

2.2.2.6 Diaphorase solution, 2.5 U/ml

Dissolve 0.00146 gm of diaphorase in 10 ml of cold phosphate buffer, 0.065 M, pH 7.4.

2.2.2.7. Resazurin solution, 20 μM.

Dissolve 0.0005 gm of resazurin in 100 ml of distilled water. The solution always keeps at 4°c

2.2.2.8. Cholic acid stock standard solution, 200 μM

Dissolve 0.00086 gm of cholic acid, sodium salt in 10 ml of distilled water. This solution always keeps at 4°c.

## 2.3 DETERMINATIONS:

### 2.3.1 Determination of β-N-acetylhexosaminidase and isozymes in serum by heat inactivation method.

Principle: The substrate, 4-methylumbelliferyl-N-acetyl-β-D-glucosaminide, is cleavaged to 4-methylumbelliferone and β-N-acetylglucosaminide in citrate phosphate buffer, pH 4.4, at 37°c by enzyme, β-N-acetylhexosaminidase, in serum. The alkaline solution, glycine-carbonate buffer pH 9.9 is added to stop the reaction. The enzyme assay was performed by spectrofluorophotometry of 4-methylumbelliferone as the fluorophone in alkaline condition.

By heat inactivation method was modified from the original method of O 'Brain (30) to determine HEXA and HEXB by incubation of serum at 50°c for 3 hours. In this way HEXA was inactivated and the remaining activity, HEXB, was determined.

Procedure :

1. Dilute 0.1 ml of serum with 0.9 ml of citrate-phosphate buffer 0.04 M, pH 4.4

2. Pipet 50 µl each of diluted serum into three tightly stoppered plastic tubes. One tube are used to determine total HEX, one tubes are heated at 50°c for three hours (HEXB determination), and the last tube for blank. The tubes for total HEX and blank are frozen immediately and keep frozen (thaw before assay)

3. Add 100 µl freshly preparation of 4-methyllumbelliferyl-N-acetyl-β-D-glucosaminide solution to all assay tubes except blank tube used citrate-phosphate buffer and incubate at 37°c for one hour.

4. Stop reaction with 5 ml of glycine-carbonate buffer 0.17M, pH 9.9

5. The resultant fluorescence of 4-methylumbelliferone was measured emission at 448 nm with the excitation at 360 nm

6. The relative fluorescence was obtained from difference between the sample and blank tubes. Read the activity of β-N-acetylhexosaminidase and HEXB from calibration curve of 4-methylumbelliferone. The activity are expressed in units per minute (μmole of substrate hydrolyzed per litre per minute)

7. Calibration curve for determination of β-N-acetylhexosaminidase was prepared by plotting fluorescence intensity with serial dilution of 4-methylumbelliferone standard solution. This dilution comprised of the final concentration of (31.2,62.5,125,250, 500 μM) by diluting 4-methylumbelliferone stock solution with glycine carbonate buffer and were analyzed as described under assay procedure (step 3-5) by using standard instead diluted serum. Because the serum was diluted ten fold. The concentration of working standard solution in unit per min are 5.2, 10.4, 20.8, 41.6, 83.2 U/min respectively.

Calculation

Concentration of HEX in U/min

$$\text{HEX (U/min)} = \frac{\text{HEX}(\mu M)\times 10}{60}$$

10= Dilution factor

60= Incubation time

Concentration of HEXA in U/min

$$\text{HEXA} = \text{TOTAL HEX} - \text{HEXB}$$



Percent HEXB

$$\text{HEXB (percent)} = \frac{\text{HEXB}\times 100}{\text{Total HEX}}$$

2.3.2 Determination of total bile acid by spectrofluorophotometry

Principle : The spectrofluorometric determination for bile acid was modified from the original method of Mashige et al(99). Bile acid are converted to 3-oxo bile acids by 3α-HSD with the concomitant reduction of NAD to NADH, and then the hydrogen of the generated NADH is transfered by diaphorase to resazurin to yield the fluorophore, resorufine. Finally, the fluorescence of resorufine is read and this is proportional to the concentration of total bile acid (Figure 6). Because,there are many enzymes in serum affectingly NAD-linked oxidoreduction that generate NADH to make the reaction proceed. Then, all enzymes were inactivated by incubation in water bath at 67°c for 30 min.

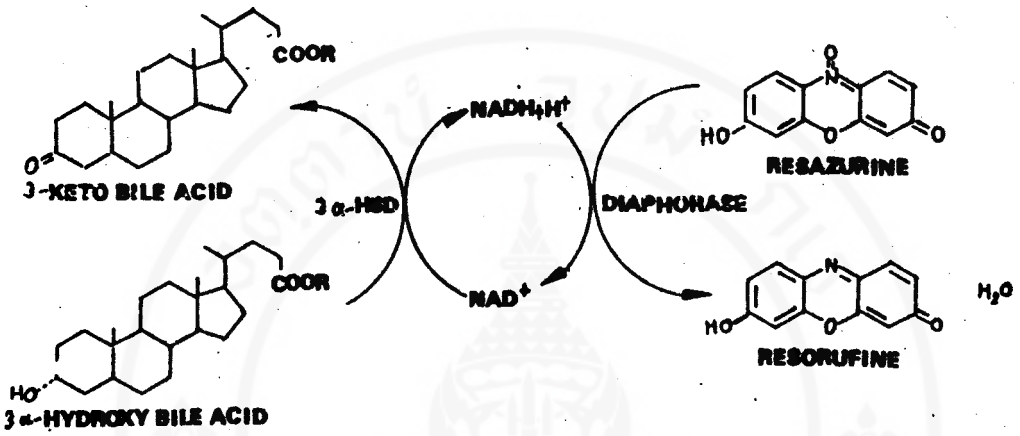


Figure 6 The principle of fluorimetric determination of serum bile acids

Procedure :

1. Pipet 100 µl each of serum into two tightly stoppered plastic tubes (one for sample tube and the other for blank tube).

2. Add 2.5 ml of Tris-HCl buffer pH 9.0 and incubated at 67°c for 30 min.

3. Add 100 µl freshly preparation of cold 3α-HSD working solution to sample tube and 100 ul cold of distilled water in blank tube.

4. Immediately add 100 µl freshly preparation of cold β-NAD solution, cold diaphorase solution and resazurin solution. Mix and incubate at 20 -25 °c for 1 hour.

5. The resultant fluorescence of resorufin was measured emission at 579.2 nm with the excitation at 571.2 nm.

6. The relative fluorescence was obtained from difference between the two tubes and read the concentration of total bile acid from calibration curve. The units are expressed as µmole per litre.

7. Calibration curve for total bile acid was prepared by plotting fluorescence intensity with dilution of cholic acid standard solution. The dilution comprised of the final concentration 3.125, 6.25, 12.5, 25, 35, 50 µM and were analyzed as described under assay procedure.

2.3.3 Determination of bilirubin in serum

Principle : Bilirubin reacts with diazotized acid to give a color that is red-violet in acid solution and in alkaline solution. It has been known for many years that not all of the bilirubin present in an aqueous solution. To obtain a complete reaction, the addition of a fairly high concentration of alcohol (ethyl or methyl) or some other solubilizing agent is necessary.

Bilirubin exists in the serum in two form- a unconjugated form and a conjugated form. The conjugated form that is more soluble in water and reacts relatively rapidly in aqueous solution. The unconjugated form is much less soluble in water and does not react in simple aqueous solution. The addition of methyl alcohol to a concentration of 40 - 50 % will dissolve the bilirubin sufficiently to react with diazo reagent. This is the basis for the commonly used Molloy - Evelyn method (100).

2.4. PRECISION :

The intra and inter assay of hexosaminidase activity and total bile acid was determined in three concentration ; high, medium, low. The intra assay based on five replicate measurements during one day. And , the inter assay was determined from daily using as control material in assayed.

2.5 RECOVERY :

Recovery of added two and three difference standards concentration of 4-methylumbelliferone and cholic acid, sodium salt were assayed after addition of the standard in order to calculate the percentages recovery.

2.6 STATISTICS :

Student's t-test and Pearson correlation were used in statistically analysis of this study.

# CHAPTER III

# RESULT

## 1. METHODS.

### 1.1.1. Determination of optimum excitation and emission wavelength

The scaning excitation and emission wavelength by Shimadzu spectrofluorophotometer of hexosaminidase determination were shown in Figure 7, 8 and 9. The maximum excitation and emission wavelength for 4-methyllumbelliferone were 360.0 nm and 448.0 nm. Then, the optimum parameters and linearity of calibration curve were depicted in Figure 10. Corresponding to the total bile acid determination were shown in Figure 11, 12 and 13. The maximum excitation and emission wavelength for resorufin were 517.2 nm and 579.2 nm. Parameters and linearity of calibration curve were depicted in Figure 14.

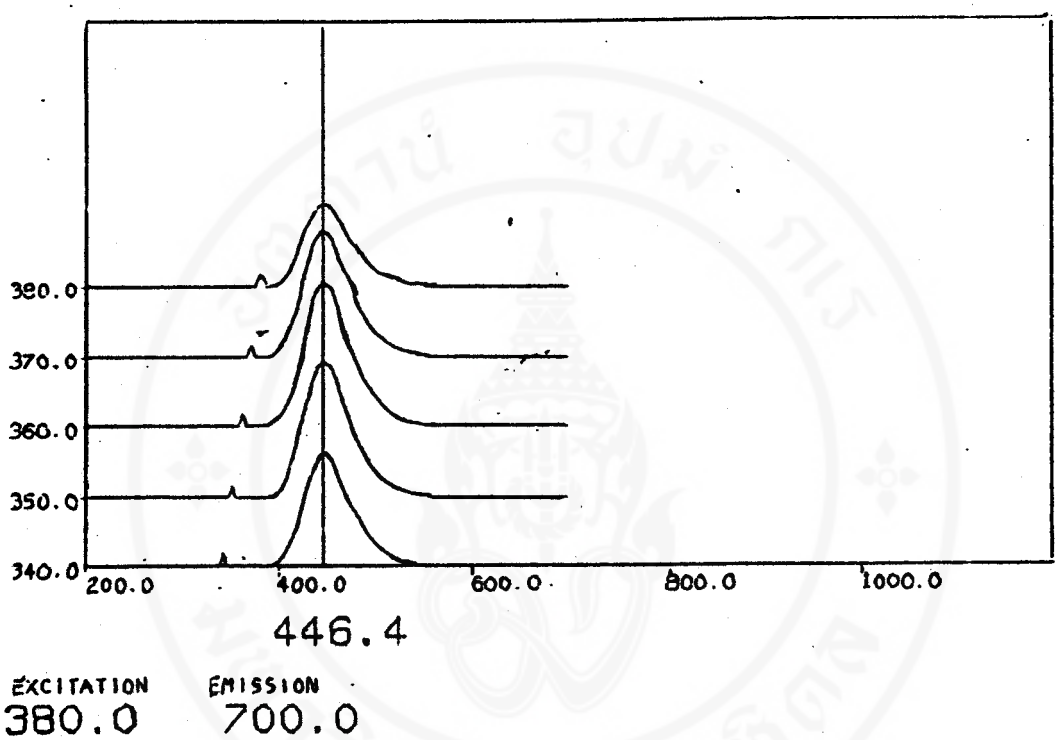


**Figure 7** The scaning emission wavelength of 4-methylumbelliferone from 200-700 nm when excitation wavelength set at 340, 350, 360, 370, 380 nm. The approximation of emission wavelength is 446.4 nm

Parameter set:

Band width 1.5 for excitation and emission

4-Methylumbelliferone concentration 500 µmole/litre

Ordinary X 1

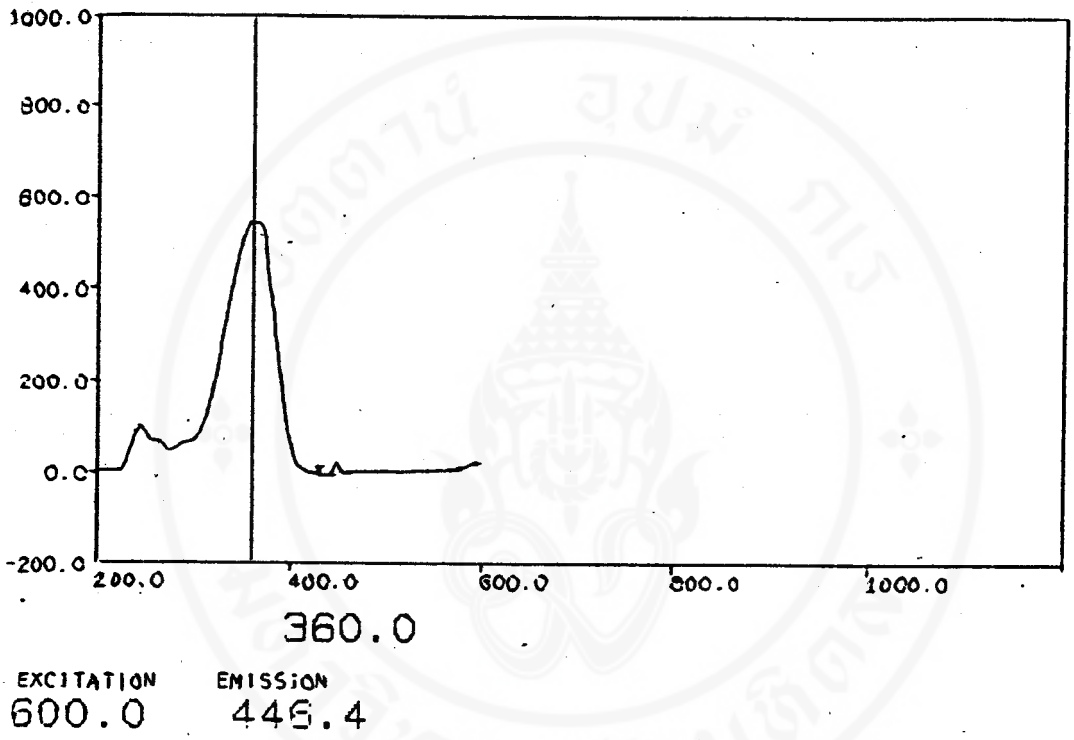


Figure 8 The scaning excitation wavelength of 4-methylumbelliferone from 200-600 nm when emission wavelength set at 446.4 nm. The maximum excitation wavelength is 360.0 nm

Parameter set:

Band width 3.0 for excitation and 1.5 for emission

4-Methylumbelliferone concentration 500 µmole/litere

Ordinary X 1

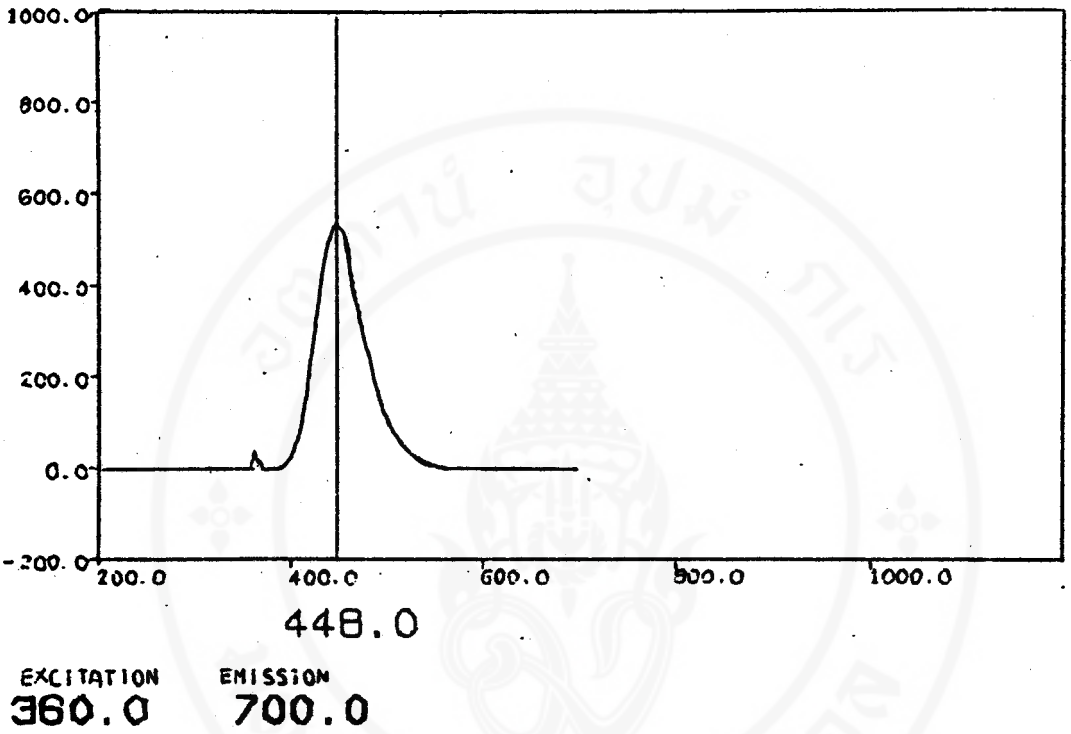


**Figure 9** The scaning emission wavelength of 4-methylumbelliferone from 200-700 nm when excitation wavelength set at 360 nm. The maximum emission wavelength is 448 nm.

Parameter set:

Band width 3.0 for excitation and 1.5 for emission

4-Methylumbelliferone concentration 500 μmole/litre

Ordinary X 1

CALIBRATION CURVE PARAMETERS

| | Ex (nm) | Em (nm) |
|---|---|---|
| FIXED: | 360.0 | 448.0 |

FACTORS:

BAND WIDTH: EX = 1.5 EM = 1.5

X-AXIS: LINEAR Y-AXIS: LINEAR

CALIBRATION CURVE

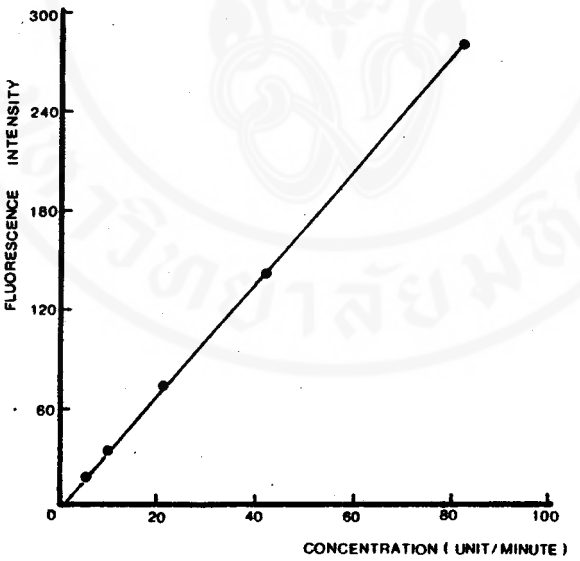


Figure 10 The parameter and calibration curve for determination hexosaminidase

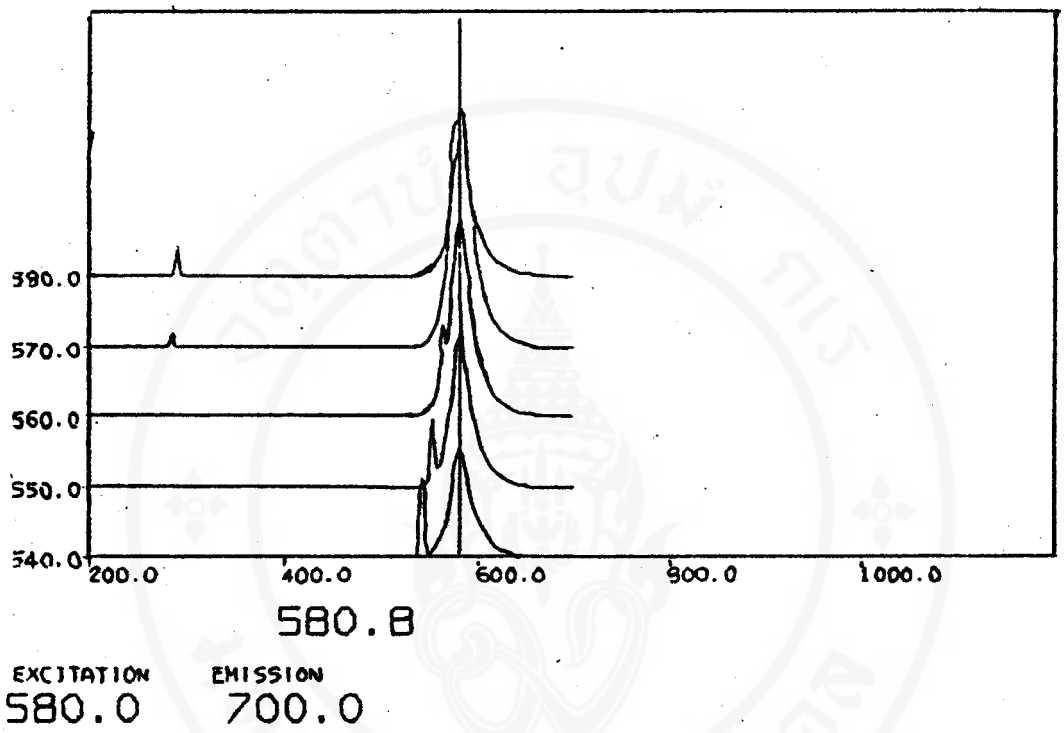


**Figure 11** The scaning emission wavelength of sodium cholate from 200-700 nm when excitation wavelength set at 540, 550, 560, 570 and 580 nm. The approximation of emission wavelength is 580.8 nm.

Parameter set:

Band width 1.5 for excitation and 3.0 for emission

Sodium cholate concentration 50.0 μmole/litre.

Ordinary X 1

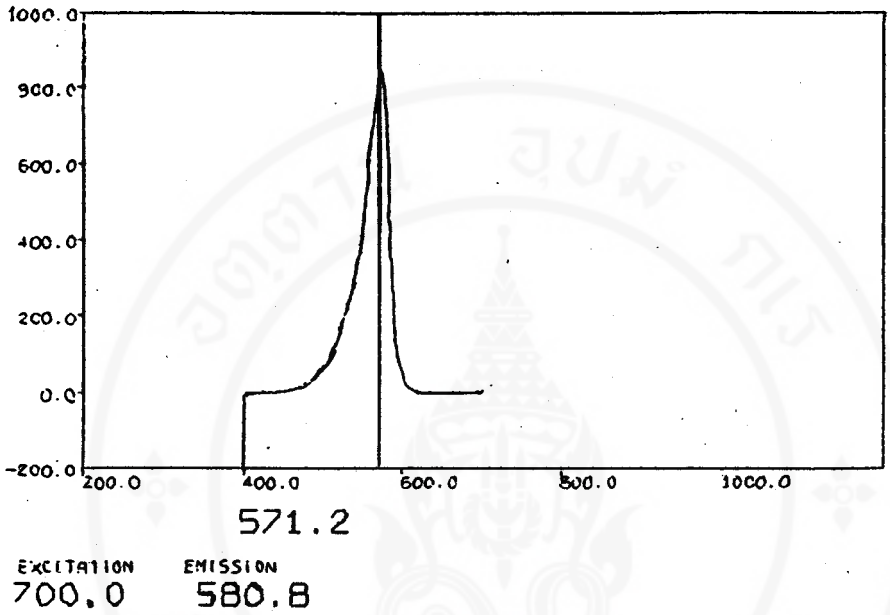


Figure 12 The scaning excitation wavelength of sodium cholate from 400-700 nm when emission wavelength set at 580.8 nm. The maximum excitation wavelength is 571.2 nm.

Parameter set:

Band width 5.0 for excitation and emission.

Sodium cholate concentration 50.0 μmole/litre.

Ordinary X 1

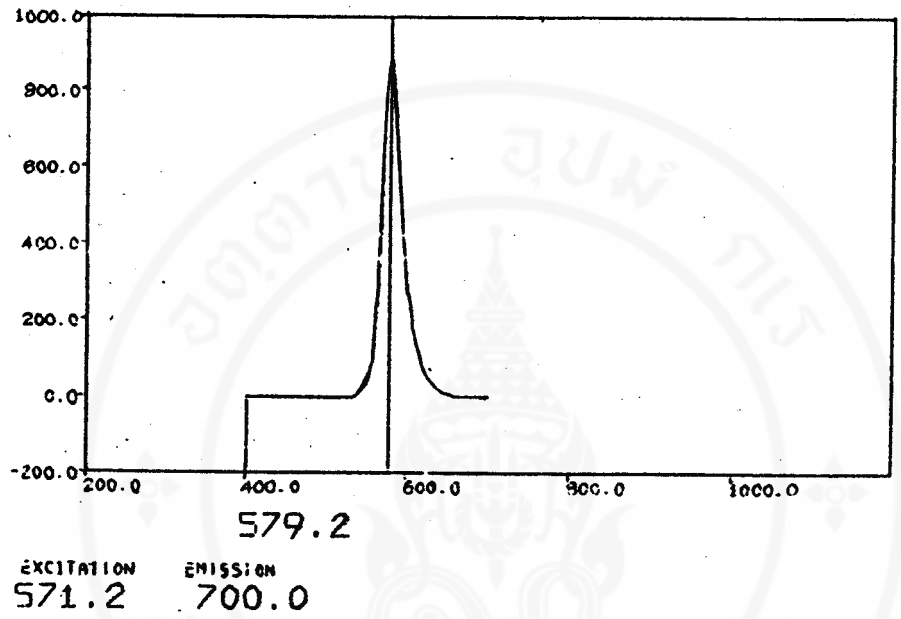


**Figure 13** The scaning emission wavelength of sodium cholate from 400-700 nm when excitation wavelength set at 571.2 nm. The maximum emission wavelength is 579.2 nm.

Parameter set:

Band width 5.0 for excitation and emission

Sodium cholate concentration 50.0 μmole/litre

Ordinary X 1

## CALIBRATION CURVE PARAMETERS

| | Ex (nm) | Em (nm) |
|---|---|---|
| FIXED: | 571.2 | 579.2 |
| FACTORS: | | |
| BAND WIDTH: | EX = 1.5 | EM = 3.0 |
| X-AXIS: LINEAR | | Y-AXIS: LINEAR |

CALIBRATION CURVE

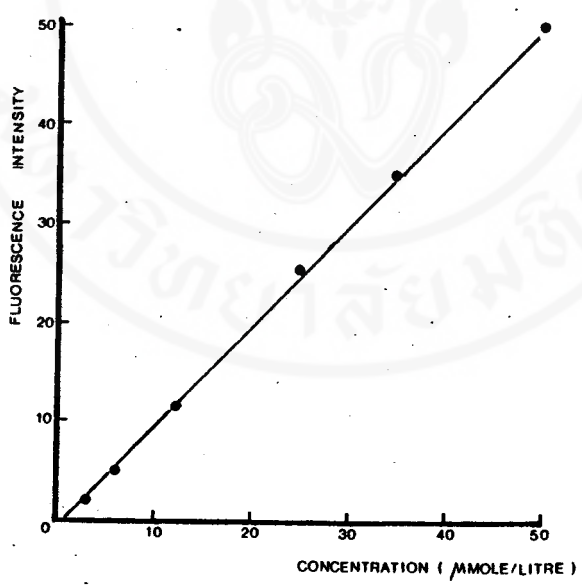


Figure 14 The parameter and calibration curve for determination total bile acid

1.2. Incubation time determination in inactivation of HEX isozyme.

To confirm heat inactivation method by O'Brien and Okada, two normal and three patient subjects were seperated in five parts, and determined HEX activity by varying incubation time 0, 1, 2, 3 and 4 hours respectively. Heat in activation curves for serum hexosaminidase at 50°c were constructed (Figure 15), the resulting curves were similar to the original heat inactivation of HEXA (30). The three hours of incubation time was sufficiency in the experiment.

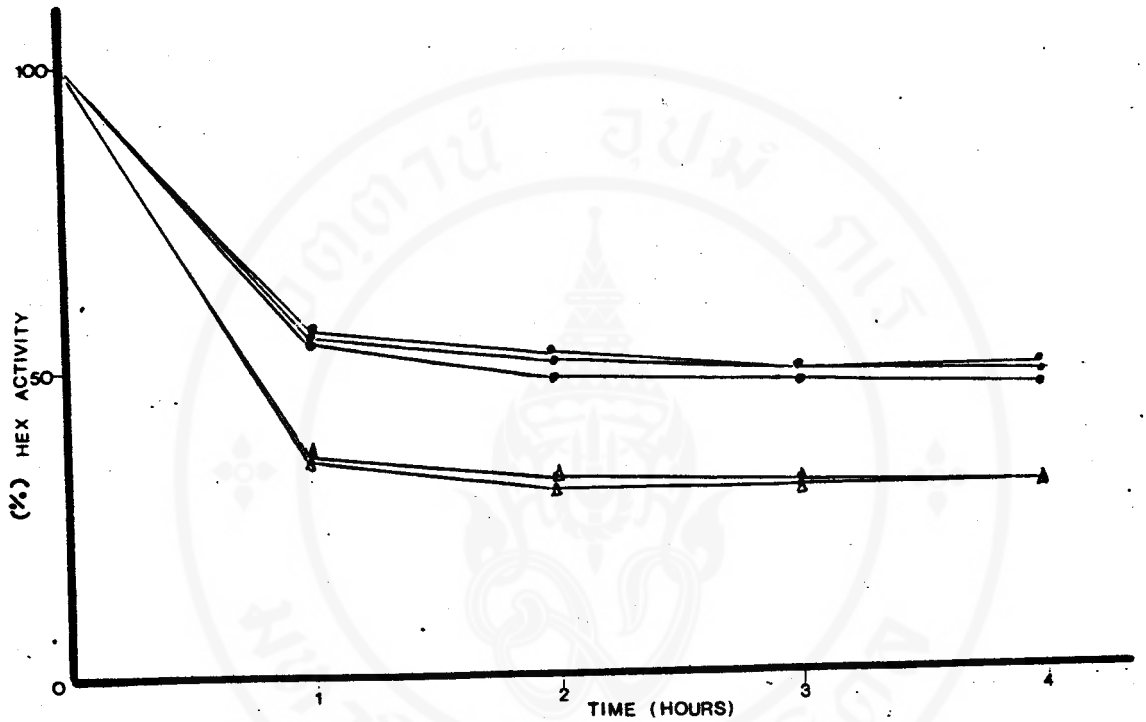


Figure 15 Heat-inactivation curve of serum HEX at 50 °c in three patient subjects (●) and two normal subjects.(Δ)

1.3. Precision and Recovery determination.

To evaluate the precision of the method, within-run and between-run were determined. The five and six of high, middle and low control serum for hexosaminidase and total bile acid respectively were investigated. The values of mean, standard deviation and coefficient (% CV) were calculated and demonstrated in Table 5 and 6.

The percent recovery of hexosaminidase and bile acid were determined by adding different concentration of 4-methylumbelliferone and sodium chlorate. The good agreement between the resulting for the amounts added and values found that depicted in Table 7 and 8.

**Table 5 The within-run precision of hexosaminidase and total bile acid in human serum**

| Intra assay | Total HEX(U/min) | | | HEXB (U/min) | | | Total bile acid (μmole/l |
|---|---|---|---|---|---|---|---|
| | H | M | L | H | M | L | |
| N | 5 | 5 | 5 | 5 | 5 | 5 | 6 |
| $\bar{X}$ | 82.2 | 54.6 | 20.4 | 42.9 | 28.2 | 9.2 | 16.4 |
| SD | 0.1 | 0.5 | 0.6 | 0.6 | 0.4 | 0.3 | 0.6 |
| % CV | 0.2 | 0.9 | 2.9 | 1.3 | 1.5 | 2.7 | 3.6 |

Table 6 The between-run precision of hexosaminidase and total bile acid in human serum

| Inter assay | Total HEX(U/min) | | | HEXB (U/min) | | | Total bile acid(μmole/l) | | |
|---|---|---|---|---|---|---|---|---|---|
| | H | M | L | H | M | L | H | M | L |
| N | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 6 | 6 |
| $\bar{X}$ | 63.1 | 43.5 | 16.1 | 33.2 | 22.4 | 7.1 | 37.7 | 16.1 | 3.3 |
| SD | 1.8 | 2.1 | 1.1 | 1.1 | 1.3 | 0.6 | 2.6 | 1.1 | 0.3 |
| %CV | 2.8 | 4.7 | 6.9 | 3.3 | 5.9 | 8.9 | 7.4 | 7.0 | 9.5 |

Table 7 Percent recovery of hexosaminidase that different amounts of 4-methylumbelliferone added to human serum

| Initially hexosaminidase (U/min) | 4-methylumbelliferone (U/min) | Measure (U/min) | Difference (U/min) | Percent recovery |
|---|---|---|---|---|
| 7.2 ± 0.3 (n=5) | 20.8 | 29.4 ± 1.9 | 22.2 ± 1.7 | 106.6 ± 8.1 |
| 7.1 ± 0.5 (n=5) | 41.7 | 52.6 ± 1.2 | 45.4 ± 0.8 | 109.1 ± 2.0 |

Table 8 Percent recovery of different amounts of sodium cholate added to human serum

| Initially total bile acid (μmole/l) | Sodium cholate added (μmole/l) | Measure (μmole/l) | Difference (μmole/l) | Percent recovery |
|---|---|---|---|---|
| 6.2 (n=3) | 12.5 | 17.0 ± 1.8 | 10.7 ± 1.8 | 85.6 ± 1.4 |
| 6.2 (n=3) | 25 | 25.5 ± 0.4 | 19.3 ± 0.4 | 77.2 ± 1.2 |
| 6.2 (n=3) | 50 | 45.8 ± 0.2 | 39.5 ± 0.2 | 79.0 ± 0.5 6 |

2. **SUBJECTS:**

The value (mean ± S.D.) of total HEX, HEXB, HEXA HEXA/HEXB ratio, percent HEXB and total bile acid in normal, control and patient subjects are summarized in Table 9. In the part of normal subject, the values of all parameters except total bile acid were compared between normal serum and normal fasting serum. The results showed no difference in enzyme activity. Moreover, the comparison between normal and control subjects showed the same results. On the other hand, all parameters in patient subject (obstructive jaundice) showed difference from normal and control subjects. The statistically significant increasing in total HEX, HEXB, HEXA and percent HEXB when compared to normal subjects ($P < 0.001$) and control subjects ($P < 0.05$). The HEXA/HEXB ratio showed decreasing when compared to both groups ($p < 0.01$).

The two group of obstructive jaundice patients (benign and malignant biliary obstruction), the values were shown in Table 9. Total HEX, HEXB and HEXA increased in malignant biliary obstruction, but the statistical significant, at $P < 0.05$, all parameter in malignant biliary obstruction were not statistical significant difference from benign biliary obstruction. Where as the P-values of total HEX = 0.095, HEXB = 0.107 and HEXA = 0.13 .

Figure 16-20 illustrated the scattergram of total HEX, HEXB, HEXA, HEXA/HEXB ratio and percent HEXB in five groups of human serum (normal subject, control subject, obstructive jaundice patient subjects, benign biliary obstructive patients and malignant biliary obstructive patients). Figure 21 showed total bile acid in three group (normal, control and patient subjects). The statistically comparison

difference between group and subgroup of subject were depicted in Table 10.

In according to tissue diagnosis (signature in section number) from pathologist, the values of total HEX, HEXB, HEXA, HEXA/HEXB ratio and percent HEXB in each patient of malignant and benign biliary obstructive patients were listed in Table 11 and 12.

The values for hexosaminidase and total bile acid were compared by Pearson product moment correlation test. In 95 normal fasting subjects showed reverse relationship, but the 17 patient fasting subjects indicated the significant, at $P < 0.05$, in total HEX and HEXA. The correlation coefficient and P-value were summerized in Table 13. The correlation curve of total HEX and HEXA versus total bile acid in patient subject were shown in Figure 22 and 23.

Table 9 The values of, mean($\bar{X}$) and standard deviation(SD) of hexosaminidase in normal, control and patient subjects.

| Parameter / Subject | NO. | Total HEX (μ/min) | HEXB (μ/min) | HEXA (μ/min) | HEXA/HEXB ratio | HEXB percent | Total bile acid(μmole/l) |
|---|---|---|---|---|---|---|---|
| NORMAL SUBJECTS | 120 | 18.5 ± 3.5 | 6.3 ± 1.9 | 12.1 ± 2.0 | 2.1 ± 0.6 | 33.7±5.6 | - |
| SERUM | 25 | 17.1 ± 3.7 | 5.9 ± 2.0 | 11.2 ± 2.1 | 2.1 ± 0.8 | 33.6±6.7 | - |
| FASTING SERUM | 95 | 18.8 ± 3.3 | 6.4 ± 1.8 | 12.4 ± 1.9 | 2.0 ± 0.5 | 33.8±5.3 | 7.1 ± 3.8 |
| CONTROL SUBJECTS (FASTING SERUM) | 49 | 20.8 ± 5.6 | 9.0 ± 3.8 | 11.8 ± 3.7 | 1.5 ± 0.7 | 42.8 ±11.4 | 6.8 ± 4.3 |
| OBSTRUCTIVE JAUNDICE PATIENT | 107 | 38.9 ± 14.6 | 18.9 ± 9.8 | 20.1 ± 6.5 | 1.2 ± 0.5 | 46.9 ±9.5 | - |
| SERUM | 90 | 38.5 ± 14.1 | 19.1 ± 9.7 | 19.5 ± 5.9 | 1.2 ± 0.5 | 47.8 ±9.7 | - |
| FASTING SERUM | 17 | 40.9 ± 17.2 | 17.7 ± 9.8 | 23.2 ± 8.4 | 1.4 ± 0.4 | 42.2 ±7.2 | 41.5 ± 29.1 |
| BENIGN BILIARY OBSTRUCTION | 10 | 31.9 ± 11.5 | 15.1 ± 10.4 | 16.8 ± 7.5 | 1.4 ± 0.6 | 44.9 ± 9.7 | - |
| MALIGNANT BILIARY OBSTRUCTION | 25 | 41.2 ± 13.1 | 20.3 ± 7.5 | 20.8 ± 6.8 | 1.1 ± 0.3 | 49.1 ± 6.8 | - |

Table 10 The P-value of comparison in groups (normal, control and patient subjects), and subgroups (malignant and benign biliary obstruction)

| Comparison in group | Total HEX | HEXB | HEXA | HEXA/HEXB ratio | HEXB percent |
|---|---|---|---|---|---|
| NORMAL SUBJECTS(n=120) VS PATIENT SUBJECTS(n=107) | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| CONTROL SUBJECTS(n=49) VS PATIENT SUBJECTS(n=107) | 0.000 | 0.000 | 0.000 | 0.008 | 0.032 |
| MALIGNANT PATIENT(n=25) SUBJECTS VS BENIGN PATIENT SUBJECTS(n=10) | 0.095 | 0.107 | 0.180 | 0.182 | 0.107 |

Table 11 The hexosaminidase in malignant biliary obstruction patients.

| Section number | Diagnosis | Age | Total HEX u/min | HEX B u/min | HEX A u/min | HEXA/HEXB ratio | HEXB percent |
|---|---|---|---|---|---|---|---|
| 4670-33 | Cholangiocarcinoma | 38 | 42.5 | 22.9 | 19.6 | 0.9 | 53.9 |
| 8101-33 | Cholangiocarcinoma | 40 | 56.3 | 27.2 | 20.0 | 1.1 | 48.4 |
| 1809-33 | Cholangiocarcinima | 37 | 34.0 | 17.6 | 16.4 | 0.9 | 51.8 |
| 41597-33 | Cholangiocarcinoma | 49 | 42.9 | 22.1 | 20.8 | 0.9 | 51.5 |
| 6728-33 | Cholangiocarcinoma | 40 | 33.2 | 17.0 | 16.2 | 0.9 | 51.2 |
| 899,941-33 | Cholangiocarcinoma | 38 | 26.6 | 12.3 | 14.3 | 1.2 | 46.3 |
| 6764-33 | Cholangiocarcinoma | 55 | 31.0 | 16.3 | 14.7 | 0.9 | 52.6 |
| 2377-33 | Cholangiocarcinoma with chronic cholecystitis | 54 | 52.1 | 24.0 | 28.1 | 1.2 | 46.0 |
| 6881-33 | Cholangiocarcinoma with chronic cholecystitis | 60 | 22.4 | 10.8 | 11.6 | 1.1 | 48.2 |
| 1140-33 | Cholangiocarcinoma with cholecystitis | 65 | 23.7 | 13.3 | 10.5 | 0.8 | 55.9 |
| 1693-33 | Cholangiocarcinoma with cholecystitis | 47 | 31.1 | 12.1 | 19.0 | 1.6 | 39.0 |
| 5927-33 | Cholangiocarcinoma,met | 46 | 53.9 | 25.2 | 28.6 | 1.1 | 46.8 |
| 7517-33 | Cholangiocarcinoma,met | 62 | 77.8 | 44.0 | 33.8 | 0.8 | 56.6 |
| 4721-33 | Carcinoma of gall bladder | 60 | 32.7 | 20.0 | 12.8 | 0.6 | 61.0 |
| 0321-33 | Carcinoma of gall bladder | 75 | 38.4 | 18.0 | 20.4 | 1.1 | 46.9 |
| 0411-33 | Carcinoma of gall bladder,met | 59 | 48.1 | 26.4 | 21.6 | 0.8 | 55.0 |
| 1372-33 | Carcinoma of gall bladder,met | 48 | 28.3 | 13.6 | 14.8 | 1.1 | 47.9 |

Table 11 (conti.)

| Section number | Diagnosis | Age | TOTAL HEX u/min | HEX B u/min | HEX A u/min | HEXA/HEXB ratio | HEXB percent |
|---|---|---|---|---|---|---|---|
| 2759-33 | Carcinoma of common bile duct | 56 | 38.8 | 21.8 | 17.0 | 0.8 | 56.3 |
| 7707-33 | Carcinoma of common bile duct with chronic cholecystitis | 67 | 32.7 | 10.2 | 22.4 | 2.2 | 31.3 |
| 5842-33 | Carcinoma of common bile duct with chronic cholecystitis | 56 | 39.4 | 19.1 | 20.3 | 1.1 | 48.4 |
| 6388-33 | Carcinoma of common bile duct with chronic cholecystitis | 65 | 60.6 | 22.5 | 38.1 | 1.7 | 37.2 |
| 12293-32 | Carcinoma of pancrease,met | 40 | 51.5 | 26.4 | 25.2 | 1.0 | 51.2 |
| 0363-33 | Carcinoma of pancreatic duct | 73 | 55.9 | 32.2 | 23.7 | 0.7 | 57.7 |
| 11951-33 | Carcinoma of periampullary | 50 | 38.7 | 17.3 | 21.5 | 1.2 | 44.6 |
| 98844-33 | Carcinoma of ampulla of vater | 45 | 37.2 | 16.3 | 20.9 | 1.3 | 43.7 |

Table 12 The hexosaminidase in benign biliary obstructive patients

| Section number | Diagnosis | Age | TOTAL HEX u/min | HEX B u/min | HEX A u/min | HEXA/HEXB ratio | HEXB percent |
|---|---|---|---|---|---|---|---|
| 10690-32 | Cyst of head of pancreas with chronic cholecystitis | 76 | 15.8 | 8.1 | 7.7 | 1.0 | 51.3 |
| 11988-32 | Common bile duct stone with chronic cholecystitis | 57 | 32.7 | 16.2 | 16.5 | 1.0 | 49.4 |
| 12676-32 | Common bile duct stone with chronic cholecystitis | 67 | 11.9 | 4.4 | 7.5 | 1.7 | 37.0 |
| 0690-33 | Common bile duct stone with chronic cholecystitis | 45 | 34.3 | 13.3 | 21.0 | 1.6 | 38.7 |
| 12861-32 | Common bile duct stone with chronic cholecystitis | 24 | 16.2 | 4.4 | 11.8 | 2.6 | 27.4 |
| 1452-33 | Common bile duct stone with chronic cholecystitis | 47 | 20.8 | 6.7 | 14.1 | 2.1 | 32.3 |
| 1778-33 | Common bile duct stone with chronic cholecystitis | 71 | 71.3 | 38.1 | 33.2 | 0.9 | 53.4 |
| 6769-33 | Common bile duct stone with chronic cholecystitis | 75 | 33.6 | 16.1 | 17.5 | 1.1 | 47.9 |
| 10330-33 | Common bile duct stone with chronic cholecystitis | 68 | 41.5 | 22.9 | 18.6 | 0.8 | 55.2 |
| 10210-33 | Common bile duct stone with chronic cholecystitis | 28 | 40.8 | 21.0 | 19.9 | 0.9 | 51.3 |

Table 13 The correlation coefficient(r) and p-value(p) of total bile acid and hexosaminidase in fasting serum of normal and patient subjects.

| Subject | NO | | Total | HEXB | HEXA | HEXA/HEXB | Percent HEXB |
|---|---|---|---|---|---|---|---|
| NORMAL | 95 | r | -0.229 | -0.223 | -0.184 | -0.150 | -0.143 |
| | | p | 0.013 | 0.015 | 0.037 | 0.073 | 0.083 |
| PATIENT | 17 | r | 0.511 | 0.348 | 0.644 | 0.261 | -0.271 |
| | | p | 0.018 | 0.086 | 0.003 | 0.156 | 0.146 |

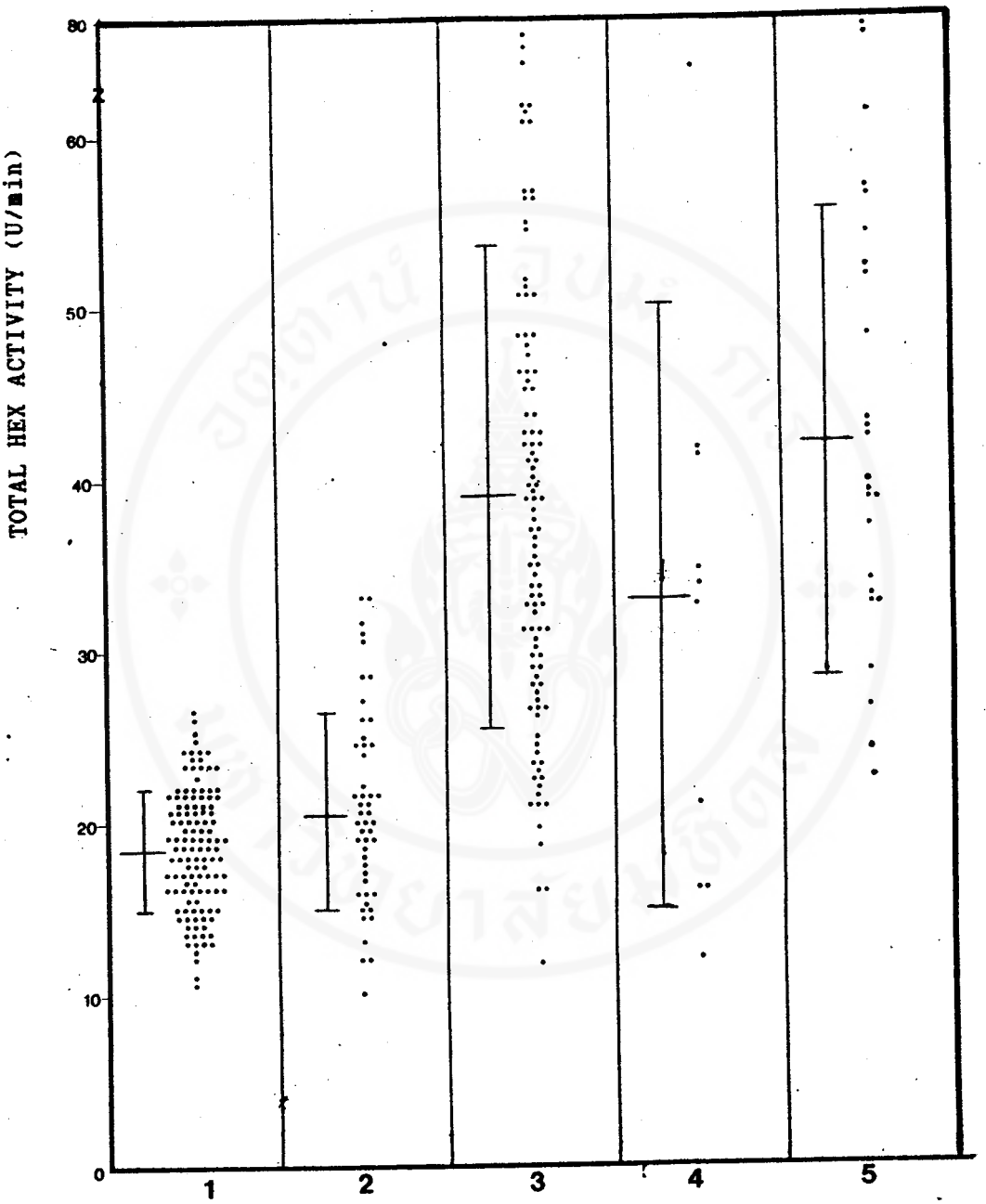


Figure 16 Scattergram of total HEX activity in human serum

1 = Normal subjects (n=120)

2 = Control subjects (n=49)

3 = Obstructive jaundice patient subjects (n=107)

4 = Benign biliary obstructive patients (n=10)

5 = Malignant biliary obstructive patients (n=25)

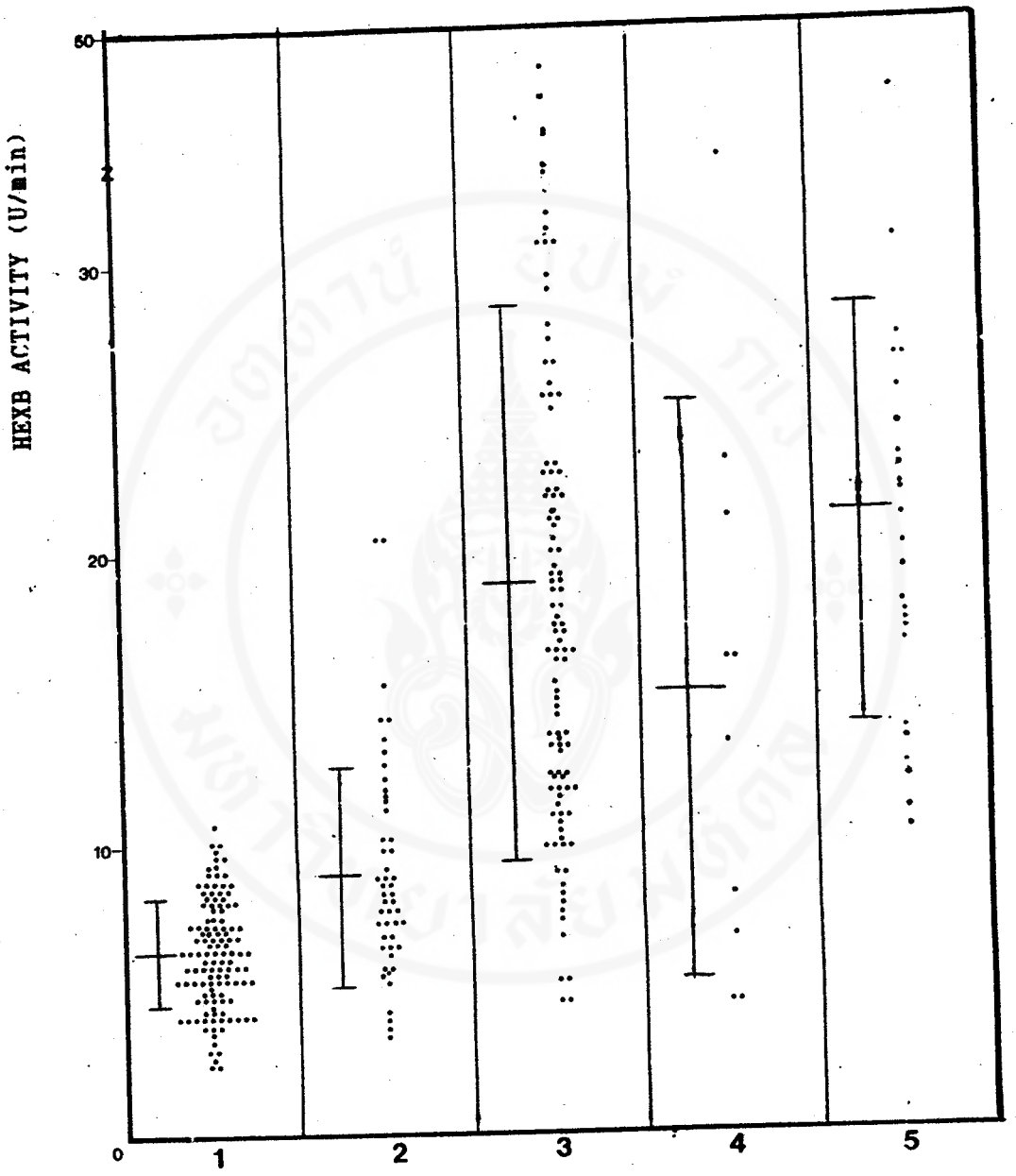


Figure 17 Scattergram of HEXB activity in human serum

1 = Normal subjects (n=120)

2 = Control subjects (n=49)

3 = Obstructive jaundice patient subjects (n=107)

4 = Benign biliary obstructive patients (n=10)

5 = Malignant biliary obstructive patients (n=25)

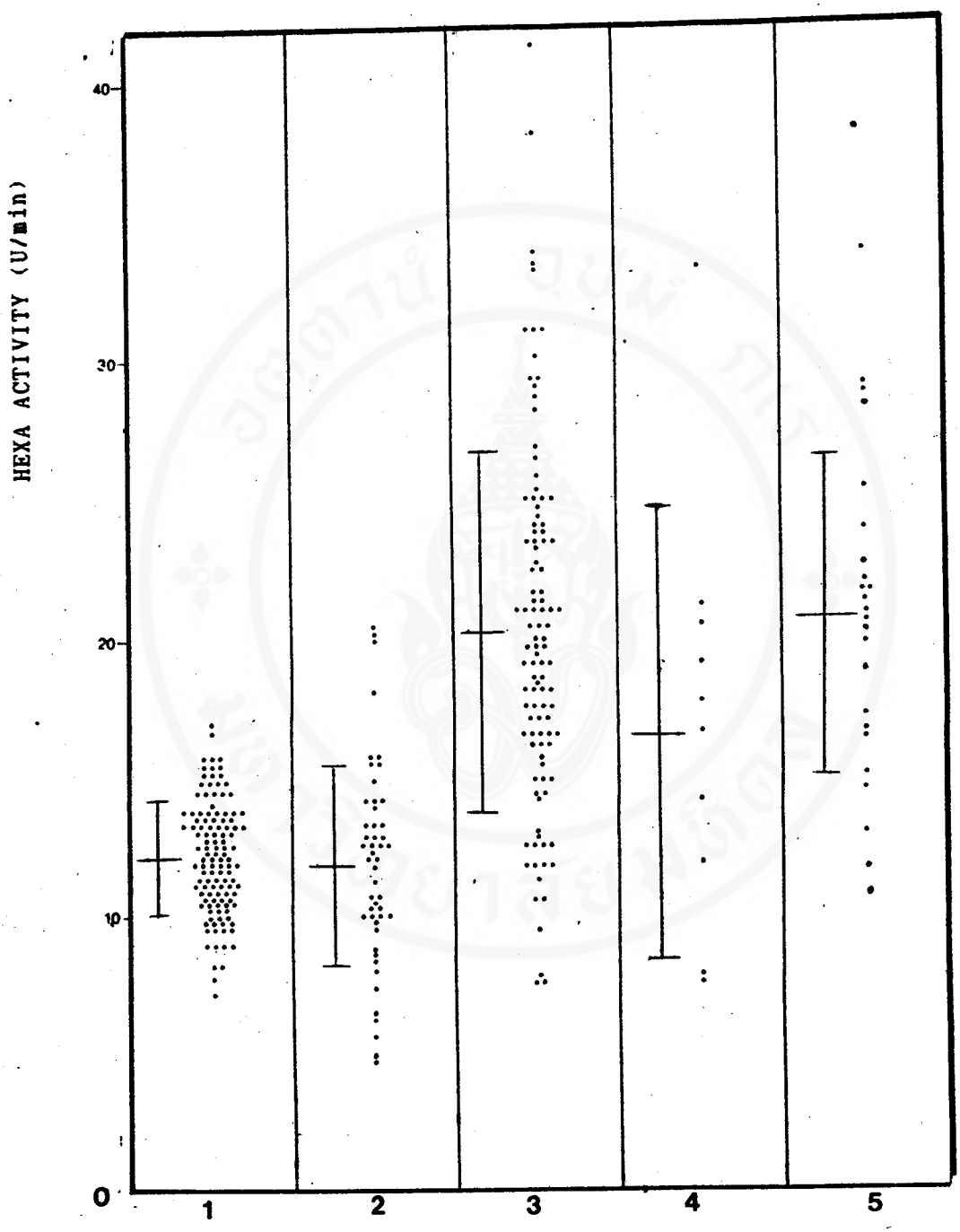


Figure 18 Scattergram of HEXA activity in human serum

1 = Normal subjects (n=120)

2 = Control subjects (n=49)

3 = Obstructive jaundice patient subjects (n=107)

4 = Benign biliary obstructive patients (n=10)

5 = Malignant biliary obstructive patients (n=25)

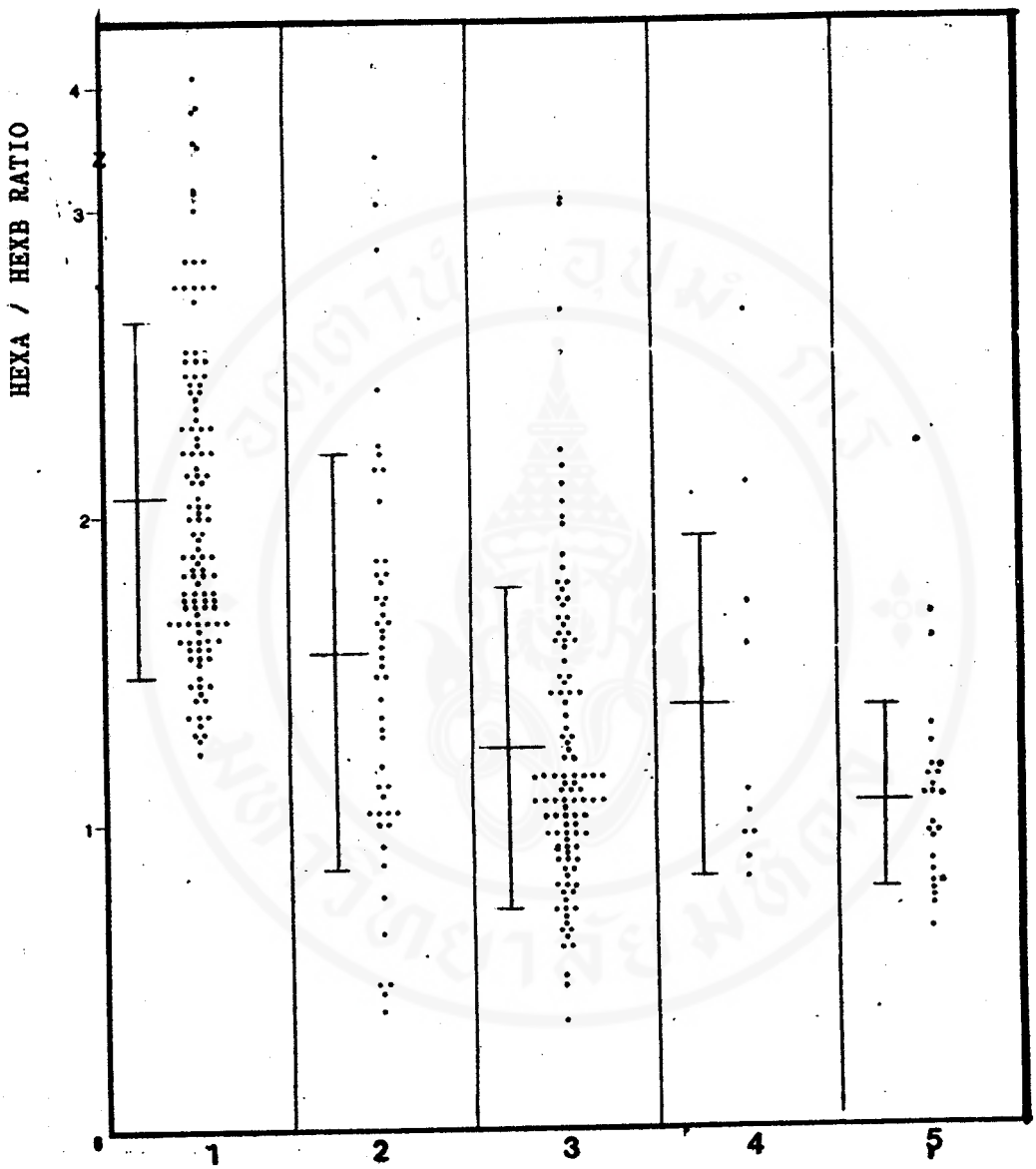


Figure 19 Scattergram of HEXA/HEXB ratio in human serum

1 = Normal subjects (n=120)

2 = Control subjects (n=49)

3 = Obstructive jaundice patient subjects (n=107)

4 = Benign biliary obstructive patients (n=10)

5 = Malignant biliary obstructive patients (n=25)

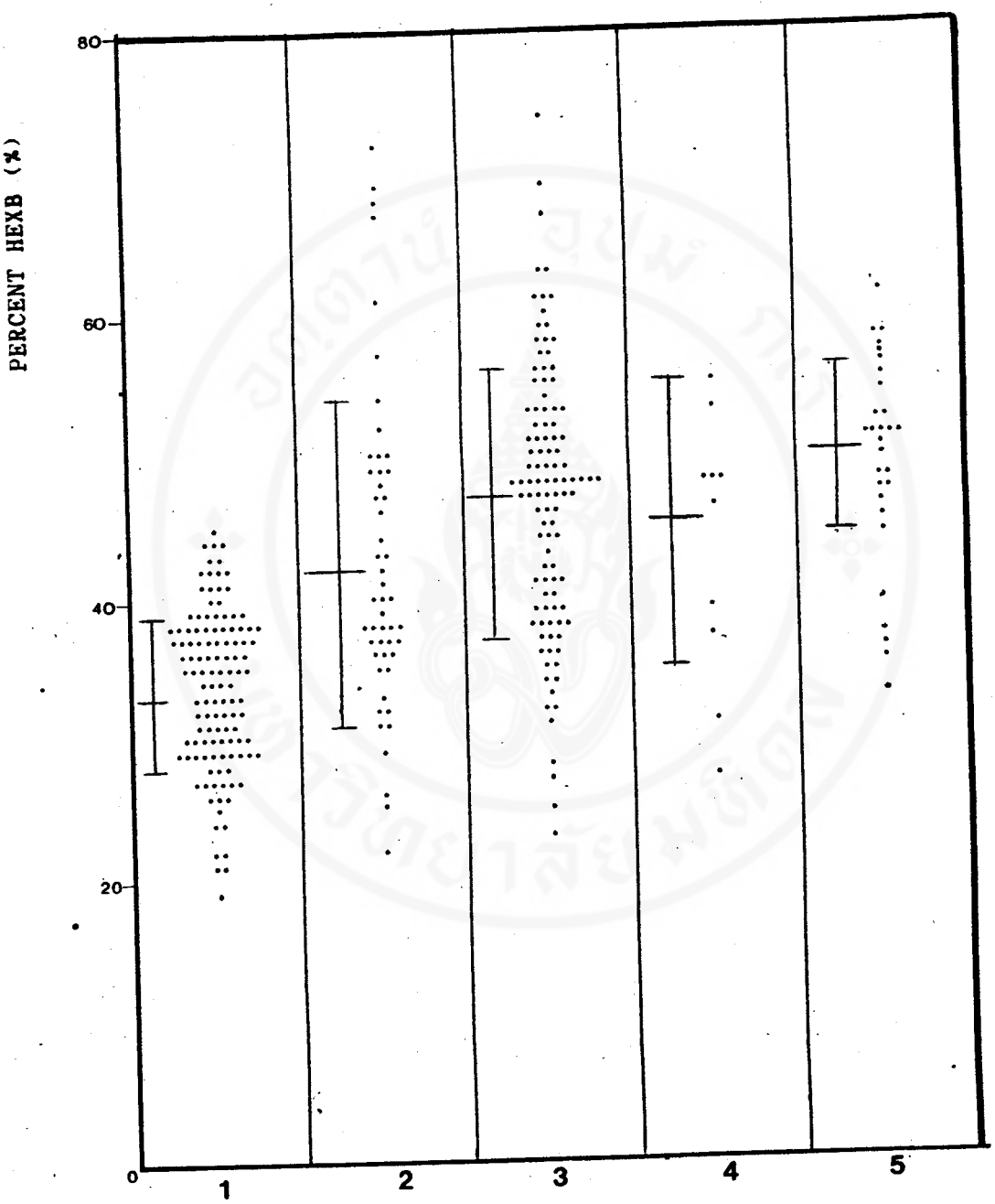


Figure 20 Scattergram of percent HEXB in human serum

1 = Normal subjects (n=120)

2 = Control subjects (n=49)

3 = Obstructive jaundice patient subjects (n=107)

4 = Benign biliary obstructive patients (n=10)

5 = Malignant biliary obstructive patients (n=25)

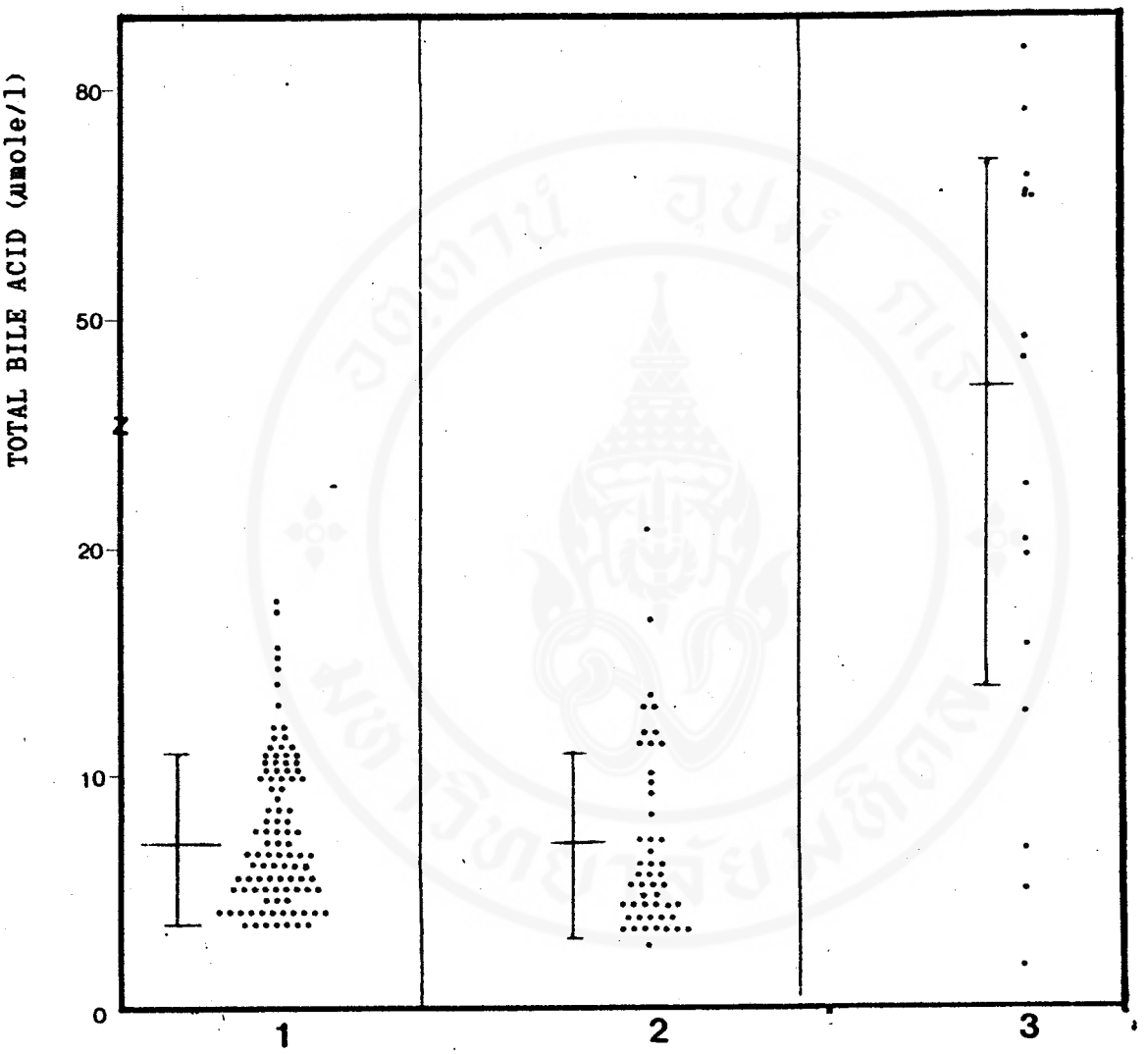


Figure 21 Scattergram of total bile acid in human serum

1 = Normal fasting subjects (n=95)

2 = Control fasting subjects (n=49)

3 = Patient fasting subjects (n=17)

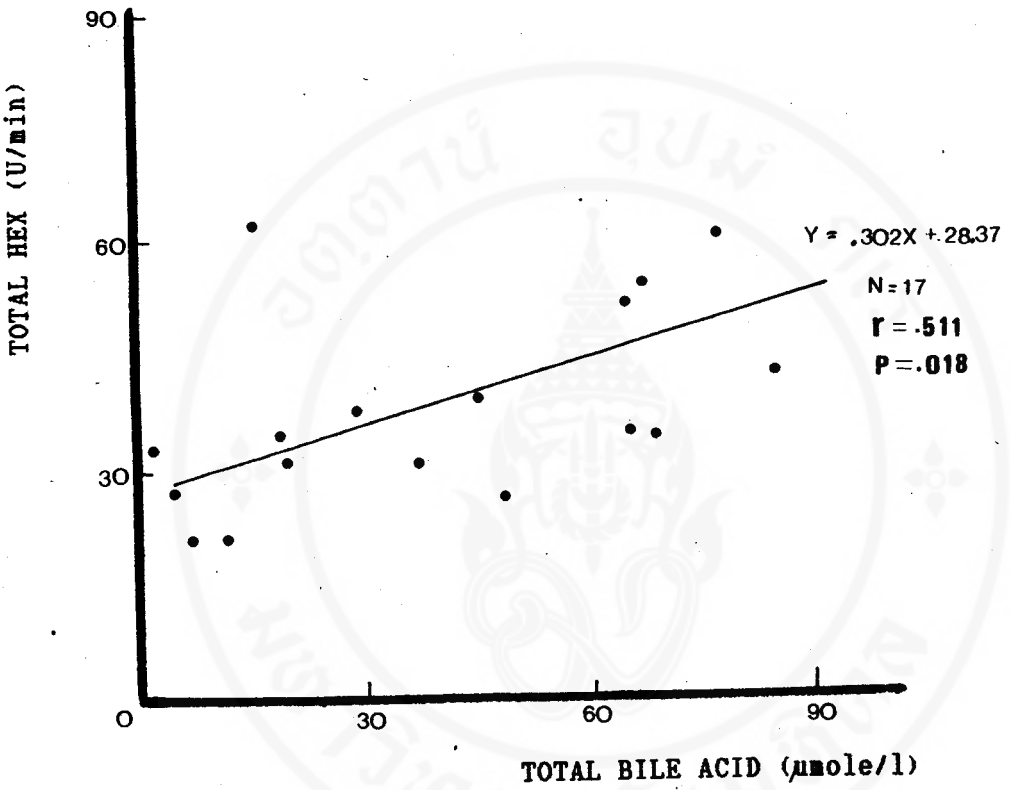


Figure 22 The correlation curve between Total HEX and total bile acid in obstructive jaundice patients

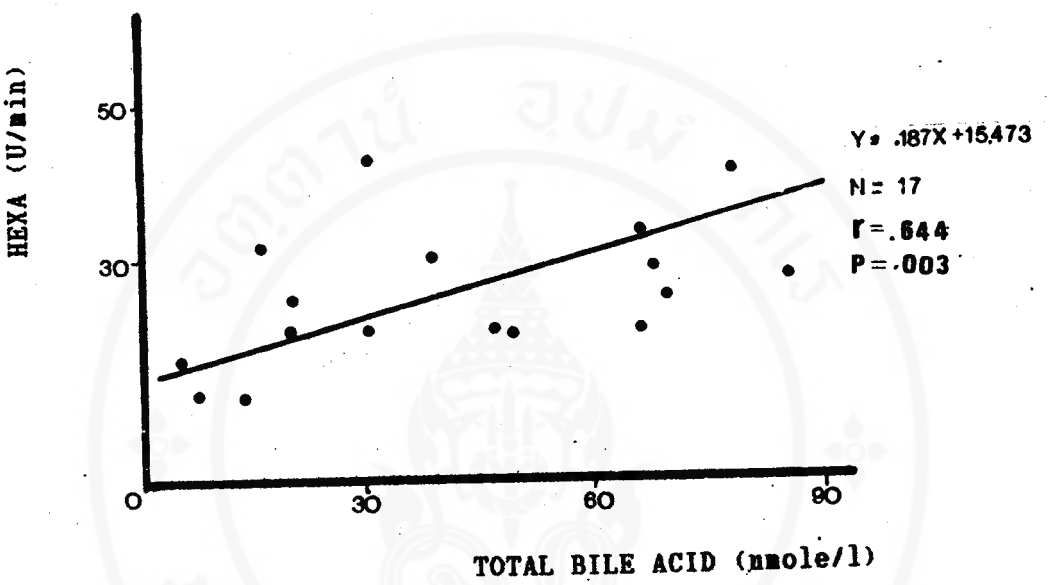


Figure 23 The correlation curve between HEXA and total bile acid in obstructive jaundice patients.

# CHAPTER IV

# DISCUSSION

The heat inactivation method was applied to determine the HEXA and HEXB isozymes in serum because the easiest and most reproduceable procedure (30,101,102). O'Brien and Okada (30) used differential diagnosis in serum of Tay-Sachs disease, and found that heating serum at 50°c for one hour; 95% or more of the activity of HEXA and 5% or less of the activity of HEXB were inactivated. Then, the values obtained were ±10% of the actual activity for each component. The important problems of colorimetric measurement were the detection limit, interference from colored serum (especially in obstructive jaundice serum) and the clouded solution from denature protien (7). The fluorometric detection by spectrofluorophotometer (Shimadzu RF-5000) is applicable because it is easy to use, high sensitivity and not give rise to such problems.

The previous study, Hultberg et al (10) compared the levels of total HEX activity in the circulation of normal subjects with liver diseases. The highest values of total HEX were found in his cholestasis group, which included patients with malignant extrahepatic biliary obstruction, choledocholithiasis, atresia of the biliary ducts and intrahepatic cholestasis due to metastasis of breast carcinoma (10). They suggested that clearance by the non-parenchymal cells was reduced because they had lost contact with much of the portal blood (10). Moreover, Scapa et al (89) showed the serum HEX level could discriminate between malignant and benign biliary obstruction. However, elevated HEX activity has been associated with

various tumors. High concentration of HEX have been reported in serum of patients with other malignancies were found to be higher than in normal control (101). The other studies have shown differences in HEX isoenzymes in patients with colonic cancer (71).

In the present study we have measured total HEX, isozymes (HEXA and HEXB) activity, HEXA/HEXB ratio and percent HEXB. The results (comparison in patient and normal subjects) showed the significant elevation of total HEX, HEXA, HEXB, and percent HEXB but depicted the significant decreasing of HEXA/HEXB ratio. It indicated that only total HEX could distinguish the liver diseases (obstructive jaundice) from normal. On the other hand, the two groups of malignant and benign biliary obstructive patients were compared. The values of total HEX, HEXB, HEXA, HEXA/HEXB ratio and percent HEXB showed no statistically significant different at $P < 0.05$.

The cause of the elevated HEX levels seen in biliary obstructed patient; interference with plasma clearance is more likely (91). Many glycoprotiens are rapidly cleared by the mamalian liver by process that appear to depend on specific hepatic glycoprotien recognition system. Ashwell and co-workers (64) have identified a hepatic receptor (in hepatocyte) that mediated the rapid clearance of galactoseterminated glycoprotien from the plasma. A second hepatic recognition system (in sinusoidal cells) that mediated the rapid clearance of glycoprotien with terminal N-acetylglucosamine or mannose residues has been described (82). Steer et al (84) experiment in rats showed that HEX (particular HEXA) is cleared from circulation by sinusoidal cells (especially Kupffer cells). Thomas et al

(87) suggested that elevated level of bile acids in the circulation or in hepatocytes may produce changes in the membranes of cell (including Kupffer cells) by acting as detergents and disrupting membrane-bound receptor function. Then, the increasing levels of bile acids in the obstructive jaundice patients, therefore, may reflect to a combination of Kupffer cells damage, diminished excretory function and portal systemic shunting (91). The results from this study shows predominant statistically correlation ($P < 0.05$) between total HEX and HEXA versus total serum bile acid levels in the liver diseases (obstructive jaundice) and HEXB mildly correlation ($r=0.348$, $P=0.086$). This correlation (especially in HEXA) still supports the idea that elevated bile acids can effect the Kupffer cell membranes and its specific receptor, although other factors may also be involved.

The possible reason why HEX levels higher in patients with malignant obstruction than in benign obstruction diseases is the longer duration of the obstruction (89). It is difficult to determine the duration of obstruction in the long-standing obstructed patients since a partial obstruction may have been present for an indeterminate length of time. Bilirubin levels provide some indication but, in general, the patients with malignant obstruction has obstruction of longer standing than those with common bile duct stones. Furthermore, confirmation by experiments in rats showed increases plasma levels of HEX following ligation of the bile duct, and levels varied directly with the duration of the obstruction (103). Another reason, possibility is that the tumor itself may be responsible for the elevated HEX levels. For example, HEX activities are elevated in ovarain adenocarcinoma tissue compared to normal ovary

(74). Extracts of human colonic carcinoma demonstrate a higher proportion of the isozymes HEXB than HEXA, while normal colonic mucosa contained a higher proportion of the A form of the enzyme (71). Lo and Kritchevsky (104) showed that in patients with solid tumors that the activity of both major HEXA and B were significantly greater in the malignant group than in the sera of healthy volunteers or patients with non-malignant ailments (HEXB>HEXA). Corresponding to the results that the mean $\pm$ SD values of HEX activity in malignant biliary obstruction were higher than in benign biliary obstruction. However, at $P < 0.05$, they were not statistically significant increasing.

# CHAPTER IV

# CONCLUSION

1. In the liver diseases (obstructive jaundice), the hexosaminidase activity and isozymes predominantly increased higher than in normal and control subjects ($P < 0.001$).

2. The differential comparison between benign and malignant biliary obstructive patients showed that the total HEX , HEXB and HEXA in malignant biliary obstruction were not statistically significant higher than benign biliary obstruction at $P < 0.05$.

3. The significant correlation between hexosaminidase with total bile acid illustrated in total HEX ($r = .511$, $P = 0.018$) and HEXA ($r = .644$, $P = .003$) and little correlation in HEXB.

# BIBLIOGRAPHY

1. Price RG, Dance N. The demonstration of multiple heat stable forms of acetyl β-glucosaminidase in normal human serum. Biochem Biophys Acta 1972; 271: 145-153.

2. Isaksson A, Hultberg B. Immunoassay of β-hexosaminidase isoenzymes in serum in patients with raised total activities. Clin Chim Acta 1989; 183: 155-162.

3. Isaksson A, Hultberg B, Sundler R, Akesson B. Uptake of β-hexosaminidase by nonparenchymal liver cells and peritoneal macrophages. Enzyme 1983; 40: 18-24.

4. Alhadeft JA, Prorok JJ., Dura PA, Plucinsky MC, Beesburg RY. Atypical β-hexosaminidase in sera of cancer patients with liver metastases. Cancer Res 1984; 44: 5422-5426.

5. Plucinsky MC, Prorok JJ, Alhadeff JA. Variant serum β-hexosaminidase as a biochemical marker of malignancy. Cancer 1986; 58: 1484-1487.

6. Alhadeft JA, Holzinger RT. Presence of an atypical thermolabile species of β-hexosaminidase B in metastatic-tumor tissue of human liver. Biochem J 1982; 201: 95-99.

7. Mahuran D, Novak A, Lowden JA. The lysosomal hexosaminidase isozymes. Isozymes. Curr Top Biol Med Res 1985; 12: 229-228.

8. Flowers HM, Sharon N. Glycosidases-properties and application to the study of complex carbohydrates and cell surface. Adv Enzymol 1979; 48: 29-95.

9. Zuhlsdorf M, Imort M, Hasilik A, Figura KV. Molecular forms of β-hexosaminidase and cathepsin D in serum and urine of healthy subjects and patients with elevated activity of lysosomal enzymes. Biochem J 1983; 213: 733-740.

10. Hultberg B, Isaksson A, Jansson L. β-Hexosaminidase in serum from patients with cirrhosis and cholestasis. Enzyme 1981; 26: 296-300.

11. Pampols T, Codina J, Giros M, Sabater J, Gonzales-Sastre F. Tissue differences in human N-acetyl-β-D-hexosaminidase isoenzymatic forms. Cell Mol Biol incl Cyto-Enzymol 1980; 26: 187-95.

12. Geiger B, Arnon R. Chemical characterization and subunit structure of human N-acetylhexosaminidase A and B. Biochemistry 1976; 15: 3484-3493.

13. Mahuran D, Lowden JA. The subunit and polypeptide structure of hexosaminidases from human placenta. Can J Bicohem 1980; 58: 287-294.

14. Tallman JF, Brady R, Cernik JM, Villaba M, Gal AE. Isolation and relationship of human hexosaminidase. J Bid Chem 1974;

249: 3489-3499.

15. Geiger B, Calef E, Arnon R. Biochemical and immunochemical characterization of hexosaminidase P. Biochemistry 1987; 17: 1713-1717.

16. Srivastava SK. Yoshida A, Awasthi YC, Bentler E. Studies on human β-D-N-acetylhexosaminidases II: Kinetic and structural properties. J Biol Chem 1974 b; 249: 2049-2053.

17. Beutler E, Yoshida A, Kuhl W, Lee JE. The subunits of human hexosaminidase A. Biochem J 1976; 159: 541-543.

18. Isaksson A, Hultberg B, Masson P, Low K, Sandgren E, Lundblad A. Enzyme immunoassay of β-hexosaminidase isoenzymes using monoclonal antibodies. Scand J Clin Lab Invest 1989; 49: 597-603.

19. Hultberg B, Isaksson A, Joelsson B, Alwmark A, Gullstrand P, Bengmarks S. Pattern of serum β-hexosaminidase in liver cirrhosis. Scand J Gastroentero 1983: 18: 877-880.

20. Vigano A, Assael B, Villa A, et al. N-acetyl β-D-glucosaminidase, (NAG) and NAG isoenzymes in children with upper and lower urinary tract infections. Clin Chim Acta 1983; 130: 297-304.

21. Whiting P, Patcrsen J, Power D, Stewart R, Catto G, Edward N. Diagnostic value of urinary N-acetyl-β-D-glucosaminidase, its isoenzymes and fractional excretion of sodium following renal transplantation. Clin Chim Acta 1983; 130: 369-376.

22. Calvo P, Barba JL, Cabezas JA. Serum β-N-acetyl-glucosaminidase, β-D-glucosidase, α-D-glucosidase, β-D-fucosidase, α-L-fucosidase and β-D-galactosidase levels in acute vital hepatitis, pancreatitis, myocardial infarction and breast cancer. Clin Chim Acta 1982; 119: 15-19.

23. Reglers A, Carretero IM, Cabezas JA. Increased serum α-L-fucosidase and β-N-acetylglucosaminidase activities in diabetic, cirrhotic and gastric cancer patients. Clin Chim Acta 1980; 103: 155-158.

24. Ellis RB, Rapron NT, Patrick AD, Greaves MF. Expression of hexosaminidase isoenzymes in childhood leukemia. New Engl J Med 1978; 298: 467-480.

25. Tanaka T, Kubayashi M, Saito O, Kamada N, Kuramoto A, Usui T. Hexosaminidase isoenzyme profile in leukemia cells. Clin Chim Acta 1983; 128: 19-28.

26. Beutler E. The biochemical genetics of the hexosaminidase system in man. Am J Hum Genet 1979; 31: 95-105.

27. Novak A, Lowden JA. Kinetics of rat liver $GM_2$ ganglioside β-D-N-acetylhexosaminidase. Can J Biochem 1980; 58: 82-88.

28. Okada S, O'Brien JS. Tay-Sachs disease: Generalized absence of a β-D-N-acetylhexosaminidase component. Science 1969; 165: 698-700.

29. Srivastava SK, Awasthi YC, Yoshida A, Beutler E. Studies on human β-D-N-acetylhexosaminidase I: Purification and properties. J Biol Chem 1974a; 249: 2043-2048.

30. O'Brien JS, Okada S, Chen A, Fillerup DL. Tay-Sachs disease: Detection of heterozgotes and homozygotes by serum hexosaminidase assay. N Engl J Med 1970; 283: 15-20.

31. Aruna RM, Basu D. Purification and properties of brain N-acetyl-β-hexosaminidase. A J Neurochem 1975; 25: 611-617.

32. Maruhn D. Rapid colorimetric assay of β-galactosidase and N-acetyl-β-glucosaminidase in human urine. Clin chim Acta 1976; 73: 453-461.

33. Woollen JW, Heyworth R, Walker PG. Studies on glucosaminidase 3: testicular N-acetyl-β-glucosaminidase and N-acetyl-β-galactosaminidase. Biochem J 1961; 78: 111-116.

34. Yuen C-T, Price RG, Chattagoon L, Richardson AC, Praill PFG. Colorimetric assays for N-acetyl-β-D-glucosaminidase and

ß-D-galactosidase in human urine using newly developed W-nitrostyryl substrates. Clin Chim Acta 1982; 124: 195-204.

35. Noto A, Ogawa Y, Mari S, et al. Simple, rapid spectrophotometry of urinary N-acetyl-ß-D-glucosaminidase, with use of a new chromogenic substrate. Clin Chem 1983; 29: 1713-1716.

36. Kanfer J, Spielvogel C.. Hexosaminidase activity of cultured human skin fibroblast. Biochem Biophys Acta 1973; 203-207.

37. Pope AJ, Mian N, Herries DG. Kinetic evidence for two types of active site in the N-acetyl-ß-D-hexosaminidase of bovine kidney. FEBS Lett 1978; 93: 174-176.

38. Pugh D, Leaback DH, Walker PG. Studies on glucosaminidase: N-acetyl-ß-glucosaminidase in rat kidney. Biochem J 1957; 65: 464-469.

39. Robinson D, Stirling JL. N-acetyl-ß-glucosaminidases in human spleen. Biochem J 1968; 107: 321-327.

40. Pugh D, Walker PG. The localization of N-acetyl-ß-glucosaminidase in tissues. J Histochem Cytochem 1961a; 9: 242-250.

41. Pugh D, Walker PG. Histochemical localization of ß glucoronidase and N-acetyl-ß-glucosaminidase. J Histochem Cytochem 1961b; 9: 105-106.

42. Hayashi M. Histochemical demonstration of N-acetyl-β-glucosaminidase employing naphol AS-BI-N-acetyl-β-glucosaminide as substrate. J Histochem Cytochem 1965; 13: 355-360.

43. Poenaru L, Dreyfus JC. Electrophoretic study of hexosaminidase: hexosaminidase C. Clin Chim Acta 1973; 43: 439-442.

44. Li S-C, Hirabayashi Y, Li Y-T. A protein activator for the enzymic hydrolysis of $GM_2$ ganglioside. J Biol Chem 1981a; 256: 6234-6340.

45. Tallman JF, Brady RO, Quirk JM, Villalba M, Gal AE. Isolation and relationship of human hexosaminidases. J Biol Chem 1974; 249: 3489-3499.

46. Conzelmann E, Sandhoff K. Purification and characterization of an activator protein for the degradation of glycolipid $GM_2$ and $GA_2$ by hexosaminidase A. Hoppe-Seyler's Z Physiol Chem 1979; 360: 1837-1849.

47. Applegarth DA, Bozoian G. Mucopolysaccharide storge in organs of a patient with Sandhoff's diseases. Clin Chim Acta 1972; 39: 269-271.



48. Schachter H. Glycoprotein biosynothesis and processing. In Callahan JW, Lowden JA (eds): "Lysosomes and Lysosomal storage disases." New York: Raven Press, 1981: 73-93.

49. Bearpark T, Stirling JL. Clearance of human N-acetyl-β-hexosaminidase from rat circulation. Biochem J 1977; 168: 435-439.

50. Tomasi LG, Fukushima DK, Kolodny EH. Steroid hexosaminidase activity in Tay-Sachs • and Sandhoff-Jatzkewitz diseases. Neurology 1974; 24: 1158-1165.

51. O'Dowd BF, Cumming DA, Gravel RA, Mahuran D. Oligosaccharide structure and amino acid sequence of the major glycopeptides of mature human β-hexosaminidase. Biochemistry 1988; 27: 5216-5226.

52. O' Dowd BF, Mahuran DJ, Cumming D, Lowden JA. Characterization by nuclear magnetic reasonance of the concarnavalin A binding oligosaccharide on the Bb chain of paiental β-hexosaminidase B: letin binding to the separated polypeptide chains of hexosaminidase A and B. Can J Biochem Cell Biol 1985; 63: 723-729.

53. De Duve C, Wattiaux R. Function of lysosomes. Ann Rev Physiol 1966; 28: 435-92.

54. Goldstone A, Koenig H. Biosynthesis of lysosomal glycoproteins in rat kidney. Life Sci 1972; 11: 511-523.

55. Hasilik A, Neufeld EF. Biosynthesis of lysosomal enzyme in fibroblasts: Synthesis as precursor of higher molecular

weight. J Biol Chem 1980; 255: 4937-4945.

56. Hasilik A, Von Figura K. Oligosaccharides in lysosomal enzymes: Distribution of high mannose and complex oligosaccharide in cathepsin D and β-hexosaminidase. Eur J Biochem 1981; 121: 125-129.

57. Gieselmann V, Pohlmann R, Hasilik A, Von Figura K. Biosynthesis and transport of cathepsin D in cultured human fibroblasts. J Cell Biol 1983; 1: 1-5.

58. Freeze H, Geiger B, Miller AL. Carbohydrate composition of human placenta N-acetyl-hexosaminidase A and B. Biochem J 1979; 177: 749-752.

59. Lowden JA. Serum β-hexosaminidases in pregnancy. Clin Chim Acta 1979; 93: 409-417.

60. Ikonne JU, Ellis RB. N-acetyl-β-D-hexosaminidase component A. Different forms in human fissues and fluids. Biochem J 1973; 135: 457-462.

61. Tucker SM, Pierce RJ, Price RG. Characterization of human N-acetyl-β-D-glucosaminidase as an indicator of tissue damage in disease. Clin Chim Acta 1980; 102: 29-40.

62. Van Elsen AF, Leroy JG. Chromatographic components of β-hexosaminidase in I-cell disease (mucolipidosis II). Hum Genet

1979; 47: 305-317.

63. Nakagawa S, Kumin S, Nitowsky M. Human hexosaminidase isozymes: Chromatographic separation as an oid to heterozyogote identification. Clin Chena Acta 1977; 75: 181-191.

64. Ashwell G, Morell A. The role of surface Carbohydrates in the hepatic recognition and transport of circulatng glycoproteins. Adv Enzymol Relat Areas Mol Biol 1974; 41: 99-128.

65. Stahl P, Schlesinger P, Rodman JS, Doebber T. Recognition of lysosomal glycosidases in vivo is inhibited by medifies glycoprotein. Nature 1976; 264: 86-88.

66. Stahl PD, Schlesinger PH. Receptor-mediated pinocytosis of mannose/N-acetyl-glucosamine terminal glycoproteins and lysosomal enzymes by macrosphages. Trends Biol Sci 1980; 5: 194-203.

67. Neufeld EF, Lim TW, Shapiro LJ. Inherited disorders of lysosomal metabolism. Annu Rev Biochem 1975; 44: 357-376.

68. Alhadeff JA, Holzinger RT. Characterization of β-hexosaminidase from liver and sera of diabetic patient and controls. Biochem Med 1982; 27: 214-225.

69. Whiting PH, Nicholls AJ, Catto GRD, Edward N, Engeset J. Pattern of N-acetyl-β-D-glycosaminidase excretion after renal

transplantation. Clin Chem Acta 1980; 108: 1-7.

70. Sorenson AW, Rosenshein N, Tepper SA, Kritchevsky D. Serum hexosaminidase as a possible biomarker for human cancer. J Med 1984; 15: 409-415.

71. Brattain MG, Kimball PM, Durant JR, Pretlow TG. β-Hexosaminidase isozymes in human colonic carcinoma. Cancer Res 1977; 37: 1833-1837.

72. Bosmann HB, Hall TC. Enzyme activity in invasive tumors of human breast and colon. Proc Natl Acad Sci U.S.A. 1974; 71: 1833-1837.

73. Okochi T, Seike H, Higashino K, et al. Alteration of hexosaminidase isozymes in human renal carcinoma. Cancer Res 1979; 39: 1829-1834.

74. Chatterjee SK, Bhattacharya M, Barlow JJ. Glycosyltransferase and glycosidase activities in ovarian cancer patients. Cancer Res. 1979; 39: 1943-1951.

75. Chatterjee SK, Chowdhury K, Bhattacharya M, Barlow JJ. Beta-hexo-saminidase activities and isoenzymes in normal human ovary and ovarian adenocarcinoma. Cancer 1982; 49: 128-135.

76. Whitehurst GB, Mashburn JP, Pretlow TG, Bradley EL, Boohaker EA. Prostatic hexosaminidase activity in patients with

benign prostatic hyperplasia and prostatic carcinoma. Cancer Res 1982; 42: 4300-4303.

77. Kimball PM, Brattain MG, White WE. Characterization of an unusual isoenzyme of N-acetyl-β-D-hexosaminidase from a human colonic carcinoma cell line Biochem J 1981; 193: 109-113.

78. Narita M, Taniguchi N, Makita A, Kodama T, Araki E, Oikawa K. Elevated activity of β-hexosaminidase and sulfhydryl modification in the B-variant of human lung cancer. Cancer Res 1983; 43: 5037-5042.

79. Varani J, Orr W, Ward PA. Hydrolytic enzyme activities, migratroy activity, and invivo growth and metastatic potential of recent tumor isolates. Cancer Res 1979; 39: 2376-2380.

80. Warren L, Buck CA, Tuszynski GP. Glycopeptide changes and malignant transformation. A possible role for carbohydrate in malignant behavior. Biochim Biophys Acta 1978; 516: 97-127.

81. Narita M, Taniguchi N, Makita A, Kodama T, Araki E, Oikawa K. Elevated activity of β-hexosaminidase and sulfhydryl modification in the B-variant of human lung cancer. Cancer Res 1983; 43: 5037-5042.

82. Schlesinger PH, Doebber TW, Mandell BF, et al. Plasma clearance of glycoprstenis with terminal mannose and N-acetyl-glucosamine by liver non-parenchymal cells. Biochem J 1978; 176: 103-109.

83. Hubbard AL, Wilson G, Ashwell G, Stukenbiok H. An election microscope autoradiographic study on the carbohydrate recognition systems in rat liver. J Cell Biol 1981; 83: 47-64.

84. Steer CJ, Kusiak JW, Brady RO, Jones EA. Selective hepatic uptake of human β-hexosaminidase A by a specific glycoprotein recognition system on sinusoidal cells. Proc Natl Acad Sci USA 1979; 76: 2774.

85. Wolter J, Lichr H, Grun M. Hepatic clearance of endotoxins: differences in arterial and portal venous infusion. J reticuloendoth Soc 1978; 23: 145-152.

86. Cooksley WGE, Powell LW, Holliday JW. Reticuloendothelial phagocytic function in human liver diseases and its relationship to haemolysis. Br J Haemat 1973; 25: 147-164.

87. Thomas P, O'Neil PF, Zamcheck N. Inhibition of specific Kupffer cell clearance of carcinoebryonic antigen from the serum of rats with bile duct obstruction. Biochem Soc Trans 1982; 10: 459-460.

88. Hultberg B, Isaksson A. A possible explanation for the occurance of increased beta-hexosaminidase activity in pregnancy serum. Clin Chim Acta 1981; 113: 135-140.

89. Scapa E, Thomas P, Loewenstein MS, Zamcheck N. Serum β-N-acetyl hexosaminidase (β-NAH) as a discriminant between malignant and benign extrahepatic biliary obstruction: Comparison with carcinoembryonic antigen (CEA). Eur J Cancer clin oncol 1985; 21: 1037-1042.

90. Hultberg B, Braconier JH, Isoksson A, Jansson L. β-Hexosaminidase level in serum from patients with viral hepatitis as a measure of reticuloendothelial function. Scand J Infect Dis 1981; 13: 241-245.

91. Scapa E, Novis BH, Loewenstein MS, Thomas p, Zamcheck N. Serum β-N-acetyl hexosaminidase and bile acid levels in patients with benign and malignant biliary obstructon. Dig Dis Sci 1988; 33: 189-192.

92. อรุณ เผ่าสวัสดิ์. ศัลยกรรมทางเดินน้ำดี. กรุงเทพ: ยูนิตี้พับลิเคชั่น, กรุงเทพเวชสาร, 2529: 118-119.

93. Bruce FS. Jaundice. In: Gastrointestinal diseases, vol 1. Edited by Morvin HS, John SF, 4 th ed. W.B. Saunders CO., 1989: 454-467.

94. Popper H, Schaffner F. Pathophysiology of cholestasis. Hum Pathol 1970: 1: 1-24.

95. Popper H, Schaffner F. Cholestasis. In: Bockus Gastroenterology, vol 5. Edited by berk JE. 4 the ed. W.B. Saunders Co., 1985: 2697-2731.

96. Schoenfield LJ. Diseases of the gall bladder and biliary system. New York: John Wiley and Sons, 1977. 105. Dworken HJ. Gastroenterology: pathophysiology and clinical applications. London: Butterworth Inc., 1982: 369-389.

97. Dworken HJ. Gastroenterology: pathophysiology and clinical applications. London: Butterworth Inc., 1982: 369-389.

98. Sherlock S. Diseases of the liver and biliary system. 6 th ed. Great Britain: Blackwell Scientific Publication, 1981: 194-205.

99. Mashige F, Imai K, Osuga T. A simple and sensitive assay of total serum bile acids. Clin Chim Acta 1976; 79: 79-86.

100. Molloy HT, Evelyn KA. The determination of bilirubin with the photoelectric colorimeter. J Biol Chem 1937; 119: 481-490.

101. Lo CH, Kritchevsky D. Physical properties of human serum hexosaminidase A and B: Studies in normal and cancer

patients. Clin Chim Acta 1979; 95: 105-112.

102. Dance N, Price RG, Robinson D. Differential assay of human hexosaminidase A and B. Biochem Biophys Acta 1970; 222: 662-664.

103. Thomas P, O'Neil PF, Zamcheck N: Relationship between CEA clearance rates and serum β-N-acetylhexosaminidase in the rat with biliary duct obstruction. Hepatology 1982; 2: 674.

104. Lo CH, Kritchevsky D. Human serum hexosaminidase elevated B from isoenzyme in cancer patients. J Med 1978; 9: 313-330.