PSEUDOCHOLINESTERASE AND ITS RELATIONSHIP TO SERUM LIPOPROTEINS IN DIABETES MELLITUS

ANNAPURNA VENKATAKRISHNAN

PSEUDOCHOLINESTERASE AND ITS RELATIONSHIP TO SERUM LIPOPROTEINS IN DIABETES MELLITUS

By

©Annapurna Venkatakrishnan

A thesis submitted to the School of Graduate Studies
in partial fulfillment of the
requirements for the degree of
Master of Science

Faculty of Medicine
Memorial University of Newfoundland
January 1990

St. John's Newfoundland Canada

ABSTRACT

The activities of pseudocholinesterase (PChE) were compared in the serum of type I and type II diabetic patients and non-diabetic subjects. Both type I and type II diabetic patients showed increased serum PChE activity concomitant with the presence of hyperlipidemia. Pearsons correlation analysis showed a positive correlation between triglycerides (TG) and PChE in both type I (P=0.499) and type II (P=0.526) diabetic patients.

Induction of chemical diabetes in rats with Streptozotocin (SZ) and alloxan also increased the serum PChE activity which paralleled the development of diabetes mellitus. Serum triglycerides, apo-B containing lipoproteins (total low density lipoproteins TLDL) and glycerol concentrations also increased. When the hyperglycemia produced in streptozotocin induced diabetic rats was controlled by administration of insulin, the levels of TG, glycerol and PChE activity all reverted to pre-diabetic levels. When insulin treatment was withdrawn hyperglycemia developed again and serum levels of PChE activity, TG, glycerol and TLDL also increased. The concentration of TG in the liver of SZ-diabetic rats was significantly higher compared to the normal rats. Adipose tissue and liver PChE activity were not significantly different in these SZ-diabetic rats.

Gold thioglucose (GTG) treated mice, which are type II diabetic models, also showed a significant increase in serum PChE activity and lipids similar to the SZ induced type I diabetic models. Inhibition of PChE activity in GTG treated mice was associated with a decrease in serum glycerol, TG and

TLDL levels. Liver PChE activity was markedly higher in the GTG treated mice compared to the untreated control mice. Administration of Iso-OMPA to GTG treated mice inhibited both liver and adipose tissue PChE activity.

Heparin treatment of SZ-diabetic rats significantly reduced serum TG levels without affecting PChE activity, cholesterol, HDL-C or TLDL levels.

Administration of Iso-OMPA, a specific PChE inhibitor, to SZ-diabetic and normal rats produced a significant reduction in serum TG, glycerol and TLDL levels. Withdrawal of iso-OMPA inhibition in the diabetic rats resulted in an increase in the lipid levels to those previously present in the diabetic state. PChE activity and TG levels were also reduced in the iso-OMPA treated diabetic and normal rat livers compared to the untreated rats. Adipose tissue PChE activity was also inhibited in the iso-OMPA treated diabetic and normal rat.

Inhibition of PChE activity with iso-OMPA failed to produce an accumulation of choline or cholinesters in the liver, serum or to affect their excretion in the urine of normal rats.

The results indicate that changes in PChE activity parallel changes in TG metabolism, particularly during the perturbances that accompany diabetes mellitus. Whether PChE has a role in very low density TG (VLDL-TG) metabolism remains to be determined but is of considerable interest.

ACKNOWLEDGMENTS

I express my sincere gratitude to my supervisor Dr.K.M.Kutty and my supervisory committee members Drs. A.J. Davis, I. Senciall and J. Curtis for their suggestions and advice during the entire period of the programme.

I am very grateful to the Faculty of Medicine of the Memorial University for the financial support and The Dr. Charles A. Janeway Child Health Centre for providing me the working facilities.

My special thanks to all the technologists and Barbara English (Secretary) at the Janeway Chemistry Laboratory for their assistance and endless encouragement. I wish to thank Dennis Traverse (technician) at the Animal Care in the Health Science Centre for the invaluable help he rendered with the animal experiments. I also extend my thanks to John Read (Consultant) Dept. of Computing Services, Henriette Harvey Building, Memorial University.

This work would not have been commenced or completed without the love, inspiration and tremendous encouragement of my dear parents. I am extremely thankful to them.

Contents

1 INTRODUCTION 1

1.1 Pseudocholinesterase 1

1.1.1 Multiple forms of PChE 2

1.1.2 Properties of Purified PChE 4

1.1.3 PChE Gene 5

1.1.4 Site of synthesis of human PChE 6

1.1.5 Physiological Function of PChE 7

1.1.6 PChE in lipid metabolism 7

1.1.7 PChE and Low Density Lipoprotein (LDL) 8

1.1.8 PChE in Obesity and Hyperlipoproteinemia 9

1.1.9 PChE in Food Assimilation 9

1.1.10 PChE in Diabetes 10

1.1.11 Other Proposed Functions of PChE 10

1.2 Diabetes 11

1.2.1 Chemistry of insulin 12

1.2.2 Biosynthesis of insulin 12

1.2.3 Mode of Action of insulin 13

1.3 Lipoproteins 15

1.3.1 Lipoprotein structure 15

1.3.2 Lipoprotein metabolism 18

1.4 Lipoprotein Metabolism In Diabetes Mellitus 24

1.4.1 Type II (NIDDM) 24

1.4.2 Type I (IDDM) 26

1.5 Animal Models of Diabetes 28

1.5.1 Type I Diabetic Models 28

1.5.2 Type II Diabetic Models 30

1.6 Objective of the present study 32

2 MATERIALS & METHODS **34**

2.1 Human Study 34

2.1.1 Subjects 34

2.1.2 Sample collection 35

2.2 Experimental Animals 35

2.2.1 Animals 35

2.2.2 Induction of Diabetes with SZ and Alloxan 36

2.2.3 Sacrifice of the animals 36

2.2.4 Homogenization of tissues 37

2.2.5 Extraction of lipids from liver 38

2.3 Biochemical Methods 38

2.3.1 Determination of PChE activity, in Serum, Liver and Adipose Tissue 38
2.3.2 Serum Glucose Estimation 41
2.3.3 Serum Triglyceride Estimation 42
2.3.4 Serum Glycerol Estimation 43
2.3.5 Serum Cholesterol Estimation 45
2.3.6 Serum High Density Lipoprotein Cholesterol Estimation (HDL-C) 46
2.3.7 Serum Total Low Density Lipoprotein (TLDL) Estimation 47
2.3.8 Estimation of Choline and Cholinesters in Serum, Liver, Adipose tissue and Urine 47
2.3.9 Thin Layer Chromatography (TLC) 49
2.3.10 Estimation of Serum C-peptide 51
2.3.11 Estimation of Serum Insulin 52
2.3.12 Estimation of Glycosylated Hemoglobin (HbA_1c) . . . 53
2.3.13 Electrophoretic Separation of Serum Lipoprotein 54
2.3.14 Statistics 58

3 RESULTS & DISCUSSIONS **59**

3.1 Human Studies 59
3.1.1 Serum Pseudocholinesterase activity in Type I and Type II Diabetic Patients 59
3.2 Animal Studies 65
3.2.1 Type I Diabetic Model 65

3.2.2 Effect of Insulin Treatment on Diabetic Rats 71

3.2.3 Effect of Iso-OMPA on Diabetic and Control Rats. . . 78

3.2.4 Effect of Heparin on PChE activity and Lipids in Diabetic Rats 91

3.2.5 Type II Diabetic Model 94

3.2.6 Effect of inhibition of PChE activity in normal rats . . 99

4 CONCLUSIONS **103**

5 REFERENCES **108**

List of Figures

1.1 General pathway of lipoprotein metabolism 23

3.1 Effect of insulin treatment and withdrawal on diabetic rats . . 74

3.2 Effect of insulin treatment and withdrawal on diabetic rats . . 75

3.3 Electrophoretogram of serum lipoproteins 77

3.4 Effect of iso-OMPA treatment and withdrawal on diabetic rats 85

3.5 Effect of inhibition of PChE activity with iso-OMPA in normal rats 86

3.6 Electrophoretogram of serum lipoproteins 88

3.7 Electrophoretogram of serum lipoproteins 90

4.1 Possible Biological Functions of PChE 107

List of Tables

1.1 Classification of diabetes mellitus according to WHO 16

1.2 Criteria for the diagnosis of diabetes NDDG vs WHO 17

1.3 Composition of plasma lipoproteins in man 21

1.4 Functions of apolipoproteins 22

3.1 Clinical characteristics of the patients 62

3.2 Comparison of serum values between Control and diabetic patients (NIDDM) 63

3.3 Comparison of serum values between Control and Type I diabetic patients (IDDM) 64

3.4 Comparison of serum values before and after induction of diabetes with Streptozotocin 68

3.5 Tissue levels of TG and PChE in control and diabetic rats . . 69

3.6 Comparison of serum values before and after induction of diabetes with Alloxan 70

3.7 Effect of two dose levels of iso-OMPA on diabetic rats 82

3.8 Tissue levels of TG and PChE in Diabetic and Iso-OMPA treated diabetic rats 83

3.9 Tissue levels of TG and PChE in Normal and Iso-OMPA treated rats 84

3.10 Comparsion between the serum values of Control & Diabetic and between Diabetic & Heparin and Saline treated Diabetic rats 93

3.11 Comparison between Control, GTG and GTG + iso-OMPA treated mice 97

3.12 Comparison of tissue PChE between control, GTG treated and GTG + iso-OMPA treated mice 98

3.13 Effect of inhibition of PChE activity on choline and cholinester levels in liver, serum and urine of rats 102

NOMENCLATURE

ACh	Acetylcholine
AChE	Acetylcholinesterase
BTC	Butyrylthiocholine
Chol	Cholesterol
C-pep	C-peptide
DTNB	5,5'dithiobis-(2-nitrobenzoic acid)
DFP	Diisopropylfluorophosphate
DN	Dibucaine number
FCR	Fractional catabolic rate
FFA	Free fatty acid
G-6-P	Glucose-6-Phosphate
GTG	Gold thioglucose
HbA_1c	Glycosylated hemoglobin
HDL-C	High density lipoprotein cholesterol
HDL	High density lipoprotein or α lipoprotein
HLA	Histocompatability antigen
HS Lipase	Hormone sensitive lipase
IDDM	Insulin dependent diabetes mellitus
IDL	Intermediate density lipoprotein
iso-OMPA	tetraisopropylpyrophosphoramide
LCAT	Lecithin cholesterol acyl transferase
LDL	low density lipoprotein or β lipoprotein

LDL-C	Low density lipoprotein cholesterol
LPL	Lipoprotein lipase
NEFA	Nonesterified fatty acid
NDDG	National Diabetes Data Group
NIDDM	Non-insulin dependent diabetes mellitus
PTC	Propionylthiocholine
PChE	Pseudocholinesterase
PEPCK	phosphoenolpyruvate carboxykinase
PFK	Phosphofructokinase
PK	Pyruvate kinase
SZ	Streptozotocin
TG	Triglyceride
TLDL	apo-B containing lipoproteins
VLDL	Very low density lipoprotein or pre β lipoprotein
VLDL-TG	Very low density lipoprotein triglyceride
WHO	World Health Organization

Chapter 1

INTRODUCTION

1.1 Pseudocholinesterase

Sir Henry Dale in the early ninteen hundreds was the first to suggest the presence of an enzyme in the blood capable of hydrolyzing acetylcholine into choline and acetic acid (Silver 1974). This view was confirmed over the years by several workers. Stedman *et al.* (1932) prepared the first crude extract of the acetylcholine hydrolyzing enzyme from horse serum and called it " Choline esterase". A few years later Alles and Hawes (1940) demonstrated the existence of two choline esterases in man: one in the erythrocytes and the other in the plasma. Subsequently, Mendel and Rudney (1943) showed that the serum choline esterase was a non-specific enzyme that hydrolyzed not only choline esters but also non-choline esters like tributyrin, tripropionin and methylbutyrate. This non-specific enzyme they called " Pseudocholinesterase". Thus these two investigators distinguished between the two cholinesterases as true cholinesterase

(acetylcholine hydrolase: EC.3.1.1.7: acetylcholinesterase AChE) and Pseudocholinesterase (acylcholine acylhydrolase: EC.3.1.1.8: PChE). AChE and PChE in vertebrates can be differentiated from other esterases by being inhibited by 10^{-5}M eserine and physostigmine. Furthermore PChE can be differentiated from "simple" esterases and AChE by the selective and irreversible inhibition with tetraisopropylpyrophosphoramide (iso-OMPA) (Aldridge 1953). In the sera of most mammals, cholinesterase activity is due solely to PChE (Silver 1974).

1.1.1 Multiple forms of PChE

The early work of Surgenor and Ellis (1954) suggested the PChE activity of human serum was due solely to a single protein. But Harris *et al.* (1962) resolved four bands of activity (termed C1-C4) in the region between α_2 and β-globulins using two-dimensional filter paper starch gel electrophoresis. In 5-10% of normal population a slow moving fifth band (C5) was observed (Juul 1968). C1 band moved the fastest and C4 the slowest. C4 was reported to be the major component as it contained 90-95% of the esterase activity found in the plasma. C4 had the highest molecular weight (340,000-365,000 daltons), the weights of the other fractions decreasing in the order C3 170,000; C2 110,000; and C1 82,000 (La Motta *et al.* 1970). Isoenzymes of PChE have also been detected in the horse serum (Reiner *et al.* 1965). The number of isoenzymes of PChE reported varies with the type of method employed for its separation. Hess *et al.* (1963) obtained 2 bands with acetylthiocholine as substrate for rabbit

serum on starch gel but no bands were detected with butyrylthiocholine (BTC) as substrate. Juul (1968) separated 12 activity bands from human serum on polyacrylamide disc electrophoresis with butyrylthiocholine and copper staining, while Das and Liddell (1970) using the same electrophoretic method but a different stain (α-naphthyl acetate) detected only 4 bands. La Motta *et al.* (1968, 1970) suggested that the different isoenzymes could be interconverted under experimental conditions, thus the multiple forms were just molecular aggregates of a common polypeptide subunit or degradation products (Lockridge and La Du 1982)

Several genetically determined variants of PChE have been found in man. These are the atypical PChE variants like (a) the dibucaine resistant, (b) fluoride resistant and (c) the silent-gene type. Homozygous for these variants suffer from prolonged apnea when administered succinylcholine (suxamethonium) (Silver 1974). These variants differ in their activity towards their substrates and inhibitors. The *atypical* or Dibucaine-resistant variant is unable to hydrolyze a number of substrates, including succinylcholine at a normal rate and is resistant to PChE inhibitors. This resistance is expressed in terms of Dibucaine number (DN) which is the % inhibition of the enzyme produced by dibucaine (10^{-5}M) (Kalow and Genest 1957). The homozygous for usual PChE have a DN of about 80. Those with a DN below 20 were reported homozygous for the *atypical* enzyme and a DN between 52-69 were heterozygous for the usual and *atypical* PChE. The DN number does not depend on the cholinesterase level and the degree of inhibition is constant for any one indi-

vidual. McGuire *et al.* (1989), demonstrated the replacement of aspartic acid by glycine at position 70 in the primary sequence of the usual PChE polypeptide. This substitution of a neutral amino acid for the acidic one may account for the reduced affinity of the "atypical" form for choline esters and dibucaine.

Harris and Whittaker (1961) identified a variant which had a low-fluoride number but a dibucaine number slightly lower than the normal. Its activity towards succinylcholine was greater than the dibucaine resistant but lower than the normal. This variant they called *fluoride-resistant.*

Liddell *et al.* (1962) reported a third variant. In this case the serum contained a protein very similar to normal PChE without any detectable enzyme activity. The gene responsible for such a protein was termed a *silent gene.*

1.1.2 Properties of Purified PChE

PChE has been purified from the following sources: human serum (Das & Liddell 1970, Muensch *et al.* 1976), horse serum (Lee & Harpst 1973, Main *et al.* 1974), rabbit liver (Rush *et al.* 1980) and porcine parotid gland (Tucci & Seifter 1969). PChE from bacterial sources have also been purified. Nagasawa *et al.* (1976) purified PChE from *Pseudomonas* and *P. polycolor.*

Most of the information about PChE has been obtained with purified human serum PChE. Highly purified human serum PChE has a molecular weight between 345,000 to 365,000 daltons and appears as a single band corresponding to the C4 band on polyacrylamide gel electrophoresis (Muensch *et al.* 1976, Das and Liddell 1970). Lockridge *et al.* (1979) suggested a tetrameric struc-

ture for PChE. It is a soluble, globular G4 protein with 4 identical subunits. The subunits are arranged as a dimer of dimers, with each dimer having identical subunits joined together by interchain disulfide bonds (Lockridge 1988). Each subunit has 574 amino acids and a molecular weight of 85,000 daltons (Muensch *et al.* 1976). The interchain disulfide bonds seem to stabilize the enzyme against heat inactivation and are not necessary for the tetrameric organization of the subunits or for the activity of the enzyme molecule.

1.1.3 PChE Gene

Two genetic loci determine PChE activity in the plasma. Locus E1 located on chromosome 3 controls most of the genetic variants, like the usual, atypical, fluoride resistant and the silent gene type (Brown *et al.* 1981). The second locus E2 controls the appearance of the C5 variant, the electrophoretically slow moving tetramer present in 10% of the caucasians (Whittekar 1986). Gene E_2 which determines the production of the C5 component of PChE is nonallelic to the E_1 gene of PChE (Harris & Robson 1963). Harris & Robson (1963) also reported that subjects whose serum contain the C5 component have 30% more plasma activity than others.

Liddell *et al.* (1963) investigated tissues from a patient with the "atypical" type of serum PChE and found that the tissue PChE was also similarly "atypical". Thus, these investigators believed that only one gene was responsible for the synthesis of the various isoenzymes. Human serum PChE has been sequenced and cloned. The amino acid sequence of PChE from the liver,

serum and brain are identical (Prody *et al.* 1987, McTiernan *et al.* 1987). The human embryonic and adult PChE also appear to be identical. This confirms that PChE has only one gene. Comparison of the amino acid sequence of human PChE and Torpedo AChE shows 54% homology. Human PChE has 38% sequence homology with Drosophilia AChE, 28% with thyroglobulin and 48% with electric eel AChE (Lockridge *et al.* 1987). There is evidence that monoclonal antibodies to human PChE do not cross react with red cell AChE or with rat PChE (Brimijoin *et al.* 1983).

1.1.4 Site of synthesis of human PChE

It is generally believed that in most species plasma PChE is synthesized in the liver (Silver 1974). Evidence for such a conclusion is based on the observation that low levels of PChE activity are associated with liver diseases (Antopol, Schrifrin & Tuchman 1938) and also with experimental liver damage (Brauer & Root 1946). Decreased levels of PChE was interpreted as a reflection of decreased protein synthesis or the functional state of the hepatocytes (Brown *et al.* 1981). Khoury *et al.* (1987) have also reported a change in the genetic character from the *atypical* to the normal form of PChE in the serum of a patient after liver transplantation. The amino acid sequence deduced from the cDNA clones showed that the liver and serum PChE were identical (McTierann *et al.* 1987, Prody *et al.* 1987).

1.1.5 Physiological Function of PChE

Though this enzyme has a ubiqitous distribution its exact physiological function is unclear. However, a number of possible roles have been put forth. The potential role of PChE in hyperlipoproteinemia and diabetes is of great importance and is the main subject of the present thesis.

Funnell & Oliver (1965) proposed that PChE is involved in the homeostatic mechanisms controlling the level of choline in plasma and acetylthiocholine in the brain. This proposal was made on the assumption that PChE hydrolyses cholinesters and thereby controls the plasma choline levels. But the majority of the cholinesters found in the plasma are in a bound form, like lecithin, lysolecithin and sphingomyelin. None of these are substrates for PChE (Kutty 1980). Thus the function of PChE in the homeostatic mechanism to regulate plasma choline is unclear.

1.1.6 PChE in lipid metabolism

Clitherow *et al.* (1963) proposed that PChE is involved in fatty acid metabolism in the liver. Ballantyne (1968) also proposed a similar function for the enzyme in the adipose tissue. This assumption was made on the fact that PChE hydrolysed butyrylcholine, a toxic compound that could be formed from butyrylCoA and choline. However, to date there is no evidence for the presence of water soluble acylcholine in serum or tissues of humans or animals. This is further supported by my finding that inhibition of PChE with iso-OMPA, a specific PChE inhibitor, did not cause accumulation of any acylcholine esters

either in the serum or liver nor excreted in the urine (Kutty, Annapurna & Prabakaran 1989).

1.1.7 PChE and Low Density Lipoprotein (LDL)

Lawrence and Melnick (1961) suggested that serum LDL forms an unstable complex with PChE by a physical interaction there by resulting in the enzyme being carried in an inactive form in the circulation. This hypothesis was supported by Dubbs (1966) who found an increase in serum PChE activity after ultrasonication of serum. However, Kutty, Rowden & Cox (1973) proposed that the association between PChE and LDL was very similar to the interaction between AChE and acetylcholine (ACh). They hypothesized that phosphorylcholine, the polar head group of phosphotidylcholine, like acetylcholine, can interact with the esteratic and anionic site of PChE, there by blocking its activity. Alternatively, Chu *et al.* (1978) reported that only 10% of the total serum PChE could be recovered from LDL, which accounts for only a fraction of the total PChE in circulation and thus does not assign a role for the remaining unbound PChE. Kutty *et al.* (1977) demonstrated that rats treated with neostigmine, a PChE inhibitor, showed a significant decrease in serum β-lipoprotein (LDL or low density lipoprotein), associated with a decreased incorporation of ^{3}H-lysine into β-lipoprotein but an increased incorporation into α-lipoprotein (HDL or high density lipoprotein). Thus, they proposed that LDL is formed from VLDL (very low density lipoprotein) in the presence of PChE. A similar close relationship between PChE and LDL was

found in the serum of a patient with parathion poisoning who had low levels of both. But during recovery both returned to normal with a concomitant decrease in HDL (Kutty *et al.* 1975).

1.1.8 PChE in Obesity and Hyperlipoproteinemia

Increased serum PChE activity is common in conditions associated with abnormal lipid metabolism like hyperlipoproteinemia (Cucuianu *et al.* 1975, 1976, Chu *et al.* 1978), obesity (Cucuianu *et al.* 1968) and diabetes (Antopol *et al.* 1937). The subjects studied also had elevated serum TG levels. Increased PChE activity was more common in persons with type IIb and type IV hyperlipoproteinemia (Kutty *et al.* 1981a, Jain *et al.* 1983).

Increase in PChE activity has been reported in animals. Experimentally fattened pigs showed an increased PChE activity (Popescu *et al.* 1976). Similarly Kutty *et al.* (1981b) found increased serum and liver PChE activity in genetically obese (ob/ob) mice. Lean mice treated with gold thioglucose (GTG) to induce obesity also exhibited increased PChE activity (Kutty *et al.* 1981b).

1.1.9 PChE in Food Assimilation

There have been reports that suggest the level of PChE activity may be influenced by the nutritional status. A significant decrease in PChE was reported by Henderson *et al.* (1971) in the serum and liver of rats when starved. Similarly, liver PChE activity was found to be depressed in malnourished children

but returned to normal with improved nutrition (Waterlow 1950).

1.1.10 PChE in Diabetes

In 1937, Antopol and associates detected an elevated PChE activity in the plasma of diabetics. This observation was later confirmed by Faber (1943). Kutty *et al.* (1981b) have demonstrated that genetically diabetic (db/db) mice have higher serum and liver PChE activities than lean controls. Deshmukh (1986) reported an increase of about 75% PChE activity over the pre-diabetic values in rats made diabetic with alloxan with a concomitant increase in cholesterol (LDL+VLDL) and decreased HDL-cholesterol level.

1.1.11 Other Proposed Functions of PChE

It has been postulated that PChE has more than one function as indicated by its presence in several tissues. Salvador & Kuntzman (1965) found PChE in the adipose tissue. Ballantyne (1968) reconfirmed this in the fat cells of the adipose tissue with histochemical and biochemical methods that employed specific substrates and inhibitors of PChE. The possibility that PChE behaves like a lipase was suggested by Szendzikowki *et al.* (1961/62) using anticholinesterases. By using nonspecific (eserine) and specific (Iso-OMPA) inhibitors of PChE these investigaters observed an *in vivo* and *in vitro* inhibition in the lipolytic activity in the rat's aorta. Similarly a decrease in the concentration of nonesterified fatty acid (NEFA) was also found in the plasma of dog treated with cholinesterase inhibitors (Colville *et al.* 1964).

In a recent study hormone sensitive lipase was also found to exhibit esterase activity (Tsujita *et al.* 1989). This esterase was able to hydrolyse nitrophenyl butyrate and was inhibited by Diisopropylfluorophosphate (DFP). Nitrophenyl butyrate is a substrate and DFP an inhibitor of PChE.

Lockridge (1982), Chatonnet & Masson (1985) and Boopathy & Balsubramanian (1987) demonstrated that purified human PChE has both peptidase and amidase activities. Lokridge *et al.* (1980) also reported that PChE hydrolysed diacetylmorphine (Heroin) in plasma.

1.2 Diabetes

Diabetes mellitus is a metabolic disorder occuring as a result of lack of insulin secretion or tissue resistance to insulin and evidenced by changes in plasma concentrations of glucose, lipids and lipoproteins. It is often characterised by fasting hyperglycemia and glycosuria. Himsworth in 1936 was the first to propose that two types of diabetes existed, differentiated by the presence or absence of plasma insulin. The two types of diabetes classified according to the National Diabetes Data Group (NDDG 1979) are type I (insulin dependent diabetes) and type II (non-insulin dependent diabetes). Type I diabetics are ketosis prone. The type II diabetics are non ketotic and often associated with obesity. Classification of diabetes according to the world health organization (WHO) is shown in Table 1.1 and the criteria for the diagnosis of diabetes in Table 1.2.

1.2.1 Chemistry of insulin

In all species studied the insulin molecule consists of 2 polypeptide chains A and B connected by 2 interchain disulfide bonds. It has a total of 51 amino acids and a molecular weight of 5800 daltons. Species differences occurs with insulin isolated from various sources, with pig insulin being most similar to human insulin. Porcine insulin differs from human insulin only in the terminal amino acid of the B chain, where alanine replaces threonine. There are three differences in the structure between bovine and human insulin ie at positions 8 (alanine replaces threonine) and 10 (valine replaces isoleucine) of the A chain and amino acid 30 on B chain. Splitting of insulin into its constituent A and B chains by cleavage of the disulfide bonds results in complete loss of its biological activity.

Discovery of insulin was hailed as a cure for diabetes. But recent studies show that it controls only the early symptoms of diabetes and does not entirely prevent the long term complications like coronary heart disease (CHD), nerve, eye, kidney and skin disorders.

1.2.2 Biosynthesis of insulin

Insulin is synthesized by the ribosomes of the endoplasmic reticulum of the β-cells of the islets of Langerhans. The product is the insulin precursor-proinsulin a single polypeptide with an approximate molecular weight of 9000 daltons. Proinsulin is known to be synthesised from large single-chain precursor pre-proinsulin which has a molecular weight of 11,500 daltons. It is converted to

proinsulin by microsomal proteases within minutes of its synthesis. The proinsulin is converted to the active insulin in the golgi apparatus by proteolytic cleavage of the connecting peptide (C-peptide) and stored in the secretory granules. The free biologically inactive C-peptide is secreted in equal molar ratio with the mature/active insulin. The human C-peptide consists of 23 amino acid residues and has a molecular weight of 3021 daltons. It differs from the porcine C-peptide by 10 residues and contains 2 amino acids less. Release of insulin involves migration of the secretory granules to the plasma membrane of the β cells where its membrane fuses with the plasma membrane and discharges its contents (both C-peptide and insulin) into the extracellular spaces. This process has been termed as emiocytosis (Randle & Hales 1972). The amount of insulin secreted by the pancreas per day has been estimated as 1 unit/kg body weight, which for a 60 kg normal adult amounts to 2.5 mg protein. Circulating insulin is degraded mainly in the liver and a small portion in the kidney by the enzyme glutathione insulin transhydrogenase by the cleavage of the disulfide bonds that connect the A and B chains. The A and B chains are further degraded by proteolysis. Half life of insulin is 3-5 min.

1.2.3 Mode of Action of insulin

The major metabolic action of insulin occurs in the muscle, adipose tissue and the liver. It seems to be comparatively inactive in the erythrocytes and gastrointestinal tract. Insulin firmly binds to a specific receptor which is a glycoprotein on the plasma membrane of its target tissues. In the adipose

tissue it facilitates the transport of glucose across the cell membrane and thus its phoshorylation to glucose-6-phosphate (G-6-P). The metabolism of G-6-P to acetyl-CoA affects fat metabolism in a number of ways: (a) metabolism of glucose along the hexose monophosphate shunt provides the coenzyme NADPH required for fatty acid synthesis, (b) the metabolism of glucose via the Embden-Meyerhof pathway generates α-glycerol phosphate which is used for the esterification of fatty acids to form TG and (c) the metabolism of glucose also provides two-carbon fragments for fatty acid synthesis (Smith 1989).

Insulin is also known to clear the circulating TG by activation of LPL. LPL hydrolyses the TG in the chylomicrons and VLDL which results in the release of free fatty acids (FFA). These FFA enter the adipocytes where they combine with the glycerol generated by glucose metabolism to form TG which is deposited in the adipocytes. Insulin is known to inhibit adipose tissue lipolysis. Thus in short insulin affects the adipose tissue metabolism by promoting glucose transport into the cell, stimulates fatty acid synthesis, inhibits lipolysis and activates LPL.

Insulin is not necessary for the transport of glucose into the liver since the hepatocytes are freely permeable to glucose. Within the cell insulin activates glycogen synthetase and directs the flow of glucose to glycogen formation. Insulin increases glucokinase activity which phosphorylates glucose to G-6-P and its metabolism via the glycolytic pathway. Insulin deficiency reduces phosphofructokinase (PFK), the second rate limiting enzyme of the glycolytic pathway and impairs glucose entry into the metabolic pathway.

Insulin inhibits gluconeogenesis by the supression of pyruvate kinase (PK) and phosphoenolpyruvate carboxykinase (PEPCK), which are rate limiting enzymes. In the liver insulin stimulates fatty acid synthesis by increasing the activity of acetyl-CoA carboxylase and generating coenzyme NADPH (hydrogen donor) by induction of pentose phosphate pathway and $NADP^+$-malate dehydrogenase (Shafrir, Bergman & Feling 1987).

In insulin deficiency the release of fatty acids from the adipose tissue is greatly increased. The liver disposes them in a number of ways. Some are completely oxidized to CO_2 and water. The majority are oxidized to two carbon fragments, these are directed into ketogenesis or glyceride synthesis.

Insulin facilitates the transport of a variety of substances across the plasma membrane other than glucose, like Na^+, K^+, inorganic phosphate ions, amino acids and calcium ions. It also helps in protein synthesis. Insulin is thus an anabolic hormone causing increased carbohydrate metabolism, glycogen and lipid synthesis, amino acid up take and protein synthesis (Shafrir, Bergman & Feling 1987).

1.3 Lipoproteins

1.3.1 Lipoprotein structure

Basically all lipoproteins have the same fundamental structure. From electron microscopic studies it appears that they are spherical particles, with the nonpolar triglycerides and cholesterol esters forming the central lipid core,

Table 1.1:

†Classification of diabetes mellitus according to WHO

A. Clinical classes

Diabetes mellitus (DM)
Insulin-dependent diabetes mellitus (IDDM)

Non-insulin-dependent diabetes mellitus (NIDDM)
(a) Non-obese
(b) Obese

Malnutrition-related diabetes mellitus (MRDM)

Other types of diabetes associated with certain conditions and syndromes:
(1) pancreatic disease
(2) disease of hormonal etiology
(3) drug-induced or chemical-induced conditions
(4) abnormalities of insulin or its receptors
(5) certain genetic syndromes
(6) miscellaneous

Impaired glucose tolerance (IGT)
(a) Non-obese
(b) Obese
(c) Associated with certain conditions and syndromes

Gestational diabetes mellitus (GDM)

B. Statistical risk classes (subjects with normal glucose tolerance but substantially increased risk of developing diabetes)

Previous abnormality of glucose tolerance

Potential abnormality of glucose tolerance

†Taken from "The Diabetes Annual" Vol 3.

Table 1.2:

†Criteria for the diagnosis of diabetes NDDG vs WHO

Plasma glucose mg/100 ml (mmol/l)*

	Oral glucose tolerance test				
Class	Fasting		Mid-test		2-Hour test
NDDG					
Normal	<115(<6.4)	and	<200(<11.1)	and	<140(<7.8)
IGT	<140(<7.8)	and	≥200(≥11.1)	and	140-199(7.8-11.1)
Diabetes**	≥140(≥7.8)	or	≥200(≥11.1)	and	≥200(≥11.1)
Non-diagnostic	all other combinations of fasting, mid-test and 2-h values				
WHO					
Normal***	<140(<7.8)			and	<140(<7.8)
IGT	<140(<7.8)			and	140-199(7.8-11.1)
Diabetes**	≥140(≥7.8)			or	≥200(≥11.1)

*The mmol/l values were computed by dividing mg/100 ml values by 18.016 the number of mg of glucose in 100 ml of a 1-mM solution) and rounding to the nearest 0.1 mmol/l. WHO obtained mmol/l values by dividing mg/100 ml values by 18 and rounding to the nearest 1 mmol/l.

**NDDG and WHO require both the fasting and 2-h values to classify a subject, except when the fasting is ≥ 140 mg/100 ml, which by itself is diagnostic of diabetes.

***Although WHO does not define a 'normal' OGTT, the term is used here to include subjects who do not meet criteria for diabetes or IGT.

†Taken from "The Diabetes Annual" Vol 3.

surrounded by an amphipathic layer of phospholipids, free cholesterol and apolipoproteins which keeps the particle in solution in the plasma (Jackson *et al.* 1976). Composition and functions of lipoproteins are as shown in Tables 1.3 and 1.4 respectively.

1.3.2 Lipoprotein metabolism

Chylomicron metabolism

Lipoproteins in man are synthesized by the liver and secreted into the plasma. Fig 1.1 presents the general pathway of lipid metabolism. Chylomicrons, the largest and lightest of the lipoproteins, are synthesised mainly in the small intestine and transport the exogenously derived fats (triglycerides) to the liver. It contains apo-B_{48} as its main protein. Following secretion into the plasma these particles rapidly acquire apo-CII and apo-E from the HDL in circulation. Triglycerides (TG) in the chylomicrons are hydrolysed by the lipoprotein lipase (LPL) present on the endothelial surfaces of the adipose tissue, cardiac and skeletal muscle. Apo-CII in the chylomicrons activates LPL (Havel *et al.* 1970). The hydrolysis of TG by LPL results in the liberation of fatty acids and monoglycerides. The fatty acids are taken up by the muscle or the adipocytes where they are oxidized or re-esterified and stored. As the TG core is depleted, the chylomicrons decrease in size. The surface materials, mainly phospholipids, free cholesterol and apo-C are transfered to HDL. These chylomicron particles known as chylomicron remnants (Redgrave 1970) are proportionately rich in cholesterolesters, apo-B and apo-E. The remnants are taken up by the liver

cells, by receptor (which recognises apo-B and apo-E) mediated endocytosis (Brown, Kovanen & Goldstein 1981) and degraded in the lysosomes. Chylomicrons in man are rapidly cleared from the blood with a half life of 4-5 min.

Very low density lipoprotein (VLDL) metabolism

VLDL's are smaller than chylomicrons and are the major transport vehicles to the extrahepatic tissues for the endogenously synthesised TG. They contain apo-B_{100} as the chief apoprotein. The fatty acids (FA) used for the synthesis of TG are derived from two sources: (1) from acetyl-CoA derived from carbohydrate metabolism and (2) uptake of FA from the circulation. Under normal conditions the first source is dominant when circulating FA is low. But in conditions like diabetes mellitus, fasting or high fat diet, the circulating FA is high, and forms the main source of TG fatty acids. Shortly after secretion, VLDL particles, like the chylomicrons, acquire apo-CII and apo-E from the circulating HDL. VLDL-TG is hydrolysed by LPL. VLDL decreases in size, its density increases and its surface phospholipids and cholesterol are transfered to the circulating HDL. The product formed is termed intermediate density lipoprotein (IDL). Lecithin cholesterol acyl transferase (LCAT) esterifies the cholesterol transfered to HDL from VLDL. The newly synthesised cholesterolester is transfered back to IDL. In rats IDL is taken up entirely by the liver and about 10% is converted to LDL (Eisenberg 1979). The IDL particles in man are finally converted to low density lipoproteins (LDL). This

lipoprotein fraction is made of mostly cholesterolester and apo-B. LDL delivers cholesterol to the extrahepatic tissues and liver. According to Goldstein and Brown (1974) these LDL particles are removed from the circulation by receptor mediated endocytosis in the peripheral tissues. The number of LDL binding receptors on the cell surface appears to be regulated by the need for cholesterol by the cell. VLDL has a half life of about 6 h in the circulation (Eisenberg 1979).

The HDL particles are synthesized and secreted both from the liver and intestine as discoid particles containing phospholipids, cholesterol, apo-AI and apo-AII as the major apoproteins (Hamilton *et al.* 1976). Studies on perfused rat liver by Hamilton *et al.* (1976) indicate that these discoid particles are rapidly transformed into spherical forms in the plasma. The most important feature of this transformation is the esterification of its cholesterol by lecithin cholesterol acyl transferase (LCAT) upon activation by apo-AI (HDL). HDL functions in the transport of cholesterol from the various peripheral tissues to the liver to be disposed. This function of HDL is refered to as "reverse cholesterol transport" (Glomset 1980). HDL has a half life of 3-6 days (Schaefer 1979).

†Table 1.3: Composition of plasma lipoproteins in man

Fraction	Source	Diameter (nm)	Density	Sf	Composition						
							Percentage of Total Lipid				
					Protein (%)	Total Lipid (%)	Triacyl-glycerol	Phospho-lipid	Choles-teryl Ester	Choles-terol (Free)	Free Fatty Acids
Chylomicrons	Intestine	100-1000	<0.96	>400	1-2	98-99	88	8	3	1	...
Very low density lipoproteins (VLDL)	Liver and intestine	30-80	0.96-1.006	20-400	7-10	90-93	56	20	15	8	1
Low density lipoproteins LDL 1 or IDL	VLDL chylo-microns	25-30	1.006-1.019	12-20	11	89	29	26	34	9	1
LDL 2		20-25	1.019-1.063	2-12	21	79	13	28	48	10	1
High density lipoproteins HDL 1*	Liver; ? intestine	20	1.063	0-2							
HDL 2		10-20	1.063-1.125		33	67	16	43	31	10	...
HDL 3		7.5-10	1.125-1.210		57	43	13	46	29	6	6
Albumin-FFA	Adipose tissue		>1.2810		99	1	0	0	0	0	100

IDL, intermediate density lipoprotein; FFA, free fatty acids. *not significant

†Taken from "Harper's Review of Biochemistry" 19th Ed.

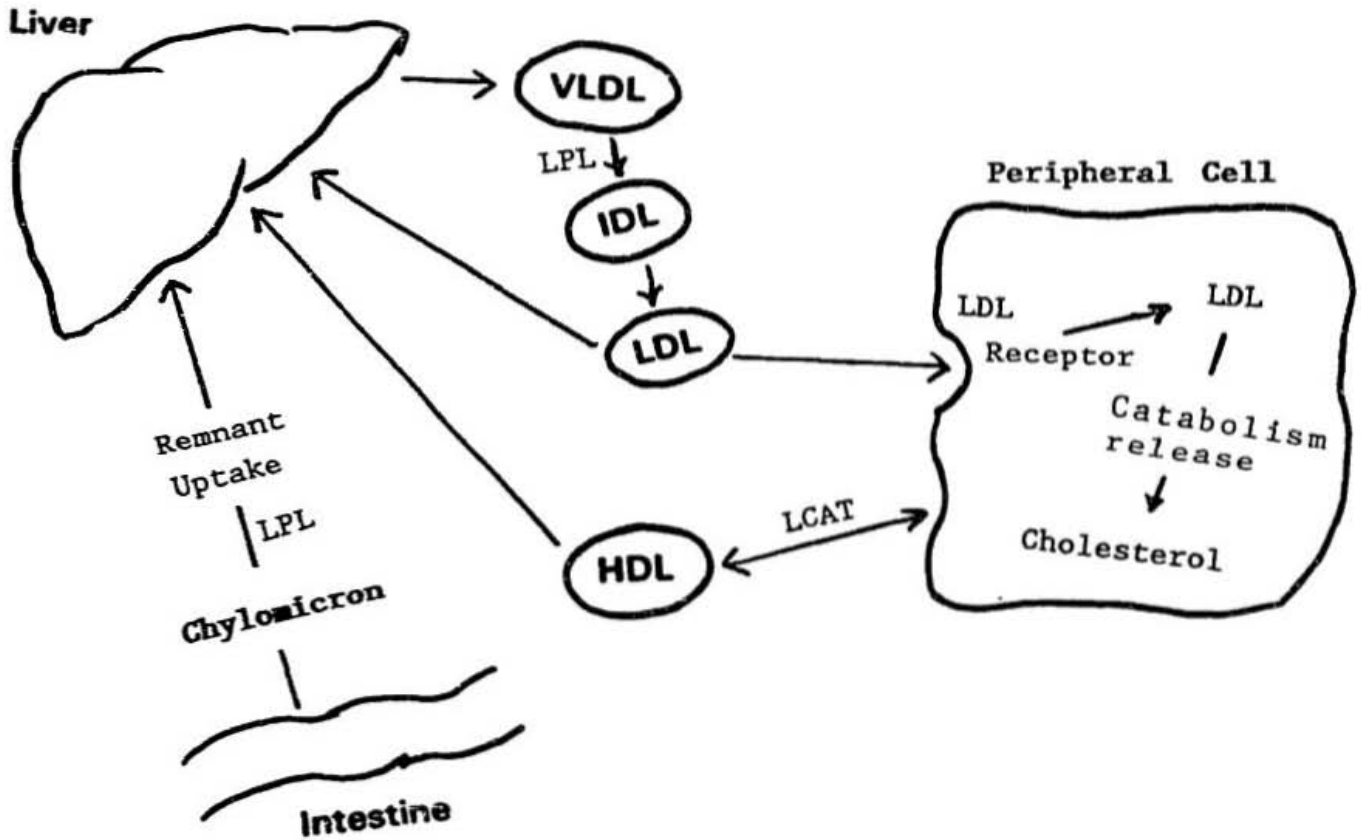
Table 1.4:

†Functions of apolipoproteins

Apolipoprotein	Approximate molecular weight	Major density class	Major sites of synthesis in man	Major function in lipoprotein metabolism
AI	28,000	HDL	liver-intestine	activates lecithin cholesterol acyltransferase
AII	18,000	HDL	liver-intestine	-
AIV	45,000	Chylomicrons	intestine	-
B-100	250,000	VLDL-IDL-LDL	liver	binds to LDL receptor
B-48	125,000	Chylomicrons-VLDL-IDL	intestine	-
CI	6,500	Chylomicrons-VLDL-HDL	liver	? activates lecithin cholesterol acyltransferase
CII	10,000	Chylomicrons-VLDL-HDL	liver	activates lipoprotein lipase
$CIII_{0-2}$	10,000	Chylomicrons-VLDL-HDL	liver	inhibits lipoprotein uptake by the liver
D	20,000	HDL	?	? cholesteryl ester exchange protein
E_{2-4}	40,000	Chylomicrons-VLDL-HDL	liver, macrophage	binds to E receptor system
F	30,000	HDL	?	-
G	75,000	VHDL	?	-
H	45,000	Chylomicrons	?	-

†Taken from "Monographs on Atherosclerosis" Vol. 14.

Fig 1.1: General pathway of lipoprotein metabolism



1.4 Lipoprotein Metabolism In Diabetes Mellitus

1.4.1 Type II (NIDDM)

Abnormal lipoprotein metabolism is a common feature in type II diabetes, which contributes significantly to the increased risk for atherosclerotic heart disease. In the majority of the studies on type II diabetic patients, the most typical lipoprotein alteration is the elevation in serum VLDL and TG. (Nikkila & Kekki 1973, Kissebah *et al.* 1982, Dunn *et al.* 1984, Ginsberg & Grundy 1982). The reason for the increase in serum VLDL-TG has been suggested to be increased synthesis and decreased clearance (Nikkila 1985). Taskinen (1986) reported increased levels of plasma fatty acid in the type II diabetic patients and concluded that the overproduction of VLDL-TG was due to the increased flux of fatty acid to the liver. Brunzell *et al.* (1985) also reported that the major cause of hypertriglyceridemia in type II patients may be due to increased synthesis of VLDL-TG as a result of increased flux of fatty acid to the liver due to obesity and insulin resistance. However, Howard *et al.* (1983) found no significant rise in VLDL-TG in subjects with mild hyperglycemia. This suggested that increased VLDL-TG production was in some way related to uncontrolled hyperglycemia. This view was supported by the observations that overproduction of VLDL decreases upon glycemic control, regardless of the type of therapy (Nikkila & Kekki 1973, Kissebah *et al.* 1982, Ginsberg & Grundy 1982, Taskinen *et al.* 1986). Dunn *et al.* (1984) also reported

that poor glycemic control could contribute to VLDL-TG overproduction and reduced fractional catabolic rate (FCR). A decrease in VLDL-TG overproduction produced by effective glycemic control could be related to decreased fatty acid mobilization from the adipose tissue. Reaven (1987) suggested that elevated plasma fatty acid in the presence of circulating insulin levels may lead to excessive production and secretion of VLDL-TG by the liver and hence to elevated plasma TG concentrations.

Decreases in lipoprotein lipase (LPL) in the adipose tissue and skeletal muscle have been observed in uncontrolled diabetes (Pykalisto *et al.* 1975). Decrease in LPL activity is consistent with decreased VLDL clearance in type II diabetes (Taskinen *et al.* 1986, Brunzell *et al.* 1979). These findings are also consistent with the finding that glycemic control increased VLDL clearance and LPL activity regardless of the type of therapy (Taskinen *et al.* 1986). The clearance of VLDL-TG could also be affected by the abnormal composition of VLDL in type II diabetes (Taskinen *et al.* 1986).

LDL metabolism

Type II diabetic patients may exhibit normal or slightly increased levels of LDL (Kostner & Karadi 1988). An increase in the concentration of TG in LDL has been reported by Taskinen *et al.* (1982). Gonen *et al.* (1985) showed that LDL from type II diabetic patients had an increased ratio of cholesterol to apo-B, with altered metabolic properties. This is supported by *in vitro* studies showing a decreased binding of LDL isolated from hypertriglyceridemic dia-

betic patients to skin fibroblasts (Hiramatsu *et al.* 1985). These investigaters showed that the decrease in binding was inversely related to TG/protein ratio in LDL.

HDL metabolism

Another characteristic finding in type II patients is decreased levels of HDL-Cholesterol (HDL-C) (Reaven 1987). A great deal of information is available suggesting an inverse relationship between plasma TG and HDL-C concentration (Nikkila 1981). Thus the causes that elevate plasma TG may also be responsible for the lowering of HDL-C levels. According to Nikkila (1981) low levels of HDL levels in type II diabetes are associated with obesity and hypertriglyceridemia. Since most of the type II diabetic patients are obese and hypertriglyceridemic, alterations in HDL levels may be due to insulin deficiency or more commonly to insulin resistance and hyperinsulinemia (Nikkila 1981). Glomset (1980) suggested that HDL-C levels are inversely related to both body weight and serum TG, therefore obesity and hypertriglyceridemia may play a role in maintaining HDL-C levels. Hepatic lipase is elevated in the type II diabetic patients and could accelerate removal of HDL-C from the plasma and thus reduce HDL levels (Nikkila 1981).

1.4.2 Type I (IDDM)

These patients are usually not obese and characterised by insulin deficiency. They therefore require insulin therapy and untreated diabetic patients char-

acteristically develop severe ketoacidosis. In addition, this type of diabetic patient also develops severe hyperlipidemia in the absence of glycemic control. Metabolic control is usually judged on the basis of blood glucose, HbA_1c and urine glucose levels. It has been well established that plasma lipid abnormalities are related to the degree of glycemic control (Sosenko *et al.* 1980, Lopes-Virella *et al.* 1981, Glasgow *et al.* 1981). Short term improvement in glycemic control by continuous subcutaneous insulin infusion (CSII) therapy by Hershcopf *et al.* (1982) reduced plasma lipid concentrations (TG and cholesterol) within 2 weeks. According to Sosenko *et al.* (1980) "poor control of blood glucose is conducive to accelerated atherosclerosis in diabetes mellitus" due to hyperlipoproteinemia. Extreme elevations in VLDL levels are reported to be characteristic of diabetic ketoacidosis, a state in which insulin concentrations are minimal (Brunzell *et al.* 1985). Hypertriglyceridemia in uncontrolled type I diabetes is due to combination of increased production and decreased clearance (Nikkila *et al.* 1977). Increased production of VLDL in type I diabetes is due to the increased mobilization of fatty acids from adipose tissue.

Decreased activity of LPL in type I diabetic patients affects the clearance of VLDL-TG from the circulation (Brunzell, Porte & Bierman 1979). LPL activity is reported to be decreased in adipose tissue and skeletal muscle in diabetic patients (Taskinen 1987). Post heparin lipolytic activity has been found to be decreased in type I diabetic patients which normalizes after treatment with insulin (Nikkila *et al.* 1977).

Sosenko *et al.* (1980) observed an increase in fasting plasma cholesterol, TG, LDL and VLDL levels with hyperglycemia. LDL has been reported to be increased in uncontrolled type I diabetic patients (Glasgow *et al.* 1981, Sosenko *et al.* 1980, Lopes-Virella *et al.* 1981). Lopes-Virella *et al.* (1982) demonstrated that the LDL from uncontrolled diabetic patients are taken up and degraded by the normal fibroblasts at a lower rate than LDL from the normal or controlled diabetic patients. Hence these investigators speculated that LDL had an altered composition in patients with poor metabolic control.

A large number of reports show a correlation between low levels of plasma HDL and untreated diabetes (Sosenko *et al.* 1980, Lopes-Virella *et al.* 1981, Glasgow *et al.* 1981). However HDL increases on insulin therapy reflecting a degree of glycemic control (Kostner & Karadi 1988). Decrease in LPL activity as a result of insulin deficiency could be one of the factors responsible for the reduced HDL levels (Taskinen 1987).

1.5 Animal Models of Diabetes

1.5.1 Type I Diabetic Models

A model resembling human insulin dependent or type I diabetes can be created in animals by treating them with agents like alloxan, streptozotocin (SZ) or anti-insulin serum. Alloxan is known to function by the inhibition of the enzyme glucokinase, present in both the liver and pancreatic β-cells. Alloxan oxidises the -SH groups at the sugar binding site resulting in the inhibition

of glucose induced insulin secretion and produces necrosis of the pancreatic β-cells due to its toxic effect (Lenzen & Panten 1988). *Alloxan diabetes* in animals, presents the typical symptoms of type I diabetes in man such as weight loss, polydipsia, polyuria, glycosuria, ketonuria, hyperglycemia and ketonemia (Malaisse 1982). Factors such as diet, age and animal species affect the dose required for the induction of a diabetic state with alloxan (Webb 1966). Streptozotocin has largely replaced alloxan, as it is less toxic and its effects are reproducible. It allows the induction of diabetes of predictable severity (Junod *et al.* 1969). The diabetogenic action of SZ is mainly due to its specific cytotoxic action on the β-cells of the islets of Langerhans where it produces rapid and irreversible necrosis (Junod *et al.* 1969). The intensity of the damage caused can be graded according to the dosage used, thus resulting in the production of diabetes of graded severity (Junod *et al.* 1969). The response to SZ action on the β-cells is triphasic: initially insulin secretion is inhibited leading to hyperglycemia with increase in plasma free fatty acids and ketone bodies, followed by extreme hypoglycemia and finally an irreversible hyperglycemia within 24 hr after the treatment (Junod *et al.* 1969). High doses of SZ (> 100 mg/kg body wt.) produce hyperglycemia, hyperketonemia and hyperlipidemia in rats (Schein *et al.* 1971), while low doses produce hyperglycemia but rarely ketonuria or hyperketonemia. Streptozotocin induced diabetes is known to produce hyperlipidemia (Schein *et al.* 1971). Bar-On *et al.* (1976) reported an increase in all the plasma lipoprotein fractions, while Blackshear & Alberti (1974) reported high levels of plasma non-esterified fatty acids (NEFA)

and ketone bodies in severely insulin-deficient rats. Hypertriglyceridemia is a common occurence in severely insulin-deficient rats and the degree of hypertriglyceridemia depends on the duration of insulin deficiency and diet (Reaven & Reaven 1974). It has been proposed that the increased fatty acid flux leads to increased endogenous synthesis of triglycerides (TG) and hence increases VLDL-TG production in these animals similar to the diabetic patients (Nikkila & Kekki 1973).

Von Tol (1977) reported defective clearance as a major factor responsible for hypertriglyceridemia in type I diabetes. Decreased clearance of TG is attributed to reduced lipoprotein lipase (LPL) activity (Robinson & Speake 1989). Decreased adipose tissue LPL activity has been reported in type I diabetes (Schnatz & Williams 1963). The lipase activity of liver, adipose tissue and heart are under hormonal regulation by insulin (Nomura *et al.* 1984). According to O'Looney *et al.* (1983) decrease in the LPL activity in diabetes could be due to decreased synthesis of the enzyme or its activator. Insulin treatment could restore the activity by stimulating protein synthesis. Insulin treatment of diabetic rats restores the LPL activity (Schnatz & Williams 1963).

1.5.2 Type II Diabetic Models

Diabetes, obesity and hyperlipidemia are frequently associated pathological conditions. Most of the type II or non-insulin dependent type of diabetic patients are obese and hypertensive. SHR/N-corpulent rats are a good model for the study of type II diabetes. These corpulent rats are hyperinsulinemic,

hyperlipidemic, have abnormal glucose tolerance and develop hyperglycemia when fed a high carbohydrate diet (sucrose) (Michaelis *et al.* 1984). According to these investigators superimposing obesity on an already existing insulin-resistant genetic background could be the cause for the hyperglycemia.

Iwase *et al.* (1986) have reported a new model of type II diabetes with hypertension. This was experimentally developed by giving various doses of SZ to the spontaneously hypertensive rats 2 days after birth. This successfully produced an animal model of mild to severe type II diabetes with hypertension.

Genetically obese rodents have often been used as models of human obesity and diabetes (Bray & York 1979). Some strains of genetically obese rats have features resembling type II human diabetes like hyperglycemia and hyperinsulinemia (Clark *et al.* 1983). Obesity can also be induced experimentally by ventralmedial hypothalamic lesions (VMH). These animals are known to exhibit hyperglycemia, hyperinsulinemia along with abnormal glucose tolerance, indicating the presence of insulin resistance similar to type II diabetic patients (Jeanrenaud 1977).

Hyperlipidemia in these animals is primarily associated with endogenous hypertriglyceridemia and is mainly due to increased VLDL synthesis. Hepatic overproduction of VLDL and increased secretion of TG or VLDL into the plasma of obese Zucker rats (Schonfeld & Pfleger 1971) and sand rats has been reported (Robertson *et al.* 1973). In the sand rats VLDL secretion was reported to be closely correlated with plasma insulin levels, and insulin levels correlated with the body weight (Robertson *et al.* 1973). VLDL secretion

rate was proportional to the fatty acid uptake in rats (Robertson *et al.* 1973).

Reaven & Greenfield (1981) found that liver exposed to elevated levels of insulin secrete more TG at a given concentration of FFA. They observed that the hyperinsulinemic ob/ob mouse when treated with small dose of streptozotocin, had a hepatic TG secretion rate that was similar to that observed in the lean controls. Similar observations of elevated TG were noted in hyperisulinemic patients with impaired glucose tolerance. (Reaven & Greenfield 1981). Fatty acid levels are increased in obesity and this could be the cause of the higher TG levels in obese patients with impaired glucose tolerance (Sailer *et al.* 1966).

PChE is also increased in the serum and liver of genetically obese (ob/ob) rats and diabetic (db/db) mice as well as in GTG induced obese mice (Kutty *et al.* 1981b). These investigators suggested that an increase in PChE activity is a result of their increased caloric intake.

1.6 Objective of the present study

The objective of this study was to find out the reasons for PChE increase in Diabetes Mellitus.

The following questions were posed:

(1) is the increase in PChE specific for one type of diabetes or common to both types?

(2) is there a relationship between serum PChE increase and abnormal lipid and lipoprotein metabolism?

(3) is there a relationship between the control of hyperglycemia and serum PChE activity in diabetes?

(4) does inhibition of PChE affect lipid and lipoprotein metabolism in diabetic and normal rats?

(5) does the clearance of TG affect PChE activity in diabetes?

(6) does the inhibition of PChE in normal rats result in the accumulation of choline or cholinesters in the serum, liver and their excretion in the urine?

Chapter 2

MATERIALS & METHODS

2.1 Human Study

2.1.1 Subjects

Both type I (insulin dependent IDDM) and type II (non-insulin dependent NIDDM) diabetic patients were studied after obtaining written informed consent. The protocol was approved by the Human Investigation Committee (Memorial University of Nfld. St. John's).

Type I patients were recruited from among the patients who attended the diabetic clinic at the Janeway Child Health Centre. The type II diabetic patients were recruited from the diabetic clinic at the Grace General Hospital.

The non-diabetic/control subjects (age matched) were chosen at random from the out patients and were confirmed as non-diabetic on the basis of normal fasting and post meal blood glucose and normal lipid profile.

2.1.2 Sample collection

Venous blood samples (fasting) were collected from the control subjects and patients for the assay of the various biochemical parameters. Blood for glucose was collected in fluoride tubes, in EDTA for HbA_1c and in plain tubes for insulin, C-peptide, lipids (triglyceride, TLDL, HDL-C and cholesterol) and lipoprotein analysis.

2.2 Experimental Animals

Animal experiments were conducted after obtaining approval from the Presidents Committee on Animal Bioethics and Care (Memorial University of Nfld. St. John's).

2.2.1 Animals

Male Sprague-Dawley rats weighing 200-250 g were purchased from Charles River Breeding Laboratories (Halifax, Nova Scotia). These animals were housed in the Animal Care Unit of the Health Science Centre at the Memorial University of Newfoundland, St. John's. Each rat was kept in an individual cage which contained heat treated wood chips for bedding. These cages were kept in a light/heat controlled room. All the animals had free access to food (Purina rat chow # 5012) and water.

Male CD-I albino mice (Swiss-Webster strain, 60 days old) were purchased from Charles River (Halifax, Nova Scotia). These animals were also housed

in the Animal Care Unit. Each mouse was kept in an individual cage which contained heat treated wood chips for bedding. All the animals had free access to food (Purina mouse chow # 5015) and water.

2.2.2 Induction of Diabetes with SZ and Alloxan

Fasting (14-15 hr) blood samples were obtained from the animals prior to injection of alloxan/SZ to obtain prediabetic levels of the biochemical parameters of interest. Diabetes was induced by a single intraperitoneal injection of freshly prepared SZ (100 mg/kg body wt.) dissolved in 0.01M disodium citrate buffer, pH 4.5 (Blackshear & Alberti 1974). For the induction of diabetes with alloxan, rats were injected intraperitoneally with alloxan (100 mg/kg body wt.) dissolved in saline. Development of diabetes was confirmed by the presence of glycosuria 72 hr after SZ injection. Urine was checked for glucose with a multistix. Urine was collected from rats in metabolic cages fitted with a steel collecting funnel and a fine wire mesh screen to retain the feces. The urine was collected in plastic containers and stored at -20°C until analysed.

2.2.3 Sacrifice of the animals

Collection of tissues and blood

The animal was placed in a 4L beaker containing gauze pads moistened with Ethrane (2-chloro-1,1,2-trifluoroethyl ether) and covered with a lid. Within 1-2 min the animal was mildly anesthetized. Blood samples (always 14-15 hr fasting) were collected by heart puncture using a 5 ml syringe with a 22

gauge, 25 mm needle. The blood was allowed to clot at room temperature and the serum was separated by centrifugation. After blood collection the animals were sacrificed under deep anesthesia. A mid-line incision was made to expose the required organs. Livers were first perfused with cold normal saline and blotted dry between gauze. Epididymal fat pads were carefully dissected out avoiding blood vessels, rinsed in saline and blotted dry with filter paper. Sera, liver and the adipose tissue were stored at -20^{0}C until ready to use.

2.2.4 Homogenization of tissues

Materials

Potassium chloride and Triton X100 were purchased from Sigma Chemical Co. USA. "Airfuge" Ultracentrifuge and Beckman TJ-6 centrifuge (Beckman Instruments, USA). Tissue grinders (Pyrex # 7727, USA).

Method

Livers were homogenized (with a tissue grinder) in 1% Triton X100 in 0.155 M KCl solution (4 ml/g wet wt. of tissue). The crude homogenate was centrifuged at 2500 rpm at 4^{0}C for 15 min in a Beckman TJ-6 centrifuge. The supernatant was removed with a Pasteur pipette and ultracentrifuged in a Beckman "Airfuge" at 110,000 rpm for 5 min at room temperature. The supernatant was saved to assay PChE activity. Adipose tissue was ground in a tissue grinder using 2 ml cold water per gram of tissue. The crude homogenate was centrifuged at 2500 rpm at 4°C for 15 min in a Beckman

TJ-6 centrifuge. The infranatant was further ultracentrifuged in a Beckman "Airfuge" at 110.000 rpm at room temperature for 5 min (Lech & Calvert 1968). The clear supernatant was used for the assay of PChE activity.

2.2.5 Extraction of lipids from liver

Method

Lipids were extracted by homogenizing the tissue (1 g) with 2:1 chloroform-methanol (20 ml, v/v), such that 1 ml of extract corresponded to 0.05 g of the tissue or the homogenate concentration equals 5% (Folch *et al.* 1957). The crude homogenate was filtered through a fat free filter paper. The filtrate was washed with 0.2 ml of its volume of 0.05% calcium chloride solution. The two layers were mixed by gentle stirring with a glass rod taking care to avoid interfacial fluff. The two phases were separated by centrifugation. The upper phase was removed completely with a Pasteur pipette and an aliquot of the lipid extract was evaporated to dryness and analyzed for triglycerides.

2.3 Biochemical Methods

2.3.1 Determination of PChE activity, in Serum, Liver and Adipose Tissue

PChE was assayed by the method of Dietz, Rubinstein & Lubrano (1973) using propionylthiocholine iodide as the substrate for rat and mice samples.

Butyrylthiocholine iodide was used for human serum and 5,5' dithiobis-(2-nitro-benzoic acid) (DTNB) was the chromogen.

Materials

Propionylthiocholine iodide; butyrylthiocholine iodide; 5,5'dithiobis-(2-nitro benzoic acid) (DTNB); butyrylcholinesterase (horse serum); and Tris-(hydroxy-methyl)-aminomethane (Tris) were purchased from Sigma Chemical Co. (USA).

Reagents

(1) Tris-DTNB buffer (0.05 M), pH 7.4: Tris (6.055 g) was dissolved in 900 ml distilled water and the pH was adjusted to 7.4 with conc. HCl. DTNB (100 mg) was dissolved in this solution and the volume was made up to 1L with water.

(2) Substrate-buffer solution (made before use): Tris-DTNB buffer 0.05 M, pH 7.4 (60 ml) was mixed with 6 ml of 0.005 M propionyl or butyrylthiocholine iodide (substrates). To this was added 15 ml of distilled water and mixed.

Reaction Sequence

$$\text{Propionylthiocholine} + H_20 \xrightarrow{\text{PChE}} \text{Propionic Acid} + \text{Thiocholine}$$

$$\text{Thiocholine} + \text{DTNB} \longrightarrow \underset{\text{(colored complex)}}{\text{5-thio-2-nitrobenzoic Acid}} + \text{Choline}$$

Method

Appropriate aliquots of tissue preparations were added to the substrate-buffer solution (2.7 ml) and the rate of change in absorbance of the thiocholine-DTNB complex formed was recorded at 420 nm against a blank. The tissue preparations analysed were: human and mice serum (20 μl), rat serum and liver homogenate (50 μl) and rat adipose tissue homogenate (100 μl). The enzyme activity was calculated from a standard curve constructed using free thiocholine iodide as a reference (0.2 to 0.025 μmol). The enzyme activity was expressed in terms of the μmoles of thiocholine formed in 1 min by 1 ml of serum or g wet wt. of tissue.

Preparation of standard curve

Butyrylthiocholine iodide was the reference standard used. Thiocholine liberated by the action of horse serum butyrylcholinesterase on butyrylthiocholine iodide was the source of free thiocholine. A stock standard solution was prepared by dissolving 15.86 mg of butyrylthiocholine iodide in 5 ml of distilled water to give a concentration of 0.2 μmol/20 μl of solution. Into a clean test tube was pipetted equal volumes of stock standard and horse serum cholinesterase (1 U/ml). The reaction mixture was incubated for 10 min at 37^0 C. After incubation was completed, serial dilutions of the enzyme treated standard solution was made to give 0.2, 0.15, 0.1, 0.05, 0.025 μmol of thiocholine per 20 μl solution. To 2.7 ml buffer substrate reagent was added 20μl of standard solutions containing 0.2 to 0.025 μmol of thiocholine and the O.D

was read at 410 nm. The standard thiocholine curve was prepared by plotting O.D verses concentration of thiocholine in μmole per test volume.

2.3.2 Serum Glucose Estimation

Serum glucose was estimated by an enzymatic method using the IL TEST^{TM} Glucose kit (Fisher Scientific Co. USA.) on the Monarch 2000 chemistry autoanalyzer.

Reagents (Provided in the kit)

Glucose reagent was provided as a dry powder which was reconstituted with 16.0 ml of deionised water before use.

Composition

Adenosine triphosphate (ATP) $\geq$ 1.79 mM; nicotinamide adenine dinucleotide (NAD) $\geq$ 2.5 mM; hexokinase ($yeast$) $\geq$ 1000 U/l; $G-6-PD \geq$ 1500 U/l. Commercial control serum was run with each set of assay as standard and control.

Reaction sequence

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{Hexokinase}} \text{G-6-P} + \text{ADP}$$

$$\text{G-6-P} + \text{NA}D^{+} \xrightarrow{\text{Glucose-6-Phosphate dehydrogenase}} \text{6-phosphogluconate} + \text{NADH} + H^{+}$$

The increase in absorbance at 340 nm due to reduction of coenzyme NAD^+ to NADH is directly proportional to the amount of glucose present in the sample.

2.3.3 Serum Triglyceride Estimation

Serum triglyceride was estimated on the Monarch 2000 chemistry autoanalyzer using the Peridochrom Triglycerides GPO-PAP (Cat # 701904) kit from Boehringer Mannheim GmbH Diagnostica.

Reagents (Provided in the kit)

Composition

(1)Tris buffer: 0.15 M, pH 7.6; $MgSO_4$ 17.5 mM; EDTA(disodium salt) 10 mM; 4-chloro-phenol 3.5 mM; Sodium cholate 0.15%;Potassium hexacyanoferrate 6 μM; hydroxypolyethoxy-n-alkanes 0.12%.

(2)Lyophylisate: ATP $\geq$ 0.54 mM; 4-aminophen-azone 0.35 mM; lipase $\geq$ 3 U/ml; Glycerol phosphate oxidase (GPO) $\geq$ 2.5 U/ml; Glycerol Kinase (GK) $\geq$ 0.2 U/ml; peroxidase $\geq$ 0.15 U/ml.

Preparation of the working reagent: A reagent strip was immersed in a bottle of buffer. This was allowed to stand for 5 or more minutes till the reagent patches turned colorless. A control and standard was run with each set of assay.

The control and standards used were commercial serum preparations namely, Precilip EL, Precinorm and Precilip (Boehringer Mannheim Diagnostica).

Method

Enzymatic hydrolysis of TG with subsequent determination of the liberated glycerol (Bucolo & David 1973).

Reaction Sequence

$$TG + 3H_2O \xrightarrow{\text{Lipase}} \text{Glycerol} + 3\text{RCOOH}$$

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{Glycerol-3-Phosphate} + O_2 \xrightarrow{\text{Glycerol phosphate oxidase}} \text{Dihydroxyacetonephosphate} + H_2O_2$$

$$H_2O_2 + \text{4-aminophenazone} + \text{4-chlorophenol} \xrightarrow{\text{Peroxidase}} \text{4-(p-benzoquinone-mono-imino)-phenazone} + 2H_2O + \text{HCl}$$

2.3.4 Serum Glycerol Estimation

Serum glycerol was measured on the Monarch 2000 chemistry autoanalyzer using the Test-Combination Triglycerides kit (Cat # 125 032), Boehringer Mannheim GmbH Diagnostica Kit.

Reagents (Provided in the kit)

Composition

Working reagent: Reagents provided in the kit were lyophilised powders and were reconstituted with distilled water before use.

(1) Triethanolamine buffer (0.1 M, pH 7.6) containing $MgSO_4$ (4 mM).

(2) NADH/ATP/PEP, when reconstituted contained 6, 33 and 11 mM respectively.

(3) Lactate dehydrogenase/Pyruvate kinase (LDH/PK) $\geq$ 800 U/ml, 130 U/ml.

(4) Glycerokinase (GK) $\geq$ 150 U/ml.

The final reaction mixtures were made as follows:

Reagent 'A' was made by mixing together 6.0 ml of buffer, 0.5 ml NADH/ATP/PEP reagent and 0.1 ml LDH/PK reagent

Reagent 'B' was made by mixing together 6.0 ml of buffer and 0.1 ml of GK.

Concentrations of Glycerol standards used were, 0.2 mM and 0.4 mM.

Reaction Sequence

$$\text{Glycerol} + \text{ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3-Phosphate} + \text{ADP}$$

$$\text{ADP} + \text{Phosphoenolpyruvate} \xrightarrow{\text{Pyruvate kinase}} \text{Pyruvate} + \text{ATP}$$

$$\text{Pyruvate} + \text{NADH} + H^+ \xrightarrow{\text{Lactate dehyrogenase}} \text{Lactate} + NAD^+$$

The change in absorbance due to oxidation of NADH to NAD^+ at 340 nm was a direct measure of the concentration of glycerol in the sample.

2.3.5 Serum Cholesterol Estimation

Serum cholesterol was estimated using IL $Test^{TM}$ cholesterol kit (Cat # 35245, Fisher Scientific Co. USA), on the Monarch 2000 chemistry autoanalyzer.

Reagents (Provided in the kit)

Composition

4-aminoantipyrene (4-AAP) 0.9 mM, phenol 21.0 mM, peroxidase (horse radish) > 24,000 U/l, cholesterol oxidase (microbial) > 1000 U/l, cholesterol esterase (microbial) > 1200 U/l.

Method

A modification of the enzymatic method established by Allain *et al.* (1974).

Reaction Sequence

$$\text{Cholesterolesters} + H_2O \xrightarrow[\text{Cholesterolesterase}]{} \text{Cholesterol} + \text{Fatty Acid}$$

$$\text{Cholesterol} + O_2 \xrightarrow[\text{Cholesterol oxidase}]{} \text{Cholesten-3-one} + H_2O_2$$

$$H_2O_2 + \text{4-aminoantipyrene} + \text{phenol} \xrightarrow{\text{Peroxidase}} \underset{\text{(colored chromogen)}}{\text{quinoneimine}} + H_2O$$

H_2O_2, in the presence of peroxidase reacts with phenol and 4-aminoantipyrene forming a red colored chromogen. Intensity of the color was proportional to the concentration of cholesterol in the sample, which was measured at 500 nm.

2.3.6 Serum High Density Lipoprotein Cholesterol Estimation (HDL-C)

HDL-C was measured on the Monarch 2000 chemistry autoanalyzer.

Reagents (Provided in the kit)

Precipitant (Cat # 543 004) from Boehringer Mannheim GmbH Diagnostica. Working reagent was prepared by diluting the stock precipitant with 20 ml water.

Composition

Contains phosphotungstic acid 0.55 mM and $MgCl_2$ 25 mM.

Method

Chylomicrons, VLDL (Very low density lipoprotein) and LDL (Low density lipoprotein) were precipitated by phosphotungstic acid and magnesium ions. Centrifugation leaves only HDL in the supernatant (Burstein *et al.* 1970).

The cholesterol content of the supernatant was determined enzymaticaly on the Monarch chemistry systems autoanalyzer, like total cholesterol.

2.3.7 Serum Total Low Density Lipoprotein (TLDL) Estimation

TLDL was estimated in the Monarch 2000 chemistry autoanalyzer.

Reagent Composition

Calcium chloride ($CaCl_2$ 1.4 g) and 1 ml heparin (10,000 U/ml) were mixed in 383 ml distilled water.

Method

TLDL ie apo-B containing lipoproteins in the serum was estimated by measuring the turbidity produced by the addition of heparin and $CaCl_2$ reagent to the sample. Commercial serum samples, like Precilip, Precinorm and Precilip E.L were used for the control and standard.

2.3.8 Estimation of Choline and Cholinesters in Serum, Liver, Adipose tissue and Urine

Free choline was estimated by the Choline oxidase method (McGowan *et al.* 1983).

Materials

Choline iodide, 4-aminoantipyrine, choline oxidase (EC 1.1.3.17: alcaligens species), peroxidase (EC 1.11.1.9: horse radish type 1) and 2-hydroxy-3,5-di chlorobenzene sulfonate were purchased from Sigma Chemicals Co. USA.

Reagents

(1) Tris/HCl buffer (50 mM) was made by dissolving 6.0558 g Tris in 900 ml H_2O and adjusting the pH to 7.8 with HCl. The volume was made up to 1L with distilled water.

(2) 4-aminoantipyrine (7.5 mM) was made by dissolving 0.152 g in 10 ml Tris/HCL buffer (50 mM, pH 7.8).

(3) 2-hydroxy-3,5-dichlorobenzene sulfonate (30 mM) was prepared by dissolving 0.0795 g in 10 ml Tris/HCL buffer (50mM, pH 7.8).

(4) Choline oxidase (7 U/ml) was dissolved in Tris/HCL buffer (50mM, pH 7.8).

(5) Peroxidase (30 U/ml) dissolved in Tris/HCL buffer (50 mM, pH 7.8).

Choline reagent

This was made by mixing together in order 2.3 ml reagent (2), 2.3 ml reagent (3), 5.46 ml reagent (4) and 3.94 ml reagent (5).

Assay procedure

Into a clean cuvette was pipetted 300 μl Tris buffer pH 7.8 and 20 μl sample, standard or blank. The O.D. at 510 nm was read until constant. To these cuvettes was added 480 μl of choline reagent. The contents were mixed by inverting and the change in absorbance was read at 510 nm for 1-2 min. Choline iodide was the standard used for plotting the standard curve with concentrations ranging from 3 to 30 μM of choline. For the determination of choline esters 20 μl of sample was incubated with 20 μl horse serum cholinesterase (1 U/ml) for 5-10 min at 37^{0}C. An aliquot of the incubated mixture (20 μl) was assayed for choline as above. This gives the absorbance for the total choline i.e. (free choline + choline obtained by the hydrolysis of cholinesters). Thus the concentrations of the cholinesters were calculated by taking the differences between the total choline and the free choline. The choline concentration was calculated from the standard curve.

2.3.9 Thin Layer Chromatography (TLC)

For qualitative determination of Cholinesters.

Materials

Ammonium reineckate (BDH), NaCl, acetone, butanol, glacial acetic acid, ethanol (reagent grade), KI and bismuth subnitrate were purchased from Sigma Chemicals Co. USA. Silica gel G TLC plates (Analtech, inc).

Reagents

(1) Saturated ammonium reineckate.

(2) NaCl 0.9%.

(3) Dragendorff's reagent: Consists of potassium iodide (0.11 M) and bismuth subnitrate 0.6 mM in 3.5 M acetic acid.

(4) Chromatography solvent: Butanol:H_2O:Glacial acetic acid:Ethanol in the ratio of 4:3:1:2 (v/v/v/v).

Procedure

Cholinesters in the sample were precipitated by ammonium reineckate. To 0.4 ml rat serum was added 1 ml saturated ammonium reineckate solution. For the blank 0.4 ml 0.9% NaCl was treated with ammonium reineckate. The solution was mixed well by vortex for 1 to 2 min and allowed to stand on ice for 5 min. This mixture was centrifuged for 10 min at 3000 xg at 4^0C. The supernatant was discarded and the reineckate precipitate was dissolved in a known minimum volume of acetone and chromatographed on silica G plates using the solvent system butanol/H_2O/glacial acetic acid/ethanol (4:3:1:2). Butyrylcholine, acetylcholine, propionylcholine and choline were used as reference compounds. The spots were visualized by spraying with Dragendorff's reagent.

2.3.10 Estimation of Serum C-peptide

Serum C-peptide was determined using the C-peptide kit (Daiichi Radioisotope Labs. Ltd).

Method

Radioimmunoassay method.

Reagents (Provided in the kit)

(1) C-peptide standards of concentration ranging from 0-30 ng/ml.

(2) C-peptide antiserum.

(3) Iodinated C-peptide (^{125}I) 2μCi.

(4) Anti-rabbit-γ-globulin.

(5) Precipitation stabilizer.

All reagents were lyophilized hence had to be reconstituted before use with distilled H_2O.

Procedure

All measurements were done in duplicate. The standard solution or patients serum (100 μl) was transfered into numbered tubes. To this was added 100 μl of iodinated C-peptide (^{125}I) solution. C-peptide antiserum (100 μl) was added to all tubes except the tubes numbered for total counts measurement. The tubes were gently shaken together with their holders and incubated at 20^0 C $\pm$ 5 for 20 $\pm$ 4 hr. After incubation was complete, 500 μl of anti-rabbit

γ-globulin serum was added to each tube. All tubes were mixed well (within 10 min) on a vortex mixer and incubated for 30-40 min at room temperature. After incubation was complete the tubes were centrifuged at 3000 rpm for 30 min at 4^0C. Supernatant was removed by aspiration. Radioactivity of each tube was measured on a gamma counter and the C-peptide concentration was obtained from a computer program in the counter.

2.3.11 Estimation of Serum Insulin

Serum insulin was determined using the Pharmacia Insulin RIA Kit.

Principle

Insulin in the sample competes with a fixed amount of ^{125}I-labelled insulin for the binding sites of the specific antibodies. The bound and free insulin are separated by the addition of a second antibody immunoadsorbent. After centrifugation the supernatant was aspirated and the radioactivity in the pellet was measured in a gamma counter.

Reagents (Provided in the kit)

All reagents were ready to use.

(1) Standard insulin (human) 0-240 μU/ml.

(2) Antibody.

(3) Insulin-^{125}I (1.0 μ Ci).

(4) Decanting suspension (sepharose-anti-guinea pig IgG).

Assay Procedure

All measurements were done in duplicate. Standard and patients samples (100 μl) were transfered into numbered tubes. To each tube was added ^{125}I (50 μl) followed by 50 μl antibody. The reaction mixture was mixed well and incubated for 30 min at room temperature. The tubes were centrifuged 10 min at 1500 g and the supernatant was removed carefully by aspiration. The radioactivity of the pellet was measured in a gamma counter. Insulin concentration was obtained from the counter by computerised calculation.

2.3.12 Estimation of Glycosylated Hemoglobin (HbA_1c)

HbA_1c in the blood was estimated with the Hemoglobin A_1c Mini Column Test kit of Bio Rad (Cat # 1919001).

Materials (Provided in the kit)

(1) Hemolysis reagent.

(2) Elution/developing reagent:

(a) Borate/phosphate buffer pH 6.7.

(b) Phosphate buffer pH 6.7.

(3) Resin columns (weakly acidic cation exchange resin).

(4) Calibrators: These were lyophilized human whole blood hemolysates.

Procedure

Separate columns were set for caiibrators, control and patient samples. The sample hemolysate was prepared by mixing together 500 μl hemolysis reagent and 100 μl of the patients sample. This was vortexed and allowed to stand for 10 min. Within 60 min of draining the columns, 100 μl reconstituted calibrators, controls and patients hemolysate was added to the column and adsorbed on to the resin by allowing it to stand for 5 min. To each column was slowly added 4.0 ml elution/developing reagent (borate/phosphate buffer pH 6.7). The eluate was discarded, 10.0 ml elution/developing reagent (phosphate buffer pH 6.7) was added to each column and the eluate was saved and labelled 'A'. To a set of test tubes labelled 'B' was added 10.0 ml elution/developing reagent (phosphate buffer pH 6.7) and 20 μl each sample hemolysate, control and calibrator. The contents of tubes labelled A and B were mixed well on a vortex before reading the absorbance at 415nm. HbA_1c concentrations was calculated from a standard curve.

2.3.13 Electrophoretic Separation of Serum Lipoprotein

(a) Polyacrylamide gel electrophoresis (PAGE)

Method

Serum lipoproteins were separated on polyacrylamide gel by the method of Frings, Foster & Cohen (1971).

Materials

Tris [tris(hydroxyethyl)aminomethane],Trizma base; TEMED (N,N,N',N'tetramethylethylenediamine); acrylamide (Bio Rad); bisacrylamide(N,N'-methylenebisacrylamide); riboflavin; sucrose; ammonium persulfate and glycine were obtained from Sigma Chemical Co. (USA) and Sudan Black B from Fisher Scientific Co.

Equipment

Gel tubes 7x75 mm glass tubes. Power pack (Electrotek^{TM} power supply. Buchler instruments N.J.).

Reagents

(A) Separating Gel:

(1) Tris (9.0 g), 0.8 ml HCl and 100 μl TEMED were dissolved in 50 ml $H_2$0.

(2) In 50 ml H_2O was dissolved 6.0 g acrylamide and 240 mg bisacrylamide.

(3) Ammonium persulfate (280 mg) was dissolved in 100 ml H_2O.

Solutions 1,2 and 3 were mixed in the ratio of 1:1:2 (v/v/v).

(B) Stacking Gel:

(1) Tris (5.98 gm) and 0.46 ml of TEMED were dissolved in 100 ml H_2O after the pH was adjusted to 6.7 with HCl.

(2) Acrylamide (2 gm) and 0.5 gm of bisacrylamide were dissolved in 20 ml H_2O.

(3) Riboflavin (8 mg) was dissolved in 100 ml H_2O.

(4) Sucrose (20 gm) was dissolved in 50 ml H_2O.

Solutions 1,2,3 and 4 were mixed in the ratio of 1:2:1:4 (v/v/v/v).

(C) Loading Gel:

Stacking gel (4 ml) and 0.5 ml working dye were mixed just before use.

(D) Dye solution:

Stock dye solution consisted of 250 mg Sudan Black B dissolved in 30 ml ethanol. Working dye solution was made before use by diluting 1.5 ml stock dye solution with 3.5 ml H_2O.

(E) Chamber Buffer:

Tris/glycine buffer was prepared by dissolving Tris (6.0 g) and glycine (28.8 g) in 800 ml H_2O. The pH was adjusted to 8.3 with conc. HCL and the final volume was made up to 1L with distilled water.

Procedure

To each gel tube was added 1.0 ml freshly prepared separating gel and the top of the gel was layered with H_2O. The gels were allowed to photopolymerize at room temperature. After polymerization was complete, H_2O used for layering the gels was removed and 100 μl of concentrating gel solution was added to each tube and the gels were topped again with H_2O and allowed to polymerize. To the polymerized gels were added 50 μl of serum and 200 μl of loading gel. They were mixed by invertion and the tubes were layered with chamber buffer, and allowed to polymerize for 30 min under intense light. The gel tubes were then inserted into the electrophoretic cell containing buffer. Electrophoresis

was run for 30 to 40 min at 5 mA per gel.

(b) Agarose gel electrophoresis

Human serum lipoproteins were separated using the Paragon Lipoprotein (Lipo) Electrophoresis Kit of Beckman (P/N 655910) USA.

Equipment

Electrophresis cell, power pack, sample applicator, wet processor station and dryer, all from Paragon, Beckman USA.

Reagents (Provided in the kit)

(1) B-2 Barbital buffer pH 8.6 was made by dissolving 5,5-diethylbarbituric acid (18.2 g) and 5,5-diethylbarbituric acid sodium salt in 1L H_2O.

(2) Lipoprotein working stain (0.07%): To 165 ml reagent alcohol was added 3 ml Paragon lipostain. To the throughly mixed solution was added deionized H_2O (135 ml).

(3) Fixative solution: To 180 ml reagent alcohol was added 90 ml deionized H_2O and 30 ml acetic acid.

(4) Destain solution: To 450 ml reagent alcohol was added 550 ml deionized H_2O and mixed.

Procedure

The compartments of the electrophoretic cell were filled with 45 ml buffer. Lipo gels were blotted with the gel blotter and the template aligned with position dots on the edges of the gel. Samples were applied into each template slot with the help of an applicator and stood for 5 min. The template was then blotted and the gels were placed into the gel bridge assembly, which was placed into the electrophoresis cell with a power supply. Electrophoresis was carried out for 30 min at 100 Volts. Upon completion the electrophoresis gels were placed in gel frames and immersed in fixative solution for 5 min. They were then completely dried in the dryer. The dried gels were stained by keeping in the working stain for 5 min. The excess dye was removed by destaining the gels for 5 min and finally dried in the dryer.The lipoprotein bands were quantitated with a densitometer at 600 nm.

2.3.14 Statistics

All values are analysed by students 't' test. Pearsons correlation analysis was done on the data of type I and type II diabetic patients.

Chapter 3

RESULTS & DISCUSSIONS

3.1 Human Studies

3.1.1 Serum Pseudocholinesterase activity in Type I and Type II Diabetic Patients

Aim

The aim of this study was to determine whether serum PChE activity is increased in diabetic patients and whether the increase is specific for a particular type of diabetes or common to both type I and type II.

Results

The levels of PChE activity and concentrations of glucose, lipids, C-peptide, insulin and HbA_1c in type I and type II diabetic patients were compared with the controls. Clinical characteristics of the diabetic patients are summarised

in Table 3.1. Approximately equal numbers of male and female subjects were closely matched with regard to age, weight and height.

Tables 3.2 and 3.3 gives a comparison of serum values between the control and diabetic patients (type II and type I respectively). Diabetics differed significantly from their age and sex matched controls. Serum PChE activity was found to be significantly higher ($p<0.001$) in the diabetic patients. The significant increase in C-peptide in the type II diabetics is consistent with identifying this group with the non-insulin dependent type. C-peptide level was significantly lower ($p<0.001$) in the type I patients which is common to this type of diabetes. Poor long term glycemic control is indicated by increased HbA_1c levels in these patients. The levels of serum lipids and lipoproteins, TG and TLDL were significantly higher in the diabetic patients. Serum cholesterol level was increased in both the types of diabetes, but was more significant in type I ($p<0.001$). HDL-C was significantly lower in the type II patients, while in type I there was no significant change ($p=0.600$).

Discussion

The present study on the diabetic patients reconfirms the observation made in the 1930's demonstrating an increase in serum PChE activity in diabetes (Antopol *et al.* 1937). But these investigators did not provide specific details regarding the type of diabetes or any contributing factor(s) responsible for the increase in PChE activity in diabetes mellitus. The present study demonstrates that PChE activity is elevated in both types of diabetes (20%

in type II and 43% in type I) as compared to the controls. Type II patients have increased serum C-peptide levels which suggests a certain degree of insensitivity to insulin. Low levels of C-peptide in the type I diabetic patients indicates decreased insulin secretion, characteristic of this type. HbA_1c is also significantly higher in the diabetic patients suggesting lack of glycemic control. Decrease in HDL-C is a common finding in patients with elevated VLDL, TG and in diabetes (Howard 1987). The common factor in both types of diabetes appears to be hyperlipoproteinemia especially hypertriglyceridemia. Increased serum PChE activity has been reported in type IIb and IV hyperlipoproteinemic patients (Kutty *et al.* 1981a, Jain *et al.* 1983) and hyperlipoproteinemia is known to be common in diabetes mellitus. There is a good positive correlation between PChE and TG as indicated by Pearsons correlation analysis which gave P=0.499 for type I and P=0.526 for type II diabetic patients respectively. Since the majority of TG is in the form of VLDL in the blood this suggests a relationship between PChE and VLDL. The correlation between serum PChE and the other variables like glucose, cholesterol, TLDL and HDL-C was not statistically significant.

Table 3.1:

Clinical characteristics of the patients

Type	No	Sex	Age yr	Duration of diabetes	Weight Kg	Height Cm
IDDM	53	M	12.8 ± 0.8	>1yr	48.5 ± 3.3	150.0 ± 3.7
	57	F	12.4 ± 0.6	>1yr	44.2 ± 2.8	145.4 ± 3.1
NIDDM	20	M	59.1 ± 2.5	>1yr	77.1 ± 2.9	170.5 ± 2.4
	21	F	45.1 ± 4.3	>1yr	66.2 ± 2.7	157.3 ± 1.2

Medication and other complications not recorded.

Table 3.2:

Comparison of serum values between Control

and Type II diabetic patients (NIDDM)

Analysis	Control(40)			Patients(41)*			P value
PChE (U/l)	3222.1†	±	46.4	3836	±	126	0.001
Glucose (mmol/l)	4.65	±	0.07	10.45	±	0.52	0.001
C-pep (pmol/l)	266.5	±	15.8	869.1	±	82.4	0.001
Insulin (pmol/l)	78.2	±	4.2	354.0	±	80.2	0.002
HbA_1c (%)	5.65	±	0.50	8.44	±	0.25	0.001
TG (mmol/l)	1.33	±	0.07	2.58	±	0.20	0.001
Chol (mmol/l)	5.47	±	0.12	6.02	±	0.21	0.050
TLDL (g/l)	3.47	±	0.11	4.24	±	0.22	0.025
HDL-C (mmol/l)	1.40	±	0.05	1.21	±	0.06	0.036
LDL-C (mmol/l)	3.32	±	0.11	3.63	±	0.16	0.250

† Mean ± SEM.

* Number of subjects.

Table 3.3:

Comparison of serum values between Control

and Type I diabetic patients (IDDM)

Analysis	Control(22)			Patients(110)*			P value
PChE (U/l)	3028.0†	±	95.0	4336.1	±	70.5	0.001
Glucose (mmol/l)	4.83	±	0.11	14.59	±	0.64	0.001
C-pep (pmol/l)	631.0	±	101	160	±	11.8	0.001
Insulin (pmol/l)	134.4	±	17.1	459.2	±	35.9	0.001
HbA_1c (%)	5.36	±	0.16	8.57	±	0.23	0.002
TG (mmol/l)	1.19	±	0.09	2.15	±	0.07	0.001
Chol (mmol/l)	3.59	±	0.14	4.67	±	0.07	0.001
TLDL (g/l)	1.96	±	0.09	2.92	±	0.07	0.001
HDL-C (mmol/l)	1.36	±	0.09.	1.42	±	0.03	0.600
LDL-C (mmol/l)	1.74	±	0.12	2.27	±	0.06	0.001

† Mean ± SEM.

* Number of subjects.

3.2 Animal Studies

3.2.1 Type I Diabetic Model

Induction of diabetes with streptozotocin and alloxan

Aim

This experiment was designed to determine a) whether serum PChE activity increase occurs with induction of hyperglycemia in the experimental animal that mimics the type I diabetic patient and b) whether a relationship exists between serum glycerol, TG and PChE activity.

Results

Table 3.4 shows a comparison of serum values of rats before and after induction of diabetes with SZ. The difference in the number of animals between the control (38) and test (30) is because a few died during/after blood collection and a few after the administration of SZ. Intraperitoneal administration of a single dose (100 mg/kg body wt.) of SZ resulted in a significant increase in serum glucose and serum PChE activity within 72 hr of the treatment, with a concomitant increase in TG, glycerol and TLDL levels. The decrease in cholesterol level was insignificant ($P=0.710$), while HDL-C decreased significantly ($p<0.05$). A decrease of 48% in circulating insulin levels was observed in SZ treated rats. Adipose tissue PChE activity was increased significantly in the SZ diabetic rats ($p<0.001$), while no change in liver PChE activity was

noted ($p=0.076$, Table 3.5). Liver TG showed a significant increase after SZ administration ($p<0.05$). The weights of control and diabetic rats were similar at the start of the experiment, but, on development of diabetes the SZ treated rats lost 12% of their body weight compared to the normals.

Induction of diabetes with alloxan, like SZ significantly increased the serum glucose level and PChE activity within 72 hr (Table 3.6). Serum TG, cholesterol, TLDL and glycerol levels were also significantly elevated.

Discussion

Streptozotocin was the chemical used for the induction of diabetes in all the experiments except in one. Diabetes was induced with alloxan only in one set of rats to verify whether increase in PChE activity was consistent with chemically induced diabetes irrespective of the type of diabetogenic agent used.

A 48% decrease in the circulating insulin level indicates damage to the β cell. In previous studies Schein *et al.* (1971) and Bar-On (1976) observed decreases of 50% and 60% respectively in the serum insulin levels after treatment with SZ. Increased serum PChE activity in SZ induced diabetic rats is consistent with the obsevations of Deshmukh (1986) on alloxan induced diabetic rats. The increase in TG levels in the diabetic rats appears to be a result of increased VLDL. This assumption is based on the following reasons, (a) all blood samples were collected after an overnight fast, (b) lipoprotein electrophoresis did not show the presence of any chylomicrons but gave a dense pre-β lipoprotein (VLDL) band (Fig 3.3) (c) and increased serum TLDL was

noted (a measure of lipoproteins of density < 1.006, Table 3.4). The increase in glycerol level occured in parallel with the elevation of TG and TLDL. Glycerol is a measure of the degree of lipolysis in the adipose tissue. Hence the increase of serum glycerol in the diabetic rats indicates increased lipolysis. Earlier studies suggested that the increase in VLDL-TG in the diabetic rats was due to both enhanced VLDL-TG synthesis and secretion (Nikkila & Kekki 1973). Increased fatty acid mobilization due to increased lipolysis appears to be the reason. This agrees well with the increase in serum glycerol, TG and liver TG found in the present study in the diabetic rats. Serum PChE activity increased together with TG and glycerol, indicating a possible relationship between PChE activity and VLDL metabolism. Decrease in serum cholesterol level in the SZ induced diabetic rats appears to be a result of the significant decrease in serum HDL-C concentration (Table 3.4).

The origin of serum PChE is suggested to be from the liver (Silver 1974). However the liver PChE activity in the diabetic rats did not differ significantly from the controls (Table 3.5). The reason for this could be that, though PChE is synthesized in the liver, it may be rapidly secreted into the circulation.

Table 3.4:

Comparison of serum values before and after induction of diabetes with Streptozotocin

Analysis	Before induction (38)	After induction (30)*	P value
Glucose (mmol/l)	5.09† ± 0.21	27.7 ± 1.334	0.001
PChE (U/l)	243.1 ± 7.2	381.5 ± 11.8	0.002
Insulin (pmol/l)	65.9 ± 4.7	34.8 ± 1.05	0.001
TG (mmol/l)	0.63 ± 0.05	2.43 ± 0.24	0.001
Glycerol (mmol/l)	0.33 ± 0.01	0.52 ± 0.03	0.001
Chol (mmol/l)	1.62 ± 0.06	1.58 ± 0.09	0.710
TLDL (g/l)	0.17 ± 0.01	0.27 ± 0.02	0.001
HDL-C (mmol/l)	1.40 ± 0.07	1.19 ± 0.07	0.041
Weights (g)	259 ± 1.49	229.7 ± 1.36	0.001

† Mean ± SEM.

* Number of animals.

Table 3.5:

Tissue levels of TG and PChE in Control

and Diabetic rats

Tissue	Control(12)			Diabetic(8)*			P value
Liver PChE	0.90†	±	0.04	1.01	±	0.03	0.076
Liver TG	19.85	±	0.68	23.36	±	1.19	0.024
Adipose PChE	0.35	±	0.01	0.43	±	0.01	0.001

† Mean ± SEM.

* Number of animals.

Expression of data: liver TG μmol/gm wet wt., liver and adipose tissue PChE μmol/min/g wet wt.

Table 3.6:

Comparison of serum values before and after induction of diabetes with Alloxan

Analysis	Control(26)*			Diabetic(16)			P value
Glucose (mmol/l)	5.55†	±	0.18	28.2	±	2.95	0.001
PChE (U/l)	252.9	±	10.3	372.4	±	23.7	0.001
TG (mmol/l)	0.84	±	0.05	1.95	±	0.13	0.001
Glycerol (mmol/l)	0.30	±	0.01	0.63	±	0.02	0.001
Chol (mmol/l)	1.35	±	0.05	1.81	±	0.07	0.001
TLDL (g/l)	0.13	±	0.00	0.21	±	0.01	0.001
Weight (g)	255	±	4.20	220.5	±	2.10	0.002

† Mean ± SEM.

* Number of animals.

3.2.2 Effect of Insulin Treatment on Diabetic Rats

Aim

The aim of the following experiment was to determine whether control of hyperglycemia with insulin affects serum PChE activity besides normalizing lipid levels.

Results

Figs 3.1 and 3.2 illustrate the effects of insulin treatment and withdrawal on diabetic rats. The difference in the number of animals was the result of deaths occuring due to the experimental treatments. Induction of diabetes caused a significant increase in the levels of serum glucose, PChE activity, TG, glycerol and TLDL. Changes in serum HDL-C and cholesterol levels were statistically insignificant ($p=0.330$ & 0.610 respectively). Diabetic rats were given 3 units of insulin/day subcutaneously for a week. After glycemic control was achieved as detected by monitoring urinary glucose, blood was collected and insulin treatment was discontinued. Treatment with insulin resulted in a significant reduction in the serum glucose level ($p<0.001$). Concomitant with the control of diabetes the serum levels of PChE activity, glycerol and TG also decreased significantly, while the decrease in TLDL level was not statistically significant ($p=0.820$) compared to the diabetic rats. In contrast, cholesterol and HDL-C showed a significant increase (p ~0.005). Glycosuria reappeared on the 3rd day of insulin deprivation. Following withdrawal of insulin the levels of serum

glucose, PChE activity, TG, TLDL, glycerol, cholesterol and HDL-C reverted to those observed in the diabetic state.

The electrophoretic patterns of serum lipoproteins obtained on 10% polyacrylamide gels of rat serum isolated before and after induction of diabetes and after treatment and withdrawal of insulin are shown in Fig 3.3. A dense VLDL band (pre-β lipoprotein) was visible in the serum isolated from the diabetic rat and was undoubtedly responsible for the increased serum TG concentration observed (Fig 3.1). The LDL (β-lipoprotein) and HDL (α-lipoprotein) bands were less pronounced in the diabetic compared to the normal rat serum. Insulin treatment reduced the VLDL band and increased HDL. Withdrawal of insulin increased the VLDL band again with a decrease in HDL and LDL bands.

Discussion

The decrease in serum PChE activity after insulin treatment with a concomitant decrease of TG which is essentially associated with VLDL, indicates a close relation between PChE activity and VLDL. The decrease in serum glycerol concentration due to insulin treatment suggests an inhibition of the accelerated lipolysis in the adipose tissue which is usually found in diabetes. Therefore the decrease of TG with the decrease in serum glycerol is an indication that part of the reason for hyper-pre-β-lipoproteinemia or increased VLDL may be the overproduction of VLDL in the liver. The reduction in serum TG was more marked than the PChE activity in the diabetic rats after

insulin treatment (Fig 3.1). Insulin treatment is known to activate lipoprotein lipase (LPL) which facilitates the rapid clearance of VLDL-TG from the circulation (Schantz & Williams 1963). This accounts for the marked decrease of TG in the diabetic rats treated with insulin. On the other hand, though PChE activity decreased significantly after insulin treatment it was not as marked as serum TG. The possible reason for this could be, that LPL has no effect in the clearance of PChE from the circulation.

About 60-70% of serum cholesterol is in the form of HDL-C in the rats. Insulin treatment decreased VLDL significantly but at the same time elevated HDL (Fig 3.3). This explains why, serum cholesterol was significantly ($p<0.005$) increased in the insulin treated rats.

Withdrawal of insulin elevated the serum glucose concentration to almost that level observed before insulin treatment (Fig 3.1). SZ is known to cause irreversible damage to the pancreatic β cells (Junod *et al.* 1969). This is confirmed by the 48% decrease in circulating insulin levels (Table 3.4) observed within 72 hr after injection of the diabetogenic agent. Serum TG, glycerol and TLDL levels were also increased after withdrawal of insulin. Plasma lipid levels are related to the degree of glycemic control (Sosenko *et al.* 1980, Lopes-Virella *et al.* 1981). PChE activity also increases with the reappearance of hyperglycemia and hyperlipidemia.

Fig 3.1: Effect of insulin treatment and withdrawal on diabetic rats

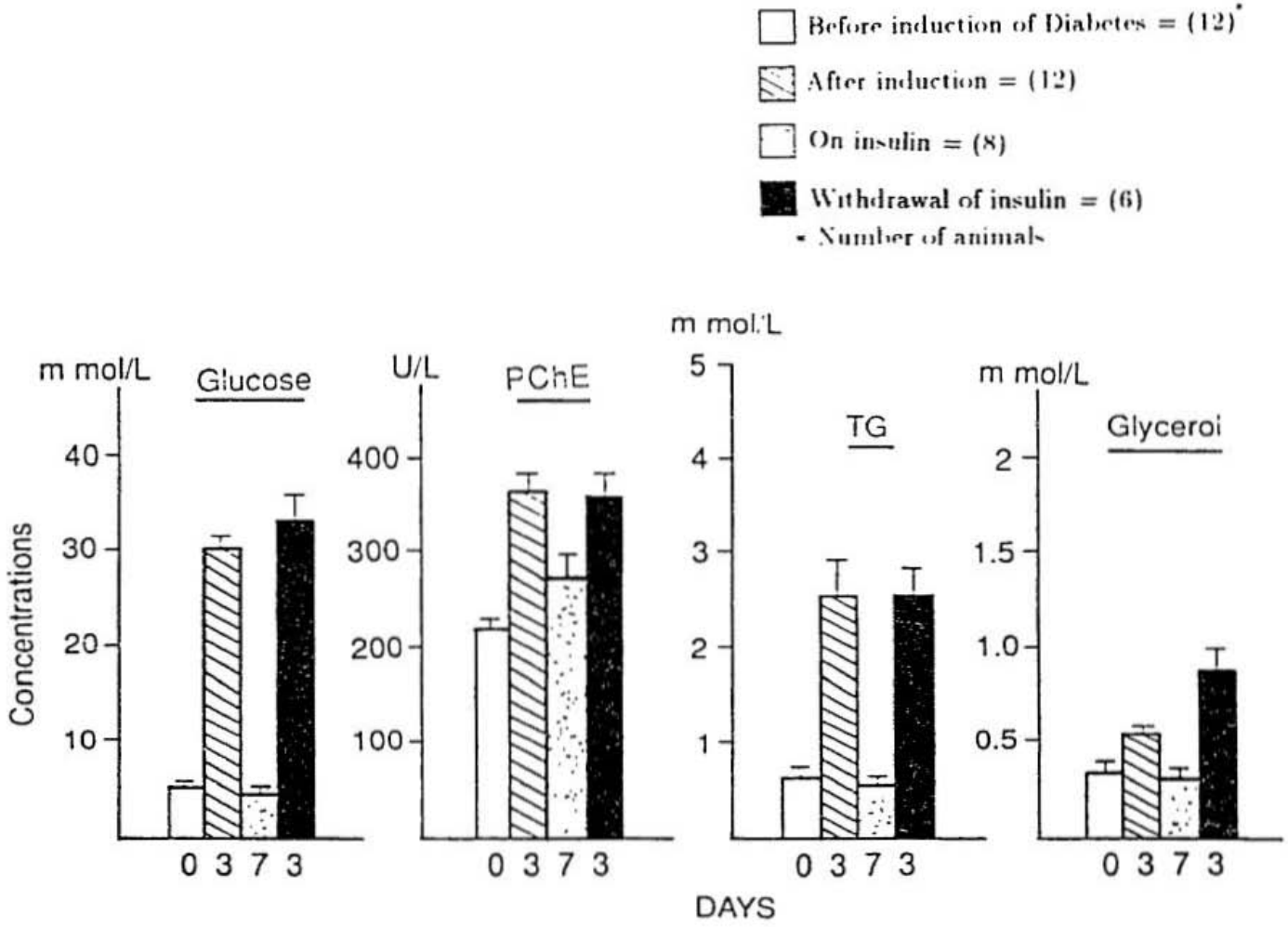


Fig 3.2: Effect of insulin treatment and withdrawal on diabetic rats

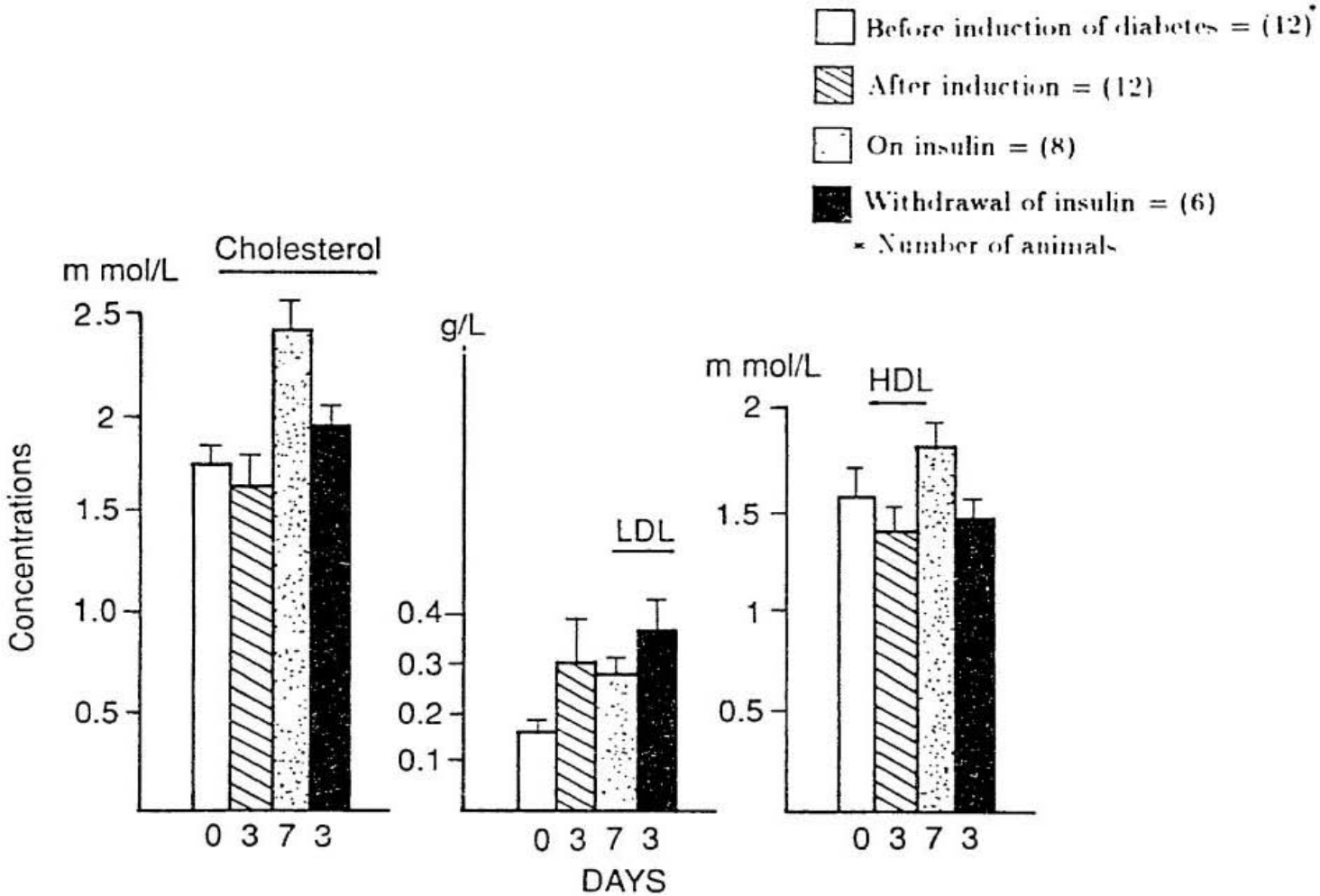


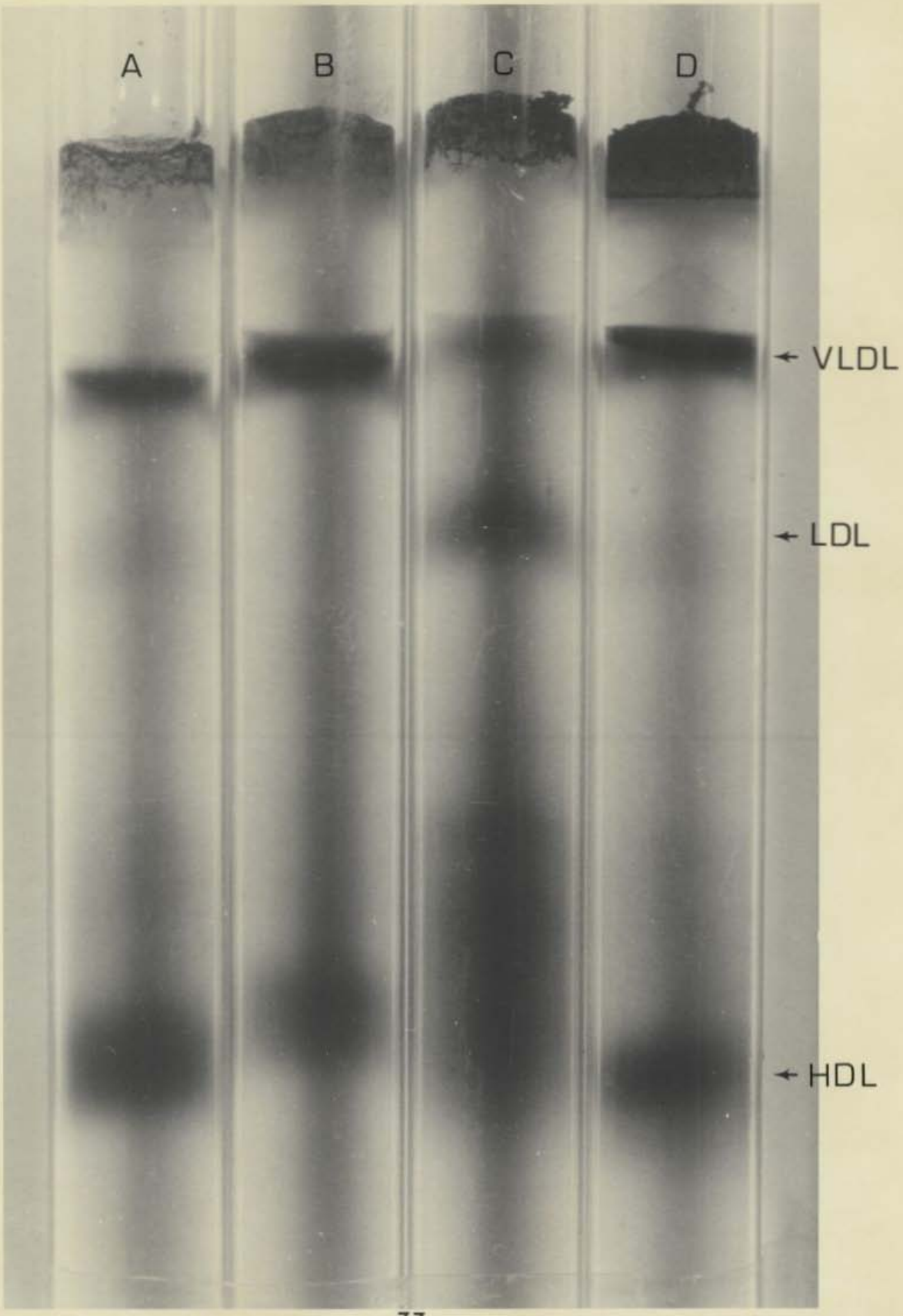
Fig 3.3: Electrophoretogram of serum lipoproteins

A - gel showing the lipoprotein pattern of normal rat serum.

B - lipoprotein pattern 72 hr after induction of diabetes with SZ.

C - lipoprotein pattern one week after insulin treatment of diabetic rat.

D - lipoprotein pattern 3 days after withdrawal of insulin treatment.


A
B
C
D
VLDL
LDL
HDL

3.2.3 Effect of Iso-OMPA on Diabetic and Control Rats.

Aim

The objective of the following study was to determine whether the inhibition of PChE activity in general and specifically in the adipose tissue can decrease lipolysis in the diabetic rat thereby decreasing the efflux of glycerol and fatty acids necessary for the TG and VLDL synthesis. If this were true it would also indicate that part of the reason for the increase in TG and VLDL levels in diabetes mellitus is due to their overproduction.

Results

Two dose levels (17.6 & 8.2 mg/rat) of iso-OMPA (specific PChE inhibitor, from Sigma Chemicals Co. USA) were administered to the diabetic rats intraperitoneally dissolved in physiological saline for a period of 6 days. Comparisons of the serum values of the normal rats with diabetic and iso-OMPA treated diabetic rats are indicated in the Table 3.7. The levels of serum PChE activity, glucose, TG, TLDL and glycerol were significantly higher in the diabetic rats than the controls, while serum cholesterol showed no significant change ($p=0.860$). These findings are similar to those observed in the previous experiments (Table 3.4; Figs 3.1 & 3.2). At both levels of iso-OMPA, PChE inhibition was highly significant. However the residual PChE activity at the lower dose of iso-OMPA was twice that of the higher dose level. The

administration of a high dose of iso-OMPA to the diabetic rats produced a significant reduction in the serum TG, glycerol, cholesterol and TLDL levels compared to the untreated diabetic rats. At lower doses there was also a significant reduction in the above mentioned variables compared to the untreated diabetic rats. But, when compared to the serum values obtained with the higher dose of iso-OMPA the only significant differences found were between serum glucose, cholesterol and TLDL. Serum glucose was significantly ($p<0.005$) reduced with the high dose of iso-OMPA treatment, whereas the low dose had no effect on serum glucose level. Liver TG and PChE activity in the adipose tissue and liver were significantly reduced in the iso-OMPA treated diabetic rats compared to the untreated diabetic rats (Table 3.8). The inhibition of PChE activity in the normal rats also resulted in a significant decrease in the serum glycerol and TG levels (Fig 3.5). A decrease in liver TG and PChE activity in both liver and adipose tissue was also noted (Table 3.9).

A second set of diabetic rats (6) were also treated intraperitoneally with iso-OMPA (17.2 mg/rat) for 6 days. At the end of the sixth day iso-OMPA treatment was discontinued for 3 days to observe the effect of withdrawal of inhibition of PChE activity on glucose, lipid and lipoprotein levels in the diabetic rats. Fig 3.4 illustrates the effect of iso-OMPA treatment and withdrawal on diabetic rats. As in the previous experiments, 72 hr after injection of SZ a significant increase in serum glucose, PChE, TG and glycerol was observed. Inhibition of PChE with iso-OMPA resulted in a significant decrease in glucose, TG and glycerol. Withdrawal of iso-OMPA treatment caused a significant

increase in PChE activity along with an increase in serum glucose, TG and glycerol levels. The reason for the excessive overshoot in serum glucose and glycerol due to the withdrawal of iso-OMPA is not clear.

The electrophoretogram of serum lipoproteins on 10% polyacrylamide gel (Fig 3.6) indicated the absence of chylomicrons. A dense pre-β-lipoprotein band was observed after induction of diabetes which decreased after iso-OMPA treatment. Diabetic and iso-OMPA treated diabetic rat serum showed β and α-lipoprotein bands which appeared lighter than the normal serum.

Fig 3.7 shows the electrophoretogram of serum lipoproteins of normal and iso-OMPA treated rats. Pre-β, β and α-lipoproteins were decreased after the iso-OMPA treatment of normal rats.

Discussion

Iso-OMPA treatment of the diabetic rats caused a marked inhibition in serum, liver and the adipose tissue PChE activity (Tables 3.7 & 3.8). A significant reduction in serum glycerol was also observed. This suggests that PChE in the adipose tissue may have a role in lipolysis. The possibility that PChE behaves like a hormone sensitive lipase was suggested by Szendzikowki *et al.* (1961/62) and Colville *et al.* (1964). They showed that specific (iso-OMPA) and non-specific inhibitors of PChE like neostigmine and physostigmine at dose levels sufficient to inhibit PChE activity decreased hormone mediated release of fatty acid from the rat aorta. Subsequent to the inhibition of lipolysis with iso-OMPA serum TG and VLDL decreased markedly (Figs 3.4 & 3.6).

Decrease in the glycerol and TG levels in the serum (Table 3.7) together with a reduction in liver TG (Table 3.8) provides substantial support that VLDL-TG production is higher in diabetic rats when compared to the normal rats. Within a short period of 3 days, withdrawal of iso-OMPA treatment caused all the serum values to rise almost to those observed in the diabetic state. A decrease in serum glucose was also observed but only with the higher dose of iso-OMPA. It is not possible to explain this observation.

Treatment of normal rats with both high and low doses of iso-OMPA also decreased serum TG, VLDL and TLDL, while glycerol did not show any significant change. This suggests that PChE may have a role in VLDL assembly and secretion and its final transformation into LDL. Earlier studies have indicated a stablizing effect of PChE on LDL (Kutty *et al.* 1973). The exact biochemical and molecular mechanism associated with the changes in PChE activity and its relationship to lipid metabolism in general and lipoprotein metabolism in particular needs to be studied thoroughly to explain this finding.

Table 3.7:

Effect of two dose levels of iso-OMPA on diabetic rats

Analysis	Control (14)*	Diabetic (14)	Higher dose(7)	Lower dose(7)
Glucose (mmol/l)	4.19† ± 0.09	32.77 ± 1.33[a]	25.8 ± 1.928[b]	32.74 ± 1.98[d]
PChE (U/l)	230 ± 7.01	440 ± 18.5[b]	35.83 ± 0.83[a]	69.43 ± 1.94[a]
TG (mmol/l)	0.99 ± 0.08	4.62 ± 0.62[a]	0.51 ± 0.08[a]	0.62 ± 0.10[b]
Chol (mmol/l)	1.25 ± 0.07	1.2 ± 0.12[d]	0.38 ± 0.098[a]	0.7 ± 0.22[c]
Glycerol (mmol/l)	0.32 ± 0.02	0.72 ± 0.07[c]	0.39 ± 0.06[c]	0.37 ± 0.07[b]
TLDL (g/l)	0.12 ± 0.01	0.56 ± 0.12[b]	0.05 ± 0.02[c]	0.13 ± 0.02[c]

† Mean ± SEM.

* Number of animals.

P values for diabetic vs control; iso-OMPA treated diabetic vs diabetic rats: a<0.001; b<0.005; c<0.05; d= 0.83-0.86.

Table 3.8:

Tissue levels of TG and PChE in Diabetic

and Iso-OMPA treated diabetic rats

Tissues	Diabetic rats(8)			Iso-OMPA Treated(7)*			P value
Liver PChE	1.01†	±	0.03	0.40	±	0.01	0.001
Adipose PChE	0.43	±	0.01	0.13	±	0.01	0.001
Liver TG	23.36	±	1.19	13.50	±	2.00	0.001

† Mean ± SEM.

* Number of animals.

Values are expressed as: liver and adipose tissue PChE μmol/min/g wet wt.; liver TG μmol/g wet wt.

Table 3.9:

Tissue levels of TG and PChE in Normal and

Iso-OMPA treated rats

Tissues	Normal Rats(12)			Iso-OMPA treated(6)*			P value
Liver PChE	0.90†	±	0.04	0.51	±	0.03	0.001
Adipose PChE	0.35	±	0.01	0.04	±	0.01	0.001
Liver TG	19.85	±	0.68	16.93	±	0.86	0.001

† Mean ± SEM.

* Number of animals.

Values are expressed as: liver and adipose tissue PChE μmol/min/g/ wet wt.; liver TG μmol/g wet wt.

Fig 3.4: Effect of iso-OMPA treatment and withdrawal on diabetic rats

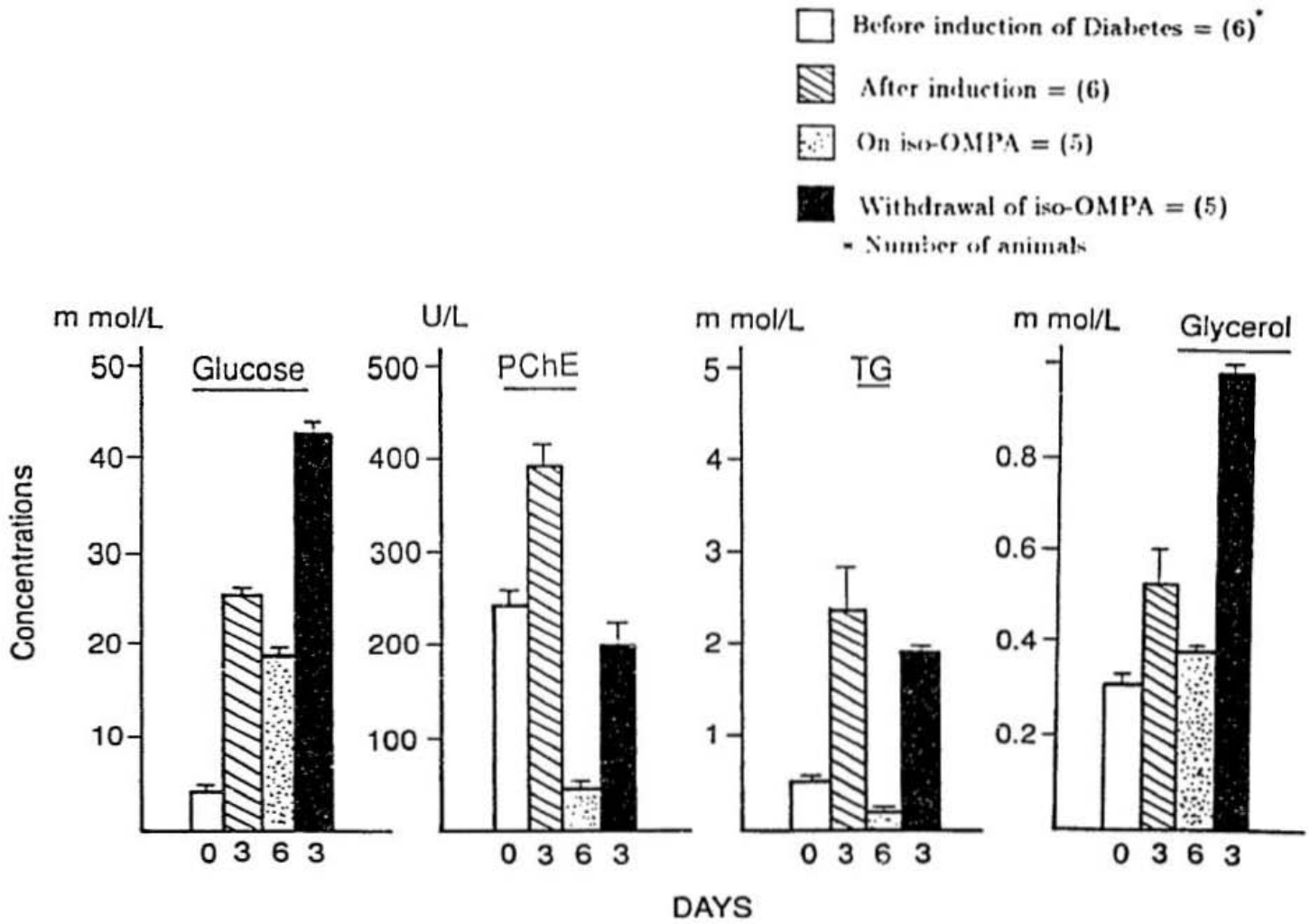


Fig 3.5: Effect of inhibition of PChE activity with iso-OMPA in normal rats

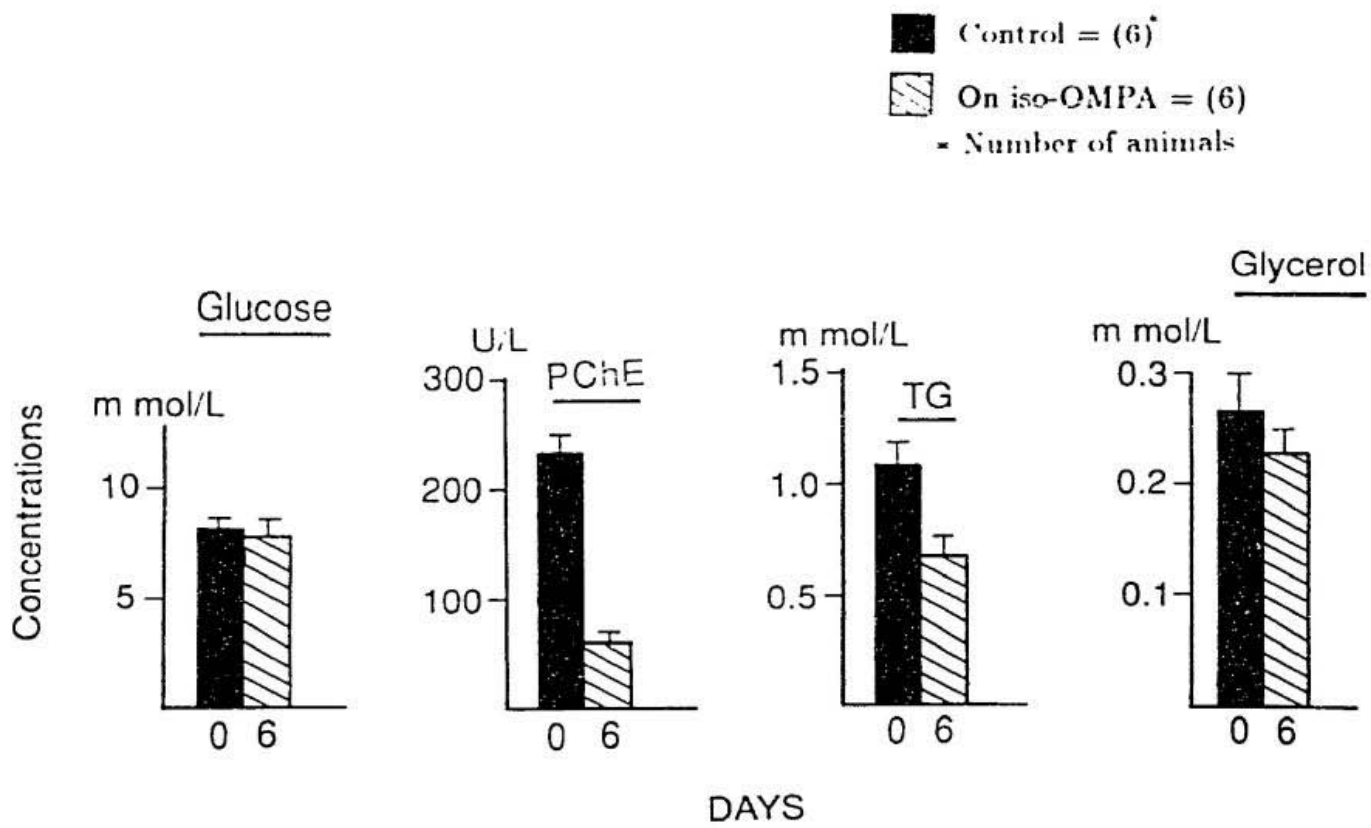


Fig 3.6: Electrophoretogram of serum lipoproteins

A - gel showing the lipoprotein pattern of normal rat serum.

B - lipoprotein pattern 72 hr after induction of diabetes with SZ.

C - lipoprotein pattern 6 days after iso-OMPA treatment of diabetic rat.

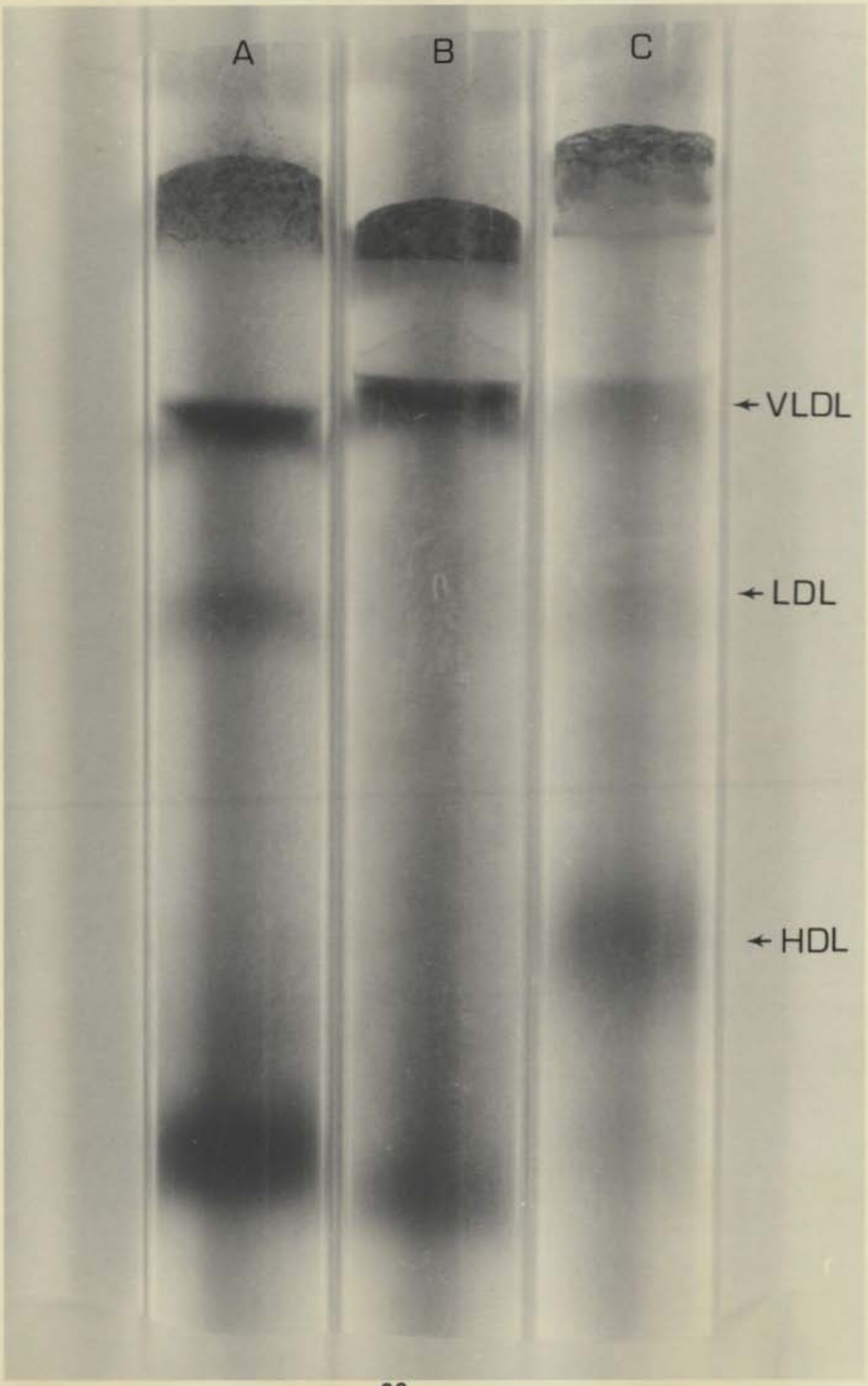

A
B
C
VLDL
LDL
HDL

Fig 3.7: Electrophoretogram of serum lipoproteins

A - gel showing the lipoprotein pattern of normal rat serum.

B - lipoprotein pattern after 6 days of iso-OMPA treatment.


A
B
VLDL
LDL
HDL

3.2.4 Effect of Heparin on PChE activity and Lipids in Diabetic Rats

Aim

The objective of this experiment was to determine whether heparin injection could decrease serum TG in the diabetic rats and concurrently to evaluate its effect on serum PChE activity.

Results

Table 3.10 shows the comparison of the levels of serum glucose, lipids and PChE activity between (1) control & diabetic, (2) diabetic and heparin treated diabetic rats and (3) diabetic and saline treated diabetic rats.

A marked increase in serum glucose 72 hr after SZ treatment was observed along with a significant elevation in the levels of PChE activity, TG and TLDL. No change in HDL-C ($p=0.090$) and cholesterol levels ($p=0.660$) was observed compared to the control. Injection of heparin (500 units) through the tail vein to 5 diabetic rats decreased serum TG levels by 85% within 30 min but PChE activity, HDL-C, TLDL and cholesterol levels remained unaffected. A rise in serum glucose was observed which was not significant ($p=0.630$).

Saline treatment of the diabetic rat had no effect on the serum PChE activity or lipid levels. However serum glucose increased significantly ($p<0.05$). The rise in the serum glucose levels in the heparin and saline treated rats could be due to the stress of handling.

Discussion

Serum TG level decreased by 85% within 30 min of heparin administration without affecting PChE activity. A decrease in TG was expected since heparin is known to release lipoprotein lipase (LPL) (Brunzell, Porte & Bierman 1979) which hydrolyses VLDL-TG, thus reducing the blood levels of both TG and TG rich-lipoproteins (VLDL) (Robinson & Speake 1989). No significant change in cholesterol ($p=0.410$) and HDL-C ($p=0.510$) was observed which agrees with the earlier report of the effects of heparin treatment (Arbeeny & Eder 1989). These investigators also used the SZ-diabetic rat model to study the effects of heparin treatment in the serum lipid and lipoprotein levels. They recorded a decrease of 67% in the serum TG level within 15 min of heparin with no change in glucose and cholesterol levels. These observations are consistent with the view that one of the reasons for hypertriglyceridemia in diabetes is due to reduced clearance of VLDL-TG (Reaven & Reaven 1974). Since serum PChE activity was not affected by heparin, this indicates that its increase in the diabetic rats is not due to the serum level of TG or decreased LPL.

Table 3.10:

Comparison between the serum values of Control & Diabetic and between Diabetic & Heparin, Saline treated diabetic rats

Analysis	Control (10)*	Diabetic (10)	Heparin treated(5)	Saline treated(5)
Glucose (mmol/l)	6.34† ± 0.33	29.81 ± 1.66 [a]	31.12 ± 2.06[c]	35.84 ± 1.69[b]
PChE (U/l)	231.6 ± 18.8	417.0 ± 26.7[a]	407.4 ± 29.2[c]	420.8 ± 41.5[c]
TG (mmol/l)	0.79 ± 0.08	4.47 ± 0.8[a]	0.65 ± 0.17[a]	5.67 ± 1.54 [c]
Chol (mmol/l)	1.71 ± 0.11	1.83 ± 0.17 [c]	1.59 ± 0.18[c]	1.79 ± 0.13[c]
HDL-C (mmol/l)	1.33 ± 0.08	1.13 ± 0.08[d]	1.04 ± 0.10[c]	1.07 ± 0.14[c]
TLDL (g/l)	0.13 ± 0.02	0.33 ± 0.04[a]	0.24 ± 0.06 [c]	0.36 ± 0.08[c]

† Mean ± SEM.

* Number of animals.

P values: a<0.001; b<0.05; d=0.090; c=0.240-0.960.

3.2.5 Type II Diabetic Model

Aim

The objective of this experiment was to determine whether serum PChE and TG increase concurrently in the type II diabetic model.

Results

Table 3.11 shows the comparison between (1) the normal and Gold thioglucose (GTG) treated mice and (2) GTG treated and GTG+iso-OMPA treated mice.

Each mouse was injected GTG (0.5 mg/g body wt) intraperitoneally (Marshall *et al.* 1955) while the control group was injected with the same volume of saline. Their weights and food intake was monitored every two days. Two weeks after the first injection of GTG, a second injection of the same dose was given to the same mice. Two weeks later the GTG treated mice were allowed water containing 10% sucrose and normal mouse chow for another two weeks. At the start of the experiment all the mice weighed 37-39 g, but at the end of the experiment the GTG treated mice gained an average of 40% more weight than the untreated. A striking elevation (72%) in the serum insulin level was also noted in the GTG treated animals fed 10% sucrose with a concomitant rise in the levels of PChE activity, TG, TLDL, glycerol and HDL-C compared to the controls.

Serum PChE activity was 91% inhibited within 24 hr after a single intraperitoneal injection of iso-OMPA (17.6 mg) of the GTG treated mice. A

significant decrease in serum TG, TLDL, and glycerol was also observed (Table 3.11). The decrease in serum glucose concentration was insignificant (p=0.086) while no change in the HDL-C and cholesterol levels was observed.

GTG significantly increased liver PChE activity but had no effect on adipose tissue PChE activity (Table 3.12). Iso-OMPA treatment of GTG treated mice caused total inhibition in adipose tissue PChE activity and 81% inhibition in the liver.

Discussion

GTG has been used experimentally to produce obesity. These animals are known to exhibit hyperglycemia, hyperinsulinemia along with abnormal glucose tolerance indicating the presence of insulin resistance similar to type II diabetic patients (Jeanrenaud 1977). Similar to the type I diabetic model, induction of diabetes with GTG caused an increase in the levels of serum PChE activity along with TG, glycerol and TLDL. However, unlike the type I diabetic rat model, these animals gained weight. Hyperphagia is a factor responsible for the development of obesity and diabetes in this model. Insulin levels were much higher in the GTG treated mice compared to the controls (Table 3.11). Increase in VLDL and TG in these animals are a result of both excess food intake and increased mobilization of fatty acids. Increase in PChE activity has been demonstrated in the genetically diabetic mice (db/db) (Kutty *et al.* 1981b) and Zuckar fat rats (Kutty *et al.* 1984); both are models of type II diabetes. Treatment with iso-OMPA of the GTG induced diabetic mice,

decreased glycerol, TG and TLDL similar to SZ diabetic rats. These results suggest that increase in PChE activity may be related to hyperlipoproteinemia and specially to overproduction of VLDL-TG.

Table 3.11:

Comparison between Control, GTG and

GTG + iso-OMPA treated mice

Analysis	Control(12)	GTG(8)	GTG+ iso-OMPA(10)*
Glucose mmol/l	10.99† ± 0.88	16.40 ± 2.82 [c]	11.43 ± 1.36 [d]
PChE U/l	3650 ± 166	4856 ± 307 [b]	427.5 ± 29.2 [a]
TG mmol/l	1.53 ± 0.19	3.56 ± 0.44[b]	1.08 ± 0.11[a]
Chol. mmol/l	3.37 ± 0.22	4.14 ± 0.27[c]	4.96 ± 0.41[d]
TLDL g/l	0.74 ± 0.16	2.19 ± 0.29[a]	0.58 ± 0.07[a]
Glycerol mmol/l	0.92 ± 0.06	1.47 ± 0.09[a]	0.74 ± 0.06[c]
HDL-C mmol/l	3.61 ± 0.21	4.46 ± 0.26[c]	5.52 ± 0.49[d]
Insulin pmol/l	61.0 ± 6.70	221.5 ± 22.0[a]	ND

† Mean ± SEM.

* Number of animals.

ND-Not done as sample quantity was insufficient.

P values: a<0.001; b<0.005; c<0.05; d= 0.08-0.12.

Table 3.12:

Comparison of tissue PChE between control, GTG treated and GTG + iso-OMPA treated mice

Tissues	Control(6)	GTG (6)	GTG + iso-OMPA(6)*
Liver	1.62† ± 0.15	2.82 ± 0.24[b]	0.55 ± 0.08[b]
Adipose	0.30 ± 0.02	0.2 ± 0.029[a]	0

† Mean ± SEM. * Number of animals.

P values: b<0.001, a<0.240.

0-PChE activity not detected.

Values are expressed as: liver and adipose tissue PChE μmol/min/g wet wt.

3.2.6 Effect of inhibition of PChE activity in normal rats

Aim

The aim of the present study was to determine whether the inhibition of PChE activity in normal rats can cause an accumulation of choline or cholinesters in the serum and adipose tissue or influence their excretion in the urine.

Results

The effects of inhibition of PChE activity on choline and cholinester levels in serum, liver and urine are reported in Table 3.13. Normal rats (5) were injected intraperitoneally with iso-OMPA (5 mg/kg body wt.) for a period of 5 days. Control rats received the same volume of physiological saline. Iso-OMPA treatment produced almost 70% inhibition in serum and 80% in the liver PChE activity in these animals. PChE activity was much lower when measured with butyrylthiocholine (BTC) as substrate compared to propionylthiocholine (PTC). No PChE activity was detected with BTC as substrate in the serum of iso-OMPA rats. Also no statistically significant difference was observed in the choline and cholinester levels in the liver, serum or urine between the test and control rats. Qualitative determination of cholinesters by thin layer chromatography (TLC) indicated that cholinester substrates were not present. The cholinesters from the samples were precipitated with aqueous ammonium reineckate. The precipitates were dissolved in acetone and chromatographed

on silica G plates. Butyrylcholine, acetylcholine, propionylcholine and choline were used as reference compounds. The spots were visualized by spraying with Dragandroff's reagent.

Discussion

Whittaker (1951) suggested that butyrylcholine may be the true substrate for PChE as it is hydrolysed more rapidly than any other choline ester. Similarly Clitherow *et al.* (1963) postulated that PChE may be involved in the hydrolysis of BuCh which is a toxic compound, that is formed from Butryl-CoA and choline during fatty acid metabolism in the liver. Funnel & Oliver (1965) have speculated that PChE was involved in the homeostatic mechanism controlling the level of choline in the plasma and acetyl choline in the brain. They made this hypothesis on the assumption that PChE hydrolyzed cholinesters, thereby controlling plasma choline levels.

If the true biological function of PChE was to hydrolize choline esters, then its inhibition should have caused an accumulation of choline or choline esters in tissues or an increased excretion in the urine. Such a finding would have been consistent with the role proposed by Clitherow *et al.* (1963) in regard to lipid metabolism and choline homeostasis by Funnell *et al.* (1965). But from these results it is clear that no qualitative or quantitative alteration of either choline or choline ester in serum, liver or urine was produced by the *in vivo* inhibition of PChE.

Based on these findings it appears that the biological function of this en-

zyme is unrelated to its cholinester hydrolysing property.

Table 3.13:

Effect of inhibition of PChE activity on choline and cholinester levels in liver, serum and urine of rats

Sample	Choline	Cholinester	PChE (*BTC*[1])	PChE (*PTC*[2])
Liver				
Control(5)*	0.91† ± 0.08	0.15 ± 0.02	0.55 ± 0.07	1.47 ± 0.14
Test (5)	0.97 ± 0.10	0.13 ± 0.01	0.18 ± 0.02[a]	0.27 ± 0.01[a]
Serum				
Control(6)	67.1 ± 13.1	40.6 ± 10.1	67 ± 1.78	336 ± 16.8
Test (5)	79.5 ± 16.4	39.5 ± 9.0	0	93.7 ± 9.03[a]
Urine				
Control(5)	25.0 ± 4.0	20.8 ± 2.7	ND	ND
Test (5)	20.1 ± 3.5	17.9 ± 3.4		

[1] Butyrylthiocholine, [2] Propionylthiocholine.

† Mean ± SEM. * Number of animals.

a ($p<0.05$). ND-Not done. 0-PChE activity not detected.

Expression of data: Serum and urine choline and cholinester mmol/l; liver choline and cholinester mmol/g wet wt; PChE activity of serum μ mol/min/l and liver μ mol/min/g wet wt.

Chapter 4

CONCLUSIONS

In 1937 it was observed that serum PChE activity was increased in the blood of patients with diabetes mellitus, but there has been very little progress made since to explain this observation. Furthermore, no specific function(s) has been assigned to this enzyme.

In the present study, a positive correlation has been found between serum PChE activity and TG concentrations in both type I and type II diabetic patients. Several reports in the literature have suggested that PChE is involved in lipid metabolism. For example, increased PChE activity has been reported to occur in humans with type IIb and type IV hyperlipoproteinemia (Kutty *et al.* 1981a; Jain *et al.* 1983), genetically ob/ob and db/db mice (Kutty *et al.* 1981b) and zucker fat rats (Kutty *et al.* 1984).

Since abnormal lipid metabolism, involving TG and VLDL, is commonly found in patients with diabetes mellitus (Howard 1987) it was of interest to determine to what extent PChE could be correlated with lipid metabolism in

the diabetic state. This has been investigated with animal models of type I and type II diabetes mellitus.

Severe hyperglycemia, induced by SZ or alloxan treatment of rats, produced marked elevations in serum TG, TLDL and glycerol concentrations as well as PChE activity. When the hyperglycemia was controlled by the administration of insulin, serum glucose, lipids and lipoprotein concentrations returned to normal. PChE activity in the serum was also reduced in parallel.

The fluctuation in the level of serum glycerol was of particular interest since it gives a measure of the degree of lipolysis in the adipose tissue. An increase in hormone mediated lipolysis is also known to occur in diabetes mellitus. In addition, LPL activity is known to be reduced in the absence of insulin and to be activated by heparin in diabetic animals (Brunzell, Porte & Bierman 1979). Heparin administration produced a marked decrease in serum TG in the present study, however, serum PChE activity was not altered.

The type II diabetic model (Gold thioglucose treated mice) also showed parallel changes in both serum PChE activity and lipid concentrations with the development of hyperglycemia.

The effect of PChE inhibition, was studied by administration of iso-OMPA to diabetic and control rats. Diabetic rats showed a reduction in serum levels of TG, TLDL and glycerol. The fall in glycerol indicates reduced lipolysis of adipose tissue TG, which may be related to inhibition of PChE activity in this tissue (or a direct effect of iso-OMPA on the hormone sensitive lipase ?).

Normal rats treated with the PChE inhibitor (iso-OMPA) also showed a

significant decrease in serum lipids and TLDL as previously reported by Kutty *et al.* (1977). Iso-OMPA treatment of diabetic mice showed similar changes to the diabetic rats.

Speculations on possible biological function(s) of PChE (Fig 4.1).

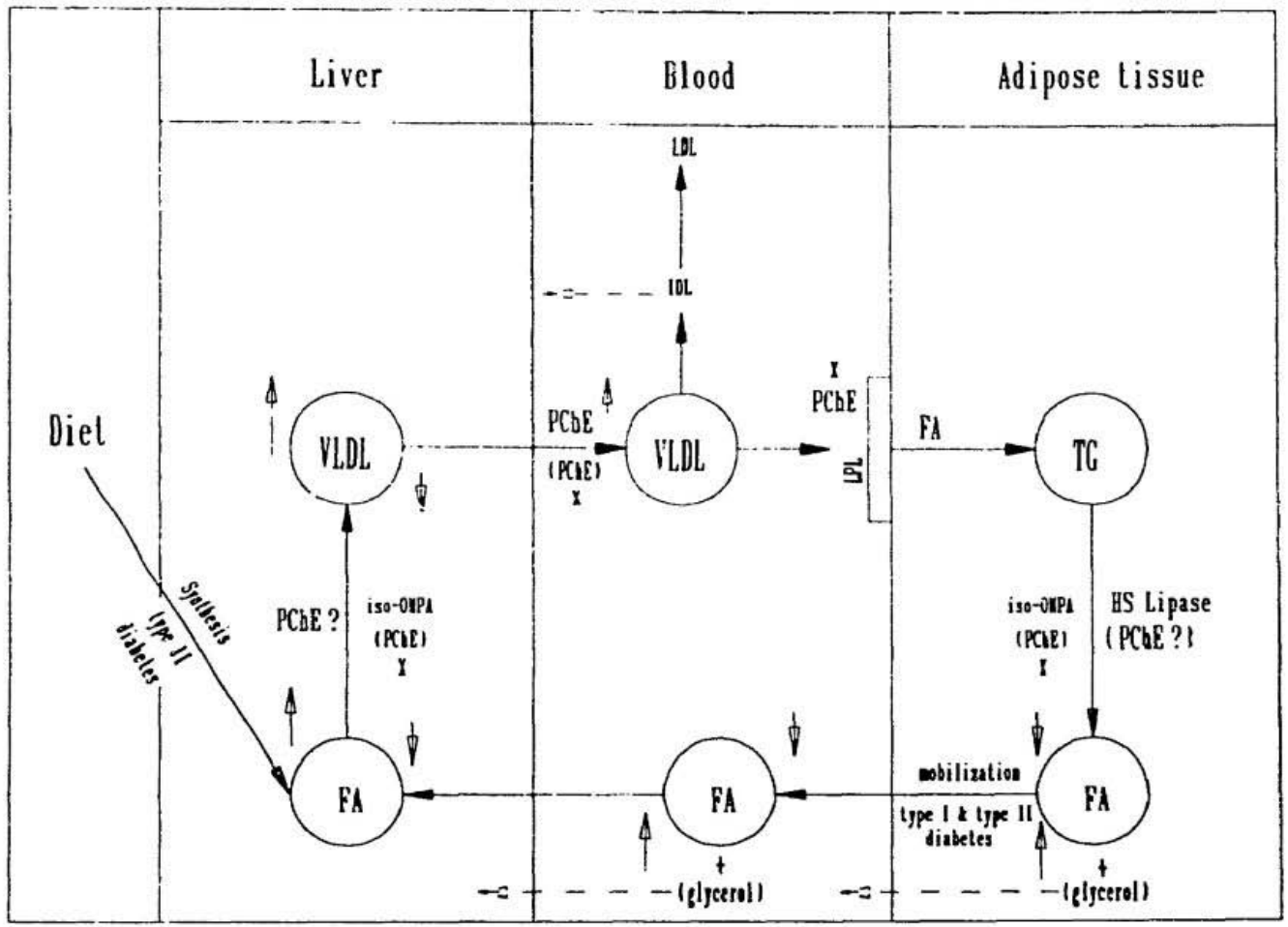
It is evident from the results presented in this thesis that there is a close relationship between the levels of PChE activity and VLDL in type I and type II diabetes and hyperlipoproteinemia. When PChE was inhibited with iso-OMPA, there was no demonstrable accumulation of cholinester substrates either in the serum or tissues. This suggests that the function of PChE is unrelated to its cholinester hydrolysing properties. So, the question is what other function(s) could it have? It may be associated with VLDL metabolism. The failure to inhibit PChE activity with heparin indicates that it is not involved in the clearance of VLDL-TG from the circulation. Therefore, it may be involved in the synthesis and secretion of VLDL. Overproduction of VLDL is known to occur in diabetes mellitus of both types (Nikkila & Kekki 1973). The PChE inhibitor, iso-OMPA, is thought to be specific for this enzyme, therefore in the absence of other known effects, the decrease in VLDL produced in the blood may be considered as related to PChE activity.

The increase in PChE activity is thought to be associated with VLDL synthesis in both types of diabetes rather than FA synthesis, since the source of FA is different in types I and II diabetes. As shown in Fig 4.1, hyperphagia in type II diabetes leads to increased synthesis of FA, TG and VLDL, whereas in type I there is increased mobilization of FA from adipose tissue stores. In

adipose tissue, inhibition of PChE appears to affect lipolysis as indicated by the fall in plasma glycerol. This suggests that PChE may have a function similar to hormone sensitive lipase (HS Lipase).

It is obvious from the above discussion that there are still many questions to be answered concerning the possible function(s) of Pseudocholinesterase. It is, however, evident that PChE could serve as a useful marker to detect abnormal lipid metabolism particularly in the diabetic patients.

Fig 4.1 Possible Biological Functions of PChE



REFERENCES

Aldridge, W.N. (1953). The differentiation of true and pseudocholinesterase by organo-phosphorous compounds. *Biochem. J.* 53, 62-67.

Allain, C.C., Poonl, S., Chan, C.S., Richmond, W. and Fu, P.C. (1974). Enzymatic determination of total serum cholesterol. *Clin. Chem.* 20, 470 475.

Alles, G. A. and Hawes, R.C. (1940). Cholinesterase in the blood of man. *J. Biol. Chem.* 133, 375-390.

Antopol,W., Tuchman, T. and Schifrin, A. (1937). Choline-esterase activity of human serum with special reference to hyperthyroidism. *Proc. Soc. Exptl. Biol. Med.* 36, 46-50.

Antopol, W., Schifrin, A. and Tuchman, L. (1938). Decreased cholinesterase activity of serum in jaundice and in biliary disease. *Proc. Soc. Exptl. Biol. Med.* 38, 363-366.

Arbeeny, C. and Eder, H.B. (1989). Effects of diabetes on the metabolism of triglyceride-rich lipoproteins. *Biochem. Soc. Trans.* 17, 51-53.

Ballantyne, B. (1968). Histochemical and biochemical aspects of cholin esterase activity of adipose tissue. *Arch. Intl. Pharmacodyn.* 173, 343-350.

Bar-On, H., Roheim, P.S. and Eder, H.A. (1976). Serum lipoproteins and apolipoprotein in rats with streptozotocin induced diabetes. *J. Clin. Invest.* 57, 714-721.

Blackshear, P.J. and Alberti, K.G.M.M (1974). Experimental diabetic ketoacidosis. Sequential changes of metabolic intermediates in blood, liver, CSF and brain after acute insulin deprivation in streptozotocin diabetic rats. *Biochem. J.* 138, 107-117.

Boopathy, R. and Balsubramanian, A.S. (1987). A peptidase activity exhibited by human serum pseudocholinesterase. *Eur. J. Biochem.* 162, 191-197.

Brauer, R. W. and Root, M. A. (1946). The effect of carbon tetrachloride induced liver injury upon the actylcholine hydrolyzing activity of blood plasma of the rat. *J. Pharmacol. and Exptl. Therap.* 88, 109-118.

Bray, G. and York, D. (1979). Hypothalamic and genetic obesity in experimental animals: an autonomic and endocrine hypothesis. *Physiol. Rev.* 59, 719-809.

Brimijoin, S., Mintz, K.P and Alley, M.C. (1983). Production and characterization of separate monoclonal antibodies to human acetylcholin esterase and butyrylcholinesterase. *Mol. Pharmacol.* 24, 513-20.

Brown, M.S., Kovanen, P.T. and Goldstein, J.L. (1981). Regulation of plasma cholesterol by lipoprotein receptors. *Science.* 212, 628-635.

Brown, S.S., Kalow, W., Pilz, W., Whittaker, M. and Woronick, C. L. (1981). The plasma cholinesterase: a new perspective. *Adv. Clin. Chem.* 22, 1-123.

Brunzell, J.D., Porte, D.Jr. and Bierman, E.L. (1979). Abnormal lipoprotein lipase mediated plasma triglyceride removal in untreated diabetes mellitus associated with hypertriglyceridemia. *Metabolism.* 28, 901-907.

Brunzell, J.D., Chait, A. and Bierman, E.L. (1985). Plasma lipoproteins in human diabetes mellitus. In *The Diabetes Annuals. K.G.M.M. Alberti and L.P. Krall editors. Elsevier, Amsterdam.* 1, 463-479.

Bucolo, G. and David, H. (1973). Quantitative determination of serum triglyceride by the use of enzyme. *Clin. Chem.* 19, 476-482.

Burstein, M., Scholnick, H.R. and Morfin, R. (1970). Rapid method for the isolation of lipoprotein from human serum by precipitation with polyanions. *J. Lip. Res.* 11, 583-595.

Chatonnet, A. and Masson, P. (1985). Study of the peptidasic site of cholinesterase: preliminary results. *FEBS Lett.* 182, 493-498.

Chu, M.I., Fontaine, P., Kutty, K.M., Murphy, D. and Redheendran, R.

(1978). Cholinesterase in serum and low density lipoprotein of hyperlipidemic patients. *Clin. Chim. Acta.* 85, 55-59.

Clark, J.B., Palmer, C.J. and Shaw, W.N. (1983). The diabetic zucker fatty rat. *Proc. Soc. Exp. Biol. Med.* 173, 68-75.

Clitherow, J.W., Mitchard, M. and Harper, N.J. (1963). The possible bilogical function of pseudocholinesterase. *Nature.* 199, 1000-1001.

Colville, K., Salvador, R., Lindsay, L. and Burnes, J. (1964). Lowering effect of neostigmine on plasma free fatty acid. *Nature.* 204, 888-889.

Cucuianu, M., Popescu, T.A. and Haragus, S. (1968). Pseudocholinester ase in obese and hyperlipemic subjects. *Clin. Chim. Acta.* 22, 151-155.

Cucuianu, M., Popescu, T.A., Opincoru, A. and Haragus, S. (1975). Serum pseudocholinesterase and ceruloplasmin in various types of hyperlipoproteinemia. *Clin. Chem. Acta.* 59, 19-27.

Cucuianu, M., Zrenghea, D., Pop, M. and Opincoru, A. (1976). Increased serum glutamyltransferase in hypertriglyceridemia: Comparison with serum pseudocholinesterase. *Clin. Chim. Acta.* 71, 419-427.

Das, P.K. and Liddell, J. (1970). Purification and properties of human serum cholinesterase. *Biochem. J.* 116, 875-881.

Deshmukh, M.B. (1986). Changes in serum cholinesterase activity and lipoprotein cholesterol levels in rats during diabetes. *IRCS Med. Sci.* 14, 234.

Dietz, A.A., Rubinstein, M.H. and Lubrano, T. (1973). Colorimetric determination of serum cholinesterase and its genetic variants by the propionyl thiocholine-dithiobis (nitrobenzoic acid) procedure. *Clin. Chem.* 19, 1309-1313.

Dubbs, C.A. (1966). Ultrasonic effects on isoenzymes. *Clin. Chem.* 12, 181-187.

Dunn, F.L., Raskin, P., Bilheimer, D.W. and Grundy, G.M. (1984). The effects of diabetic control on very low density lipoprotein metabolism in patients with type II diabetes mellitus and marked hypertriglyceridemia. *Metabolism.* 33, 117-123.

Eisenberg, S. (1979). Very low density lipoprotein metabolism. *Prog. Biochem. Pharm.* 15, 139-165.

Faber, M. (1943). Serum cholinesterase in disease. *Acta. Med. Scand.* 114, 59-71.

Folch, J., Lees, M. and Sloane Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226, 497-509.

Frings, C.S., Foster, L.B. and Cohen, P.S. (1971). Electrophoretic separation of serum lipoproteins in polyacrylamide gel. *Clin. Chem.* 17, 111-114.

Funnell, H.S. and Oliver, W.T. (1965). Proposed physiological function for plasma cholinesterase. *Nature.* 208, 689-690.

Ginsberg, H. and Grundy, S.M. (1982). Very low density lipoprotein metabolism in non-ketotic diabetes mellitus: effects of dietary restriction. *Diabetologia.* 23, 421-425.

Glasgow, A.M., August, G.P. Wellington, H. (1981). Relationship between control and serum lipids in Juvenile-onset diabetes. *Diabetes Care.* 4, 76-80.

Glomset, J.A. (1980). High density lipoprotein in human health and disease. *Adv. Intern. Med.* 25, 91-116.

Goldstein, J.L. and Brown, M.S. (1974). Binding and degradation of low density lipoprotein by cultured human fibroblasts. *J. Biol. Chem.* 249, 5153-5162.

Gonen, B., White, N., Schonfeld, G., Skor, D., Miller, P. and Santiago, J. (1985). Plasma levels of apo-B in patients with diabetes mellitus: the effect

of glycemic control. *Metabolism.* 34, 675-679.

Hamilton, R.L., Williams, M.C., Feildings, F.C. and Havel, R.J. (1976). Discoidal bilayer structure of nascent HDL from perfused rat liver. *J. Clin. Invest.* 58, 667-680.

Harper's Review of Biochemistry 19th ed. Martin, Jr. D.W., Mayes, P.A. and Rodwell, V.W. Lange Medical Publications. pg: 196.

Harris, H. and Whittaker, M. (1961). Differential inhibition of human serum cholinesterase with fluoride : recognition of two phenotypes. *Nature.* 191, 496-498.

Harris, H., Hopkinson, D.A. and Robson, E.B. (1962). Two-dimentional electrophoresis of pseudocholinesterase components in normal human serum. *Nature.* 196, 1296-1298.

Harris, H. and Robson, E.B. (1963). Fractionation of human serum cholinesterase components by gel filtration. *Biochem. Biophys. Acta.* 73, 649-652.

Havel, R.J., Kane, R.P., Balasse, E.O., Segel. and Basso, L.V. (1970). Splanchnic metabolism of VLDL in normo-triglyceridemic and hypertriglyceridemic humans. *J. Clin. Invest.* 49, 2017-2035.

Henderson, A., Hamilton, I. and King, J. (1971). The effect of starvation on the cholinesterase activity of liver and serum of young male rats. *Biochem. J.* 125, 36-37.

Hershcopf, R., Plotnick, L.P., Kaya, K., Benidict, G.W., Hadji-Georgopoulos., Margolis, S. and Kowarski, A. (1982). Short term improvement in glycemic control utilizing CSII: the effect of 24 hour integrated concentration of counterregulatary hormones and plasma lipids in IDDM. *J. Clin. Endo. & Metab.* 54, 504-509.

Hess, A.R., Angel, R.W., Barron, K.D. and Bernsohn, J. (1963). Proteins and isoenzymes of esterases and cholinesterases from the serum of different species. *Clin. Chim. Acta.* 8, 656-667.

Himsworth, H.P. (1936). Diabetes Mellitus. Its differentiation into insulin sensitive and insulin-insensitive types. *Lancet.* 1, 117-119.

Hiramatsu, K., Bierman, E.L. and Chait, A. (1985). Metabolism of low density lipoprotein from patients with diabetes hypertriglyceridemia by cultured human skin fibroblasts. *Diabetes.* 34, 8-14.

Howard, B.V. (1987). Lipoprotein metabolism in diabetes mellitus. *J. Lip. Res.* 28, 613-628.

Howard, B.V., Reitman, J.S., Vasquez, B. and Zech, L. (1983). Very low density lipoprotein-Triglyceride metabolism in non-insulin dependent diabetes mellitus. Relationship to plasma insulin and free fatty acid. *Diabetes.* 32, 271-276.

Iwase, M., Kikuchi, M., Nunoi, K., Wakisaka, M., Maki, Y., Sadoshima, S. and Fujishima, M. (1986). A new model of type II (non-insulin dependent) diabetes mellitus in spontaneously hypertensive rats: diabetes induced by neonatal streptozotocin treatment. *Diabetologia.* 29, 808-811.

Jackson, R.L., Morrisett, J.D. and Gotto Jr, A.M. (1976). Lipoprotein structure and metabolism. *Physiol. Rev.* 56, 259.

Jain, R., Kutty, K.M., Huang, S. and Kean, K. (1983). Pseudocholinest erase / high density lipoprotein cholesterol ratio in serum of normal persons and of hyperlipoproteinemics. *Clin. Chem.* 29, 1031-1033.

Jeanrenaud, B. (1977). An overview of experimental models of obesity. *In, Recent advances in obesity research II (ed. George Bray).* 111-122.

Junod, A., Lambert, A.E., Stauffacher, W. and Renold, R.E. (1969). Diabetogenic action of streptozotocin: Relationship of dose to metabolic response. *J. Clin. Invest.* 48, 2129-2139.

Juul, P. (1968). Human plasma cholinesterase isoenzymes. *Clin. Chim. Acta.* 19, 205-213.

Kalow, W. and Genest, K. (1957). A method for the detection of atypical

forms of human serum cholinesterase. Determination of dibucaine numbers. *Can. J. Biochem. Physiol.* 35, 339-346.

Khoury, G.F., Brill, J., Walts and Busutlil, R.W. (1987). Atypical serum cholinesterase eliminated by orthotopic liver transplantation. *Anesthesiology.* 67, 273-274.

Kissebah, A.H., Alfarsi, S., Evans D.J. and Adams, P.W. (1982). Integrated regulation of VLDL-TG and Apolipoprotein-B kinetics in NIDDM. *Diabetes.* 31, 217-225.

Kostner, G.M. and Karadi, I. (1988). Lipoprotein alterations in diabetes mellitus. *Diabetologia.* 31, 717-722.

Kutty, K.M. (1980). Review: Biological function of cholinesterase. *Clin. Biochem.* 13(6), 239-243.

Kutty, K.M., Rowden, G. and Cox, A.R. (1973). Interrelationship between beta-lipoproteins and cholinesterase. *Can. J. Biochem.* 51, 883-887.

Kutty, K.M., Jacob, J.C., Hutton, C.J., Davis, P.J. and Peterson, S.C. (1975). Serum beta lipoproteins: Studies in a patient and in guinea pigs after the injestion of organophosphorous compounds. *Clin. Biochem.* 8, 379-383.

Kutty, K.M., Redheendran, R. and Murphy, D. (1977). Serum cholin esterase, function in lipoprotein metabolism. *Experientia.* 33, 420-421.

Kutty, K.M., Jain, R., Haug, S. and Kean, K. (1981a). Pseudocholin esterase / High density lipoprotein cholesterol ratio as an index of risk for cardiovascular disease. *Clin. Chim. Acta.* 115, 55-61.

Kutty, K.M., Huang, S. and Kean, K. (1981b). Pseudocholinesterase in obesity: hyperchaloric diet induced changes in experimental obese mice. *Experientia.* 37, 1141-1142.

Kutty, K.M., Jain, R., Kean, K.T. and Peper, C. (1984). Cholinesterase activity in the serum and liver of zucker fat rats and controls. *Nutrition Research.* 4, 99-104.

Kutty, K.M., Annapurna, V. and Prabhakaran, V. (1989). Pseudocholin esterase - a protein with functions unrelated to its name. *Biochem. Soc. Trans.* 17, 555-556.

La Motta, R.V., McComb, R.B., Noll, C.R., Wetstone, H.J. and Reinfrank, R.F. (1968). Multiple forms of serum cholinesterase. *Arch. Biochem. Biophys.* 124, 299-305.

La Motta, R.V., Woronick, C.L. and Reinfrank, R.F. (1970). Multiple forms of serum cholinesterase: molecular weights of isoenzymes. *Arch. Biochem. Biophys.* 136, 448-451.

Lawrence, S.H. and Melnick, P.J. (1961). Enzymatic activity related to human serum beta lipoproteins: histochemical, immuno-electrophoretic and quantitative studies. *Proc. Soc. Exptl. Biol. Med.* 107, 998-1001.

Lech, J.J. and Calvert, N.N. (1968). Isolation and properties of a fluoride sensitive tributyrinase from adipose tissue. *Can. J. Biochem.* 46, 707-714.

Lee, J. and Harpst, J. (1973). Physical properties and subunit structure of butyrylcholinesterase from horse serum. *Biochemistry.* 12, 1622-1630.

Lehmann, H., Liddell, J., Blackwell, O'Cornnor, D.C. and Daws, A.V. (1963). Two further pseudocholinesterase phenotypes as causes of suxamethonium apnea. *Brit. Med. J.* 1, 1116-1118.

Lenzen, S. and Panten, V. (1988). Alloxan history and mechanism of action. *Diabetologia.* 31, 337-432.

Liddell, J., Lehmann, H. and Silk, E. (1962). A 'Silent' pseudocholin esterase gene. *Nature.* 193, 561-562.

Liddell, J., Newman, G.E. and Brown, D.F. (1963). A pseudocholinester ase variant in human tissues. *Nature.* 198, 1090-1091.

Lockridge, O. (1982). Substance P hydrolysis by human serum cholin esterase. *J. Neurochem.* 39, 106-110.

Lockridge, O. (1988). Structure of human serum cholinesterase. *Bio Essays.* 9(4), 125-128.

Lockridge, O., Eckerson, H.W. and La Du, B.N. (1979). Interchain disulfide bonds and subunit organization in human serum cholinesterase. *J. Biol. Chem.* 254(17), 8324-8330.

Lockridge, O., Jackson, N., Eckerson, H.W. and La Du B.N. (1980). Hydrolysis of diacetyl-morphine (heroin) by human serum cholinesterase. *J. Pharmacol. Exp. Ther.* 215, 1-8.

Lockridge, O. and La Du, B.N. (1982). Loss of interchain disulfide peptide and dissociation of the tetramer following limited proteolysis of native human serum cholinesterase. *J. Bio. Chem.* 257, 12012-12018.

Lockridge, O., Bartels, C.F., Vaughan, T.A., Wong, C.K., Norton, S. E. and Johnson, L. L. (1987). Complete amino acid sequence of human serum cholinesterase. *J. Biol. Chem.* 262, 549-557.

Lopes-Virella, M.F., Wohltman, H.J., Loadhott, C.B. and Buse, M.G. (1981). Plasma lipids and lipoproteins in young insulin dependent diabetic patients. Relationship with control. *Diabetologia.* 21, 216-223.

Lopes-Virella, M.F., Sherer, G.K., Lees, A.M., Wohltmann, H.J., Mayfield, R., Sagel, J., LeRoy, E.C. and Colwell, J.A. (1982). Surface binding, internalization and degradation by cultured human fibroblasts of LDL isolated from tpye I (insulin-dependent) diabetic patients, changes with metabolic control. *Diabetologia.* 22, 430-436.

Main, R., Soucie, W., Buxton, I. and Arinc, E. (1974). Purification of horse serum cholinesterase. *Biochem. J.* 143, 733-744.

Malaisse, W.J. (1982). Alloxan toxicity to the pancreatic B-cell. *Biochem. Pharmacol.* 31, 3527-3534.

Marshall, M.B., Barrett, R.J. and Mayer, J. (1955). Hypothalamic lesions in gold thioglucose injected mice. *Proc. Soc. Exp. Biol.* Med. 90, 240-244.

McGowan, M.W., Artiss, J.D. and Zak, B. (1983). Enzymatic colorimetry of lecithin and sphingomyelin in aqueous solution. *Clin. Chem.* 29, 1513-1518.

McGuire, M.C., Nogueira, C.P., Bartels, C.F., Lightstone. H., Hajra, A., Van Der Spek, A.F.L., Lockridge, O. and La Du, B.N. (1989). Identification of the structural mutation responsible for the dibucaine-resistant (atypical) variant form of human serum cholinesterase. *Proc. Natl. Acad. Sci. USA.* 86, 953-957.

McTiernan, C., Adkins, S., Chatonnet, A., Vaughan, T.A., Bartels, C.F., Kott, M., Rosenberry, T.L., La Du, B.N. and Lockridge, O. (1987). Brain cDNA clone for human cholinesterase. *Proc. Natl. Acad. Sci. USA.* 84, 8682-8686.

Mendel, B. and Rudney, H. (1943). Studies on Cholinesterase. 1. Cholin esterase and Pseudocholinesterase. *Biochem. J.* 37, 59-63.

Michaelis, O.E., Ellwood, K.C., Judge, J.M., Schoene, N.W. and Hansen, C.T. (1984). Effect of dietary sucrose on the SHR/N-Corpulent rat: a new model for insulin-independent diabetes. *Am. J. Clin. Nutr.* 39, 612-618.

Monographs on Atherosclerosis (1986), Editors: Krichevsky, D. and Pollak, O.J. Karger. Vol. 14, pg:160 & 162.

Muensch, H., Goedde, H.W. and Yoshida, A. (1976). Human serum cholinesterase subunits and number of active sites of the major component. *Eur. J. Biochem.* 70, 217-223.

Nagasawa, T., Sugisaki, H., Tani, Y. and Ogata, K. (1976). Purification and characterization of butyrylcholine-hydrolyzing enzyme from *Pseudomonas polycolor. Biochem. Biophys. Acta.* 429, 817-827.

National Diabetes Data Group (NDDG). (1979). Classification and diagnosis of diabetes mellitus and other categories of glucose intolerence. *Diabetes.* 28, 1039-1057.

Nikkila, E.A. (1981). High density lipoprotein in diabetes. *Diabetes.* 30,

82-87.

Nikkila, E.A. (1985). Very low density triglyceride metabolism in diabetes. *Monogr. Atheroscler.* 13, 44-52 (Karger Basel).

Nikkila, E.A. and Kekki, M. (1973). Plasma triglyceride transport kinetics in diabetes mellitus. *Metabolism.* 22, 1-22.

Nikkila, E.A., Huttunen, J.K. and Ehnholm, C. (1977). Postheparin plasma lipoprotein lipase and hepatic lipase in diabetes mellitus. Relationship to plasma triglyceride metabolism. *Diabetes.* 26, 11-21.

Nomura, T., Hagino,Y., Gotoh, M., Iguchi, A. and Sakamoto, N. (1984). The effects of streptozotocin diabetes on tissue specific lipase activities in the rat. *Lipids.* 19, 594-599.

O'Looney, P., Maten, M.V. and Vahouny, G.V. (1983). Insulin-mediated modifications of myocardial lipoprotein lipase and lipoprotein metabolism. *J. Biol. Chem.* 258, 12994-13001.

Polonsky, K.S. and Rubenstein, A.H. (1989). Kinetics and Metabolism of Insulin, Proinsulin and C-peptide. *In Endocrinology (2nd. Ed) Ed. Leslie, J. DeGroot.* 2, 1304-1317.

Popescu, T., Fekete, T., Popescu, E., Bojthy, I. and Lazlo, M. (1976). Serum pseudocholinesterase activity during experimental fattening. *Rev. Roumania Medica-Medica International.* 14, 71-73.

Prody, C.A., Zevin-Sonkin, D., Gnatt, A., Goldberg, O. and Soreg, H. (1987). Isolation and characterization of full length cDNA clones coding for cholinesterases from fetal human tissues. *Proc. Natl. Acad. Sci. USA.* 84, 3555-3559.

Pykalisto, O.J., Smith, P.H. and Brunzell, J.D. (1975). Determinants of human adipose tissue lipoprotein lipase. Effect of diabetes and obesity on basel and diet-induced activity. *J. Clin. Invest.* 56, 1108-1117.

Randle, P.J. and Hales, N.C. (1972). Insulin release mechanisms In *Hand-*

book of Physiology. Vol 1, 219-235.

Reaven, G.M. (1983). Insulin resistance in NIDDM. Does it exist and can it be measured? *Am. J. Med.* 74, 3-17.

Reaven, G.M. (1987). Non-insulin dependent diabetes mellitus, abnormal lipoprotein metabolism and atherosclerosis. *Metabolism.* 36, 1-8.

Reaven, G.M. and Greenfield, M.S. (1981). Diabetic hypertriglyceridemia. Evidence for three clinical syndromes. *Diabetes.* 30, 66-75.

Reaven, P.E. and Reaven, G.M. (1974). Mechanisms of the development of diabetic hypertriglyceridemia in streptozotocin-treated rats. Effect of diet and duration of insulin deficiency. *J. Clin. Invest.* 54, 1167-1178.

Redgrave, T.G. (1970). Formation of cholesterylester-rich particulate lipid during metabolism of chylomicrons. *J. Clin. Invest.* 49, 465-71.

Reiner, E., Seuferth, W. and Hardegg, W. (1965). Occurence of cholin esterase isoenzymes in horse serum. *Nature.* 205, 1110-1111.

Robertson, R.P., Gavareski, D.J., Henderson, J.D., Porte, D.Jr. and Biermar., E.L. (1973). Accelerated triglyceride secretion, a metabolic consequence of obesity. *J. Clin. Invest.* 52, 1620-1626.

Robinson, D.S. and Speake, B.K. (1989). Role of insulin and other hormones in the control of lipoprotein lipase activity. *Biochem. Soc. Trans.* 17, 40-42.

Rush, R., Main, R., Miller, S. and Kilpatrick, B. (1980). Resolution and purification of two monomeric butyrylcholinesterase from rabbit liver. *J. Bio. Chem.* 255, 7155-7160.

Sailer, S., Sandhofer, F. and Braunsteiner, H. (1966). Overweight and triglyceride level in normal persons and patients with diabetes mellitus. *Met abolism.* 15, 135-137.

Salvador, R. and Kuntzman, R. (1965). Cholinesterase of adipose tissue.

J. Pharmacol. and Exptl Therap. 150, 84-91.

Schaefer, E.J. and Levey, R.I. (1979). Composition and metabolism of high density lipoprotein. *Prog. Biochem. Pharm.* 15, 200-215.

Schein, P.S., Alberti, K.G.M.M. and Williamson, D.H. (1971). Effects of streptozotocin on carbohydrate and lipid metabolism in rat. *Endocrinology.* 89, 827-834.

Schnatz, J.D. and Williams, R.H. (1963). The effect of acute insulin deficiency in the rat adipose tissue lipolytic activity and plasma lipids. *Diabetes.* 12, 174-178.

Schonfeld, G. and Pfleger, B. (1971). Overproduction of very low density lipoprotein by liver of genetically obese rats. *Am. J. Physiol.* 222, 1178-1181.

Shafrir, E., Bergman, M. and Feling, P. (1987). The Endocrine Pancreas: Diabetes Mellitus. *In Endocrinology and Metabolism (2nd ed).* 1043-1178.

Silver, A. (1974). In, The Biology of Cholinesterase. (Ist ED), *North-Holland Publishing Co., Amsterdam, Oxford.* 443-449.

Smith, R.J. (1989) Biological actions of insulin and glucagon. In Endocrinology (2nd Ed), edited Leslie, J. DeGroot. 2, 1263-1289.

Sosenko, J.M., Breslow, J.L., Miettinen, O.S. and Gabbay, K.H. (1980). Hyperglycemia and plasma lipid levels. A prospective study of young insulin-dependent diabetic patients. *N. Engl. J. Med.* 32, 650-654.

Stedman, E., Stedman, E. and Easson, L.H. (1932). Cholinesterase, an enzyme present in the blood-serum of the horse. *Biochem. J.* 26, 2056-2066.

Surgenor, D.M. and Ellis, D. (1954). Preparation and properties of serum and plasma proteins. Plasma cholinesterase. *J. Am. Chem. Soc.* 76, 6049-6051.

Szendzikowki, S., Patelski, J. and Pearse, A. (1961/62). The influence of cholinesterase inhibitors on the lipolytic activity of rat aorta. *Enzymol.*

Biol. Clin. 1, 125-137.

Taskinen, M.R. (1987). Lipoprotein lipase in diabetes. *Diabetes Metab. Rev.* 3, 551-570.

Taskinen, M.R., Nikkila, E.A., Kwsi, T. and Harno, K. (1982). Lipoprotein lipase activity and serum lipoproteins in untreated type II (insulin-independent) diabetes associated with obesity. *Diabetologia.* 22, 46-50.

Taskinen, M.R., Beltz, W.F., Harper, I., Fields, R.M., Schonfeld, G., Grundy, S.M. and Howard, B.V. (1986). The effects of NIDDM on very low density lipoprotein-triglyceride and very low density apo-B metabolism: studies before and after sulfonylurea therapy. *Diabetes.* 35, 1268-1277.

The Diabetes Annuals/3. K.G.M.M.Alberti and L.P.Krall, editors. 1987. Elsevier Science Publishers, B.V. pg: 4 & 7.

Tsujita, T., Ninomiya, H. and Okuda, H. (1989). P-Nitrophenyl butyrate hydrolyzing activity of hormone-sensitive lipase from bovine adipose tissue. *J. Lip. Res.* 30, 997-1004.

Tucci, A. and Seifter, S. (1969). Preparation and properties of porcine parotid butyrylcholinesterase. *J. Bio. Chem.* 244, 841-849.

Von Tol, A. (1977). Hypertriglyceridemia in diabetic rat. Defective removal of serum VLDL. *Atherosclerosis.* 26, 117-128.

Waterlow, J.C. (1950). Liver cholinesterase in malnourished infants. *Lancet.* 258, 908-909.

Webb, J.L. (1966). Alloxan, *In Webb, J.L(ed). Enzyme and metabolic inhibitors. Academic Press. New York.* 3, 367-419.

Whittaker, M. (1986). Cholinesterase: *Monographs in human genetics, Vol.11 (Beckman, L. ed), Karger, Basel.*

Whittaker, V.P. (1951). Specificity, mode of action and distribution of cholinesterase. *Physiol. Rev.* 31, 312-343.