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## The Metabolic Response to Natural Diets Containing Variable Amounts of Sucrose in Hypertriglyceridemia Prone Diabetics

Walter S. Jellish  
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THE METABOLIC RESPONSE TO NATURAL DIETS  
CONTAINING VARIABLE AMOUNTS OF SUCROSE  
IN HYPERTRIGLYCERIDEMIA PRONE DIABETICS

Walter S. Jellish

A Dissertation Submitted to the Faculty of  
the Graduate School of Loyola University of Chicago  
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Also to my wife, Michelle, my family and friends, my thanks for their support and love throughout this research project.



## LIFE

Walter Scott Jellish was born in Ashland, Wisconsin on May 5, 1954. After graduating from Ashland High School in 1972, he received a Bachelor of Arts in Biology and Chemistry and graduated Magna Cum Laude with Distinction in Chemistry from Northland College, Ashland, Wisconsin. In 1977 he began his doctoral research in the Department of Biochemistry at Loyola University under the supervision of Dr. Albert A. Dietz, studying the effects of sucrose diets on the development of hyperlipemia in non-insulin dependent diabetics. While attending Loyola, he received the American Diabetes Association, Northern Illinois Affiliate Student Research Award in 1980, and served on several student committees. He was also elected a member of Sigma Xi and received a Certificate of Recognition from the society. He recently coauthored an approved grant from the Sugar Foundation in support of continued research in the field of diabetic hyperlipemia.

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## ABBREVIATIONS

1.	Ains-GP	-	Anti-insulin guinea pig antibody
2.	BUN	-	Blood Urea Nitrogen
3.	C/PL	-	Cholesterol/Phospholipid
4.	ESR	-	Electron spin resonance
5.	FFA	-	Free fatty acid
6.	g/L	-	grams per liter
7.	HDL	-	High Density Lipoprotein
8.	HMG CoA	-	Hydroxy methyl glutaryl Coenzyme A
9.	IR	-	infra red spectroscopy
10.	IRB	-	Internal Review Board
11.	LDL	-	low density lipoprotein
12.	LCAT	-	lecithin cholesterol acyltransferase
13.	$\mu$ Eq	-	microequivalents
14.	NMR	-	nuclear magnetic resonance
15.	NIH	-	National Institute of Health
16.	NSB	-	non-specific binding
17.	OD	-	optical density
18.	PAGE	-	polyacrylamide gel electrophoresis
19.	RER	-	rough endoplasmic reticulum
20.	SDS	-	sodium dodecyl sulfate
21.	SER	-	smooth endoplasmic reticulum
22.	TG	-	Triglyceride
23.	TC	-	Total cholesterol
24.	V/A buffer	-	veronal albumin buffer
25.	VLDL	-	Very Low Density Lipoprotein
26.	VLDL-TG	-	Very Low Density Lipoprotein Triglyceride

## 1. INTRODUCTION

### A. Statement of Problem

One third of North Americans will die of an atherosclerotic disease. In comparison, this disease will cause the death of three quarters of America's diabetic population. Diabetes occurs in ten percent of the U.S. Armed Forces veteran population with more than 90 percent of these veterans having type II (non-insulin dependent or maturity-onset) diabetes (1). Atherosclerotic occlusive vascular disease is the most common complication in diabetes (2-4). Epidemiological studies have indicated that other risk factors involved in atherosclerosis include age, sex, race, family history, smoking, hypertension, dyslipoproteinemia and obesity (5).

While it may be useful to consider atherosclerosis in the diabetic as a severe form of the disease found in non-diabetics, many investigators believe this approach to be simplistic. Many differences occur between groups of patients in the progression of the disease manifestations. Which risk factors make diabetics especially susceptible to atherosclerosis or whether the disease produces its own factors to increase atherosclerotic susceptibility are open to debate.

Hypertriglyceridemia and hypercholesterolemia are common problems of the diabetic. While drug therapy is, in many cases, ineffective for lowering and managing triglyceride and cholesterol concentrations, an effective and safe dietary approach would be advantageous. The major concern in treating diabetic, hypertriglyceridemic patients is the type of dietary therapy to be administered. Since increased hyper-

lipemia occurs with increased fat consumption, there has been a growing tendency to limit fat intake in diabetic patients. This results in diets that are composed of higher amounts of carbohydrate and protein relative to fat. This trend in diabetic care has received official sanction from the American Diabetes Association's Committee on Food and Nutrition (6). It is now recommended that most diabetics should consume a diet containing up to 50 or 60% carbohydrate. In a recent editorial in Diabetologia, Reaven (102) is skeptical about the effects of high carbohydrate diets. He observes that the beneficial effects of high carbohydrate diets on diabetic patients are modest at best.

The effects of a high carbohydrate diet on the diabetic population were first studied by Himsworth (7). One advantage claimed for the use of a high carbohydrate diet was the enhancement of insulin sensitivity. In a series of studies in normal patients, he demonstrated that the plasma glucose response to the oral glucose load improved when dietary carbohydrate was increased. In similar studies with both insulin-dependent and non-dependent diabetics, Himsworth (8) was able to show a sensitive and insensitive population to high carbohydrate intake. Insensitive patients showed no increase in insulin sensitivity and a significant deterioration in glucose tolerance. Brunzell and Anderson (9-10) performed similar experiments and found an improvement of 8-11% in blood glucose levels when carbohydrate was increased from 45 percent to 78-85 percent of total caloric intake.

In recent years, sources of carbohydrate have changed. The



current level of simple sugars supplied by the diet is about 200 g/person/day; of this, 62 percent is composed of the refined sugar sucrose (11). Many industrialized food preparations and processed beverages contain sucrose, accounting for more than two-thirds of the refined sugar consumed (11). Beverage products now comprise the single largest industrialized usage of refined sugar while the next largest use is in cereal and bakery products. These three food sources now account for about one-sixth the per capita consumption of refined sugar. Much of this sucrose is hidden sugar, either used in the baking process or in enhancing the sweetness of foods. Therefore, consumers (including diabetics) are unaware of their total sucrose consumption.

As early as 1950, workers with the Kemper Rice Diet reported that although high carbohydrate diets increase glucose tolerance and lowered serum cholesterol levels, there was also a sizeable increase in neutral fats (triglycerides) (12). Waddell and Fallon (13) examined the possibility that diets of high sugar content accelerate hepatic triglyceride synthesis by increasing the capacity for fatty acid esterification in rats. Rats fed diets containing 75 percent glucose or fructose increased their capacity for triglyceride formation from [ $^{13}\text{C}$ ] glycerol (14). An initial increase occurred when both sugars were administered, but a sustained increase in serum triglyceride could only be accomplished with a high fructose intake. When serum triglyceride removal was inhibited by administration of Triton WR-1339, both diets increased labelled glycerol incorporation and increased triglyceride levels. They concluded that the difference in triglyceride levels between fructose and glucose diets may be attributed to a smaller increase in

hepatic production of triglyceride when rats were given glucose. These investigators speculated that another factor involved was the rate of serum triglyceride removal. Bierman and Hamlin (14) studied the effects of low-fat, high carbohydrate diets in diabetic patients. The liquid diets were administered with rice supplements and contained 15 percent protein, 85 percent carbohydrate and virtually no fat. Their results demonstrated that insulin-treated diabetics exhibited a hyperlipemic response to isocaloric substitution of carbohydrate for fat. Several studies, however, tend to refute these observations. Kiem and Anderson (15) studied the effects of a high-carbohydrate, high-fiber diet on hyperglycemic diabetic men. Their finding showed that solid diets containing 75 percent of calories as carbohydrate resulted in a significant improvement in glucose tolerance and lowered the average fasting serum triglyceride levels.

The use of carbohydrate induction as a viable theory for hypertriglyceridemia was questioned from its inception. Antonis and Bersohn (16) reported that subjects, given an isocaloric high-carbohydrate diet, showed a rise in fasting triglycerides in the first few weeks, but the abnormally increased levels tended to recede to the prediet level after 3 weeks. If carbohydrate-induced lipemia was a physiological fact, then populations which consume high-carbohydrate diets would tend to have chronically elevated triglyceride levels. Several investigators have demonstrated that this is not the case (17-21).

In contrast, MacDonald and Braithwaite (22) in 1964, and Kuo and Bassett (23) in 1965, studied the effects of the disaccharide sucrose on hyperlipemia. Both groups found the consumption of low fat diets,

containing 500 g of sucrose as compared to corn starch, resulted in increased serum lipids. Fatty-acid patterns in adipose tissue were also affected by the high carbohydrate intake. Mann and co-workers were able to reduce fasting serum triglyceride levels by an average of 22 percent through voluntary sucrose restriction (24,25). The interpretation of the results was clouded by the effect of weight loss in most hypertriglyceridemic individuals. Thus, the effect of sucrose might be calorie-induced rather than sugar-induced lipemia.

Evidence continued to mount that increased sucrose consumption produced virtually all the clinical symptoms of diabetes. Cohen, et al. demonstrated that feeding high sucrose diets to human volunteers resulted in impaired glucose tolerance, hyperlipemia, and impaired insulin activity (26). In his experiments, he also found the feeding of sucrose to rats produced diabetes, renal arteriosclerosis and diabetic retinal angiopathy. Studies in which sucrose-restricted diets were administered, tended to confirm the effect of sucrose on hyperlipemia with no significant correlation made between changes in triglyceride levels and weight (27-28). Since sucrose is a disaccharide, studies were undertaken to determine if sucrose or its monosaccharide components, glucose and fructose, could be responsible for the hyperlipemic effect. MacDonald (29) gave patients fat-free liquid diets of sucrose, starch, or glucose-maltose. He found a decrease in serum lipids on the glucose-maltose regime, whereas with the sucrose-starch diet, the lipid level remained constant. Since previous fat-free diets had been shown to lower lipid levels, these findings suggested that the absence of fat in the diet was compensated for by the formation of lipids from carbohy-

drate when the starch-sucrose diet was consumed. Further evidence to support this possibility was the altered fatty acid pattern observed in patients on the starch-sucrose diet. The increased oleic acid content observed was consistent with the conversion of carbohydrate to fat. MacDonald (30) investigated the effects of glucose and fructose diets on blood lipid levels concluding that the fructose component of the diet gives rise to responses of lipid metabolism that are different from those found after consuming glucose. Triglyceride responses to dietary glucose appeared to be unaffected by the many factors that affect serum triglyceride responses to fructose. However, Akgun and Ertel (31) demonstrated that fructose and sorbitol meals resulted in lower plasma glucose and immunoreactive insulin levels than corresponding amounts of sucrose in a meal. This observation was directly in contrast to the ones made by MacDonald (30).

A direct relationship has been observed between the estimated magnitude of insulin secretion and the increase in plasma triglycerides produced by carbohydrate-rich diets (32). Mann and Truswell (33) found that there was no difference between concentrations of fasting serum lipids when starch replaced glucose, but the concentration of serum triglycerides was higher after mixed meals containing sucrose. They also found increased insulin release after meals of complex carbohydrate as compared to sucrose. They correlated the insulin response to a significantly smaller degree of lipemia while consuming the starch diet. MacDonald (34) found the increase in serum glucose after sucrose ingestion slightly less than that after glucose ingestion. Because sucrose is composed of 50 percent glucose, and all of the fructose

moiety could not have been interconverted, the elevated glycemic response was surprising. Their results were explained by postulating that an equal dose of sucrose would elicit a lower insulin response than an equal amount of glucose. They concluded that lipid levels would fall on high glucose diets because of increased lipoprotein lipase activation in relation to lipid response (35). This, however, was not the case, and the fall in triglyceride in three out of four experiments was greater with fructose than glucose. Hayford and Danney (36) found that elevated plasma insulins usually reflected decreased triglyceride levels, while Olefsky (37) found a positive relationship between insulin and hypertriglyceridemia. Hayford (36) felt that it was possible the insulin and triglyceride changes were both secondary to the dietary changes and were not related to one another. Since sucrose was associated with higher triglyceride concentrations, he could not resolve whether the inverse relationship was a function of the insulin response, dietary components, or a combination of both.

Most of the experiments performed to evaluate carbohydrate influence on lipemic responses utilized low fiber liquid diets. The investigation of sucrose induced carbohydrate hyperlipemia was further confused by the report of Albrink and Newman (38) in which the effect of high and low fiber diets on plasma lipids and insulin were ascertained. Two very high carbohydrate diets were administered, one with high fiber content, and one with low fiber. The glucose response to both test meals were similar while the insulin response to the low fiber meal was twice as great as that to the high fiber meal containing equivalent amounts of carbohydrate. Serum lipids increased when low fiber

meals were consumed and decreased after high fiber diets. These results suggest that carbohydrate-induced hyperlipemia does not occur if the carbohydrate diet is rich in dietary fiber. An interesting report was issued by Beck-Nielsen and Pederson in 1980 (39), examining the effects of high fructose feeding in normal subjects. They found high fructose feeding was associated with a drop in insulin sensitivity, and for the first time, increased insulin concentrations in certain individuals was observed. The high fructose diet resulted in a 30 percent reduction of insulin binding to monocytes but glucose feeding resulted in no change. They concluded that feeding a diet high in fructose can result in a reduction in the number of high-affinity receptor sites, i.e., a reduction in total receptor affinity for insulin. High-sucrose feeding can result in a 36 percent decrease in specific cell binding fractions for insulin (40). When these results are compared to the above, one can conclude that sucrose, or its monosaccharide component, fructose, causes an altered insulin sensitivity, at least in part, explained by altered insulin binding. The decrease in insulin binding is associated with reciprocal changes in plasma insulin concentration. The decrease in cellular binding of insulin by fructose was attributed to the depression of both cellular levels of cyclic AMP and protein synthesis caused by the depletion of ATP.

It is also possible to explain the higher insulin levels observed after sucrose feeding on the basis of adaptive changes of the small intestine. Sucrase activity was greater in humans who consumed isocaloric diets containing sucrose compared to glucose (41). The intestinal absorption of glucose and fructose was enhanced after rats

and baboons had adapted to diets high in sucrose (42-43). In human volunteers, a significantly greater secretion of the intestinal hormone, gastric inhibitory polypeptide (GIP), was found in response to a sucrose load when compared to a starch load (44). GIP stimulates insulin secretion of the pancreas in response to oral (but not intravenous) glucose (45). Thus sucrose feeding could produce adaptive increases in intestinal digestive enzymes and hormones which could be expected to increase secretion of insulin. This could be accomplished by increasing the rate of appearance of the component monosaccharide in the circulation or by a direct action on insulin secretion. Eaton and Nye (46) also found a positive correlation between insulin levels and triglyceride production. To ascertain if insulin was the direct cause of hyperlipemia in "sensitive" patients, direct pharmacological inhibition of pancreatic insulin secretion was used to examine the effect of acute suppression of insulin on triglyceride concentrations. Their observations imply, that in normal weight lipemic subjects, the pancreatic beta cells are in a chronic state of hypersecretion, characterized by an increased carbohydrate-stimulated insulin secretion associated with increased basal triglyceride concentrations.

Hypertriglyceridemia in diabetics, resulting from increased intake of carbohydrates, could occur via three mechanisms. In some patients, as reported above, a decreased insulin release seems to be characterized by a decrease in both the secretion and removal of triglyceride-rich lipoprotein with the defect in removal being greater than the defect in production. Even in the absence of exogenous fat, the disparity between triglyceride rich lipoprotein (VLDL) production and removal is sufficient

to result in an increase in plasma triglyceride concentrations (47). A second mechanism through which carbohydrate induced lipemia can occur results from resistance to insulin-mediated glucose uptake. The compensatory hyperinsulinemia acts upon the liver to accelerate hepatic triglyceride synthesis and secretion resulting in elevated triglyceride levels (37). A third mechanism that could induce hypertriglyceridemia was proposed by Reaven (48) who studied a non-insulin dependent diabetic patient population who were insulin resistant, but had normal fasting and postprandial insulin concentrations. Because of insulin resistance, normal insulin levels cannot stimulate proper glucose uptake or inhibit lipolysis. These subjects have marked elevations of fasting and postprandial glucose and free fatty acids levels. Thus, the increased hypertriglyceridemia could occur due to stimulation of the normally insulinized liver by increased circulating FFA concentrations (49).

Other reports have supported the concept of increased triglyceride synthesis causing hypertriglyceridemia in relation to a carbohydrate load (50-52). However, these reports deal with total blood triglyceride concentrations and the kinetic behavior of the plasma triglycerides in these patients is largely unknown. Reliable data on the transport and production of plasma triglycerides can be obtained by labeling them in vivo. It has been shown that a glycerol-labeling technique (described later) can give useful information on human plasma triglyceride turnover kinetics (53-56) and has been used to characterize aspects of plasma triglyceride metabolism in carbohydrate induced lipemia. Nikkila and Kekki (57), in a landmark paper, evaluated diabetic hypertriglyceridemia and found this syndrome to be characterized



by a primary stimulation of hepatic triglyceride secretion. Tobey et al. (58) found significant relationships between insulin-resistance, insulin-response, VLDL-TG secretion and plasma TG concentrations in both normal and hypertriglyceridemic individuals, suggesting that insulin modulates plasma TG levels in both groups. However, some additional abnormality must be present to account for the elevated plasma TG concentrations in hypertriglyceridemic individuals. Adams and Kissebah (59) were able to demonstrate that a group of hypertriglyceridemic patients were hyperinsulinemic and showed increased triglyceride synthesis with increased FFA release. These patients had an abnormality in the removal mechanism for plasma triglycerides, a decreased  $V_{max}$  and an increased  $K_m$  when compared to other hypertriglyceridemics. Adams and Kissebah (59) hypothesized that elevated FFA release due to lipolysis, because of insulin resistance, could inhibit lipoprotein lipase (the primary enzyme in plasma lipid removal) and lead to some impairment in triglyceride clearance. The primary change could be due to enzyme derangement, deficiency of insulin receptors or an abnormality in circulating insulin. Huttunen et al. (60) showed the fractional removal rate of endogenous triglyceride to be positively correlated to lipoprotein lipase activity. The mean activity of post heparin plasma lipoprotein lipase was also significantly lower in subjects with hyperprebeta-lipoproteinemia.

Persson (61) did a comprehensive study of 249 patients with different types of hyperlipidemia. His work clearly demonstrated a lower lipoprotein lipase activity per wet weight adipose tissue in type IV hyperlipidemic individuals compared to normal subjects. Brunzell and

Porte (62) studied untreated diabetic subjects and documented a defect in triglyceride removal related to lipoprotein lipase activity. They suggest an interesting mechanism, in which the increase in  $K_m$  for the lipase system could be related to a change in VLDL protein cofactors. These proteins, designated apoproteins, are structural components of lipoprotein molecules, which serve as activators for lipoprotein lipase, or function as recognition proteins which aid in the peripheral removal of the lipoprotein from the circulation (212-218). These protein components are described later in the text.

An alteration in the lipoprotein cofactors, causing an inefficient removal of triglyceride from VLDL, has long been postulated as a mechanism for the induction of hypertriglyceridemia. Gabor and Spain (63) studied the apoprotein composition of VLDL and HDL by gel electrophoresis and found the VLDL of diabetics had an increased proportion of apo C, particularly apo CIII. They also demonstrated that diabetics with increased VLDL and serum triglyceride concentration had decreased ratios of CII to CIII. Bar-On and Roheim (64) noted a relative increase in CIII<sub>1</sub> apoprotein and a decrease in CIII<sub>2</sub> apoprotein in streptozotocin induced diabetic rats fed high sucrose diets. Varying CIII components differ only in their amount of sialic acid residues. Since diabetes has been correlated to increased concentrations of other serum glycoproteins, it is tempting to speculate that altered C proteins might influence lipoprotein lipase activity (65). Witzum and Schonfeld (66) gave carbohydrate-enriched diets to rats to investigate the effect on VLDL composition and structure. A rise in triglyceride and cholesterol in the carbohydrate fed rats and a fall in apo B concentration was

observed. The arginine-rich protein (ARP) also fell while apo C rose. These investigators also studied perfused livers of these animals and found changes in apoproteins to be parallel to those seen in plasma. This suggested the action of the diet on the liver was responsible for the plasma VLDL changes seen (66). Falko (67) showed that ingestion of high carbohydrate diets (88 percent total calories) caused apo CII and apo CIII levels to increase by 30 percent in VLDL while these same levels fell in higher density lipoprotein fractions. Levels of apo C increased in proportion to VLDL-TG in normal individuals, but increased less in patients with type IV hyperlipemia. Falko postulated that short term feeding of high carbohydrate diets resulted in alterations in the metabolism of lipoproteins in hyperlipemic subjects.

Studies conducted by Lambert and Catapano (68, 69) tended to substantiate previous work. A large population of hypertriglyceridemic subjects were assessed for apo CII, CIII<sub>1</sub> and CIII<sub>2</sub> levels. Good correlations were obtained between triglyceride levels and apo CII with apo CIII<sub>1</sub> increasing with hypertriglyceridemia. Apo CIII<sub>2</sub> followed the pattern of CII, but to a lesser extent. Definite results concerning C apoprotein levels were obtained by Matsuoka and Shirai (70). These investigators found that patients who had hypertriglyceridemia were deficient in apo CII with maximum hydrolysis of VLDL occurring at 2.5 ug apo CII/mg triglyceride. The Km value of the lipoprotein lipase enzyme for apo CII deficient VLDL was 7.8 mol/L. When 2.5 ug of apo CII was added to the medium, the Km decreased to 1 mol/L. A possible explanation of the affect of apo CII to decrease the Km is that the protein enhances the interaction between lipoprotein lipase and triglyceride

within the surface monolayer of the lipoprotein.

Studies have also been conducted concerning the effect of high carbohydrate on apo B catabolism (71, 72). Ginsbert (71) found no production change in apo B during the normal carbohydrate diet in which 40 to 60 percent of VLDL apo B was converted to LDL. However, patients on high carbohydrate diets converted only 16 to 42 percent of VLDL apo B to LDL. In most cases direct catabolism of VLDL apo B occurred without conversion to higher density lipoprotein classes during the high carbohydrate diet with essentially no VLDL apo B converted directly to LDL apo B. A fall in LDL-apo B concentrations did not follow the reduced input from VLDL and was explained by a reduction in the removal rate of LDL-apo B. Thus, significant alterations in the routes of catabolism of apo B occurred with high carbohydrate diets. Others have reported that apo LDL turnover in hyperlipoproteinemic patients did not differ from the control (73). Since VLDL and LDL are in a precursor-product relationship, these results also suggest a defect in VLDL catabolism.

VLDL are usually found to migrate as a single band with pre beta mobility during electrophoresis (74-76). Recently, two electrophoretic populations of VLDL were isolated from human serum (77-79). The mobility of the slow subunit has been found to have more beta (LDL) rather than pre beta characteristics. The reduced mobility of the slower component was related to a loss of C apoproteins relative to B and E. This change in protein composition and increase in the ratio of cholesterol esters to triglyceride was remarkably similar to the structure of remnant VLDL (breakdown products of VLDL lipolysis) (80). These results suggest that many individuals who are hyperlipemic could have large

amounts of the remnant lipoproteins present. These  $\beta$ VLDL have been found to cause macrophages to become loaded with massive amounts of cholesteryl esters (81-83). This effect was attributable to a high affinity binding site on the macrophage surface that recognizes  $\beta$ VLDL. The B and E apoproteins, major constituents of  $\beta$ VLDL, interact with this high affinity receptor. Similar studies were conducted demonstrating remnant particle uptake by rat aortic smooth muscle cells (84). Aliquots of VLDL and  $\beta$ VLDL were added to smooth muscle cell cultures and remnant uptake exceeded that of very low density lipoproteins. Avid uptake of remnants by aortic smooth muscle cells might have relevance to the close association between increased levels of circulating VLDL remnants and atherosclerosis. Homologous studies were performed on cultured human aortic smooth muscle (85) in which regular VLDL uptake was measured instead of  $\beta$ VLDL. In humans, VLDL uptake approximated 320 to 1100 ng/100 ug of protein, whereas VLDL uptake by rat aortic muscle averaged only 5 ng/100 ug. Humans and rats differ markedly in their propensity to develop atherosclerosis. Since man appears to be particularly prone to accumulate lipid in arterial smooth muscle, these results are consistent with the possibility that these lipoproteins play a central role in atherogenesis.

An interesting observation has been made by Brown et al (86) concerning the accumulation of cholesterol in macrophages. LDL could not be incorporated into the cells unless the epsilon-amino groups of lysine in the lipoprotein had been removed or chemically modified, giving the protein an enhanced negative charge. Naturally occurring reactions which would modify these sites were investigated. A fascinating observation

noted was that treatment of LDL with malondialdehyde stimulated cholesterol LDL accumulation in monocytes (87). The importance of this reaction derives from the fact that malondialdehyde (MDA) is produced by platelets as a byproduct of arachidonic acid metabolism during the synthesis of thromboxanes (88). If liberated in high enough quantities (for example, regions of thrombosis), it is possible that (MDA) could modify LDL so that it is taken up by macrophages. If this is the case, platelet aggregation and developmental atherosclerosis may be closely linked. Platelet hyperactivity has been linked with diabetic maladies for some years since an increased platelet sensitivity to aggregation from ADP and epinephrine has been described many times in diabetes (89-92). Increased plasma prostaglandin E levels were found in diabetic patients administered aggregating agents, suggesting that an increase in this biosynthetic pathway is one cause of increased platelet sensitivity (93). Platelet function also seems to be dependent on dietary fat (94). It was found that diets low in saturated and high in polyunsaturated fats prolonged the aggregation times and decreased aggregability of platelets in subjects receiving these diets. Other studies have shown that arterial thrombus formation is significantly delayed by a high polyunsaturated/saturated fat ratio (95, 96).

Different types of endogenous hyperlipemia can also cause increased platelet function (97). The mechanism of platelet hypersensitivity in non-insulin dependent disorders is unknown. Alterations in platelet function may be related to an underlying metabolic abnormality in familial hyperbetalipoproteinemia. Platelets may incorporate total low-density lipoproteins or components into their membranes and thus alter

their surface characteristics. To substantiate this, Bolton (98) has isolated lipoprotein fractions that are responsible for changes in the electrophoretic mobility of platelets. Zahavi et al. (99) demonstrated enhanced in vivo platelet release reactions and malondialdehyde formation in patients with hyperlipemia. The mechanism of the enhanced release reaction and MDA formation could be related indirectly to elevated serum lipids. Besides changes in membrane constituents, changes in the cholesterol content of platelets can influence the metabolism of arachidonic acid and increase the levels of thromboxane B<sub>2</sub> (100). Finally, in a study measuring the effects of sucrose on insulin levels and platelet adhesiveness, Szanto (101) found that increased insulin levels correlated well with increased platelet adhesiveness.

Therefore, this introduction suggests a general mechanism to explain the relationships between diabetes mellitus, platelet aggregation, hyperlipoproteinemia and carbohydrate intolerance, all contributing factors in atherogenesis. Since the many reports about the effect of sucrose seem to conflict in the literature, the plan of this study is to administer both high sucrose and low sucrose diets employing natural food sources, to diabetic patients over a long term period to ascertain which atherogenic parameters are affected.

The recent recommendation of the American Diabetes Association Committee on Food and Nutrition and a greater consumption of processed foods which have variable but large proportions of added unquantitated sucrose, necessitates the need to know how hypertriglyceridemic diabetics respond to graded amounts of sucrose.

## 2. RELEVANT LITERATURE REVIEW

### A. Atherosclerosis Disease Description

Pathologically, the atherosclerotic lesion consists of a plaque-like thickening of the arterial wall. Moore (103) demonstrated that atheromata could be induced by placing a catheter into the aorta of rabbits for different periods of time, indicating that endothelial injury is important in the atherogenic process (112). The plaque consists of a thickening of proliferating smooth muscle cells surrounded by large amounts of interstitial substances that include collagen, elastin, glycosaminoglycans, and fibrin (104-106). The substance that is most detrimental to the atheromatous plaque is lipid, chiefly cholesterol. Compelling evidence has been established which include the following observations:

1. Atherosclerosis, leading to myocardial infarction, can be produced in every species studied by increased cholesterol consumption (105).
2. Endothelial damage in normal animals will usually produce a benign scar. However, if blood cholesterol and triglyceride levels are increased, lipid is deposited in the lesion and atherosclerosis develops (105).
3. In the human population, in which mean plasma cholesterol levels are lower than 160 mg/dl, atherosclerosis is reduced even when other factors, such as diabetes, smoking, and hypertension exist.
4. Myocardial infarctions are positively correlated with



serum cholesterol and triglyceride levels (107, 108).

5. Finally, genetic diseases that cause elevations in cholesterol concentration usually produce atherosclerosis in early childhood (109).

a. Cause of the Disease

In nature, injuries to the endothelium occur in a myriad of ways. Blood flow around irregularities, such as atheromata and branch points, could cause injury. Diabetes has also been associated with increased endothelial damage. Repeated immunization, such as insulin injection in insulin-dependent diabetics, could lead to antibody-antigen complexes resulting in vascular injury (110). Ditzel (111) raised the question that diabetics may have impaired erythrocyte oxygen transport and may develop arterial injury by hypoxia.

Platelets do not adhere to normal, healthy endothelium (114), but platelets interact with the collagen of injured endothelium through the effects of thromboxane. Further endothelial damage and the release of platelet-derived growth factor stimulates smooth muscle proliferation. The breakdown in the barrier function of the endothelium allows large amounts of plasma constituents, including lipoproteins, to penetrate the artery wall. Proliferating smooth-muscle cells migrate into the inner layer and remove foreign plasma constituents by endocytosis. These constituents are hydrolyzed components of lipoproteins that are metabolized to relatively soluble materials (free fatty acids, amino acids, and monosaccharides). The cholesterol and free fatty acids that are absorbed by the endothelium are esterified and can not be removed by

simple solubilization. Thus, cholesterol and triglyceride accumulate in the arterial wall. These smooth muscle cells do not possess receptor mediated uptake of lipoproteins but a type of scavenger mechanism is in operation (118) and large levels of lipid can be accumulated. Other constituents of the arterial wall such as glycosaminoglycans have been shown to trap lipoproteins entering the endothelial space (116, 117). These findings point to the fact that atherosclerosis is a multifaceted disease state in which many of the factors listed above are additive (107).

## B. SUCROSE

### a. History

Sucrose, or  $\beta$ -D-fructofuranosyl- $\alpha$ -D-glucopyranoside, is composed of one molecule of glucose and one molecule of fructose joined by a glycoside bond. It is a non-reducing sugar and is stable against boiling with very strong alkali solutions. However, it is more easily hydrolyzed by acids than are other common disaccharides. Sucrose is synthesized in the green leaves of plants and stored in the fruits and roots as a source of energy and carbon. Commercially, sucrose is obtained primarily from sugar beet or sugar cane, accounting for 40 and 60 percent respectively of world sucrose production (131).

Like fat consumption, the amount of sucrose consumed per individual increases with the average income of the country. In India, Pakistan, and China, daily consumption averages approximately 15 g of sucrose per person. In the U.S., England, and Scandinavia the daily individual consumption is 150 g of sucrose. From a nutritional point

of view, a high sucrose consumption is undesirable since it is a highly refined product containing no essential nutrients. Clearly, however, humans and animals have a strong preference for its sweet taste.

#### b. Digestion

Sucrose must be hydrolyzed into its monosaccharide components before absorption in the small intestine. There is no significant absorption of unhydrolyzed sucrose. In animal experiments, sucrose administered by injection was quantitatively excreted (140). Hydrolysis of sucrose is catalyzed by enzymes in the intestinal brush borders of the mucosal epithelial cells. Patients with congenital lack of sucrase are unable to absorb sucrose (133-135). Theoretically, the ability of a subject to absorb a specific disaccharide is dependent on the intestinal activity of the enzyme which hydrolyzes the specific disaccharide. It is possible that dietary disaccharide stimulates the corresponding disaccharidase activity by induction. It is postulated that a regulatory gene codes for a repressor protein which binds the operator region. Glucose and other sugars may bind the repressor so that the operator gene can synthesize mRNA resulting in increased enzyme production. This concept of specific dietary regulation of jejunal disaccharidase activity has been postulated by Rosenweig and Herman (136). They studied the effect of isocaloric diets of glucose and sucrose or sucrose and maltose. Intestinal biopsies were done on each subject after either glucose, sucrose, or maltose consumption. It was found that the activities of the disaccharidases were higher with sucrose diets than with glucose and maltose diets.

Intestinal absorption processes also respond to dietary sucrose. Rats that were adapted to sucrose diets demonstrated enhanced absorption of sucrose and constituent monosaccharides as compared to isocaloric diets of starch (42). Since disaccharidase activity has been shown to occur in proximal areas of the small intestine and glucose absorbed in this part of the intestine has been found to be a more efficient secretagogue for gastric inhibitory polypeptide (GIP), a more rapid absorption of glucose after a sucrose load in sucrose adapted individuals might partially explain the enhanced level of GIP in these individuals (138). GIP is an important stimulator of insulin secretion and the term enteroinsular axis has been used to describe the influence of this intestinal hormone on insulin secretion (139). The finding that sucrose is a much more efficient secretagogue for GIP might explain specific metabolic differences observed in sucrose as compared to glucose ingestion. Rats fed disaccharides (sucrose rather than monosaccharides) exhibited an increase in hepatic lipogenic activity (140). Disaccharides are hydrolyzed prior to their intestinal absorption and must have their effect at this level. The disaccharide effect could result from greater GIP stimulation. This stimulation produces hyperinsulinism, and signals a pattern of enzyme induction which directs carbohydrate metabolism to lipogenesis.

The idea that high sucrose diets led to an increase in hepatic fatty acid synthetase was supported by the work of Bruchdorfer and Khan (141). When the diet contained fructose or sucrose, a high correlation was found between hepatic synthetase and triglyceride concentration. The presence of these sugars in the diet could not be related to insulin

concentration. In another study of hyperlipemia due to sucrose ingestion, it was found that a majority of the triglycerides that were synthesized came directly from sucrose as the main carbon source (142). By using [ $^{14}\text{C}$ ] labeled sucrose Chlouverakis was able to demonstrate a greater [ $^{14}\text{C}$ ] content in triglycerides produced with sucrose feeding than with comparable glucose or starch feeding. Sucrose has also been found to cause greater weight gains in animals than isocaloric amounts of glucose or starch (143, 144). In general, these results imply that the organism metabolizes sucrose more efficiently than glucose. This agrees with theoretical calculations demonstrating that smaller amounts of sucrose are required to produce a unit of metabolizable energy than glucose (145). While the effects of sucrose on the liver and the endocrine pancreas point to increased lipogenesis, the cause of this hyperlipemia remains to be elucidated.

## C. HUMAN TRIGLYCERIDE METABOLISM

### a. Exogenous Incorporation

Triglycerides are the principal form in which long chain fatty acids are stored and transported in the blood. This is true whether they arise from intestinal absorption or hepatic secretion. To be absorbed, these lipids must first be converted into more polar compounds by processes that take place primarily in the lumen of the small intestine (119). The lipids are finely emulsified by the churning action of the small intestine, then further broken down by the action of pancreatic lipases and solubilized in detergent structures containing high amounts of free cholesterol and bile salts, called mixed micelles.

These structures are polar enough to transport lipid across the unstirred water layer that lies on the surface of the intestinal membrane. Once inside the intestine, these lipids are reesterified with glycerol and are transported to the endoplasmic reticulum (148, 149). Osmiophilic particles of the same size, shape and staining properties, called chylomicrons (large lipoproteins of intestinal origin), are assembled at the endoplasmic reticulum of the mucosal cell with triglyceride added to the protein core. These particles first appear in the apical region of the cell within vesicular and tubular cisternae of smooth surfaced endoplasmic reticulum. Thereafter they appear in cisternae with some attached ribosomes. The newly-synthesized chylomicrons appear to migrate from the smooth endoplasmic reticulum to the Golgi apparatus (supranuclear in intestinal absorptive cells) (120). The Golgi apparatus is a tubular system through which the chylomicrons must pass. The particles accumulate in secretory vesicles of the Golgi and appear at lateral extracellular spaces between absorptive cells. The lipoproteins are subsequently extruded from these vesicles into the lacteals and course through the lymphatics gaining access to the systemic circulation via the thoracic duct. This lymphatic route bypasses the liver and the bulk of the triglyceride is hydrolyzed in several extrahepatic tissues. After a fat-containing meal, the process of deposition begins when these chylomicrons enter the circulation and come in contact with the endothelial surface of adipocyte capillaries. FFA, and  $L \alpha$  glycerophosphate is then released and stored in the adipocyte.

### b. Endogenous Production

Most mammals can convert carbohydrate to fatty acids which can be deposited as adipose fat. The central argument concerning enzymatic triglyceride production from carbohydrate derived precursors concerns the role that processing of carbohydrate plays on fatty acid biosynthesis. Recent studies over the last two decades have led to a prodigious effort to uncover the mechanism involved in the physiologic regulation of fatty acid biosynthesis. There are two types of control mechanisms that regulate the complete synthesis of fatty acids by the de nova pathway. One is long term control which involves changes in the content of fatty acid biosynthetic enzymes including acetyl CoA carboxylase, fatty acid synthetase complex, citrate lyase, glucose 6-phosphate dehydrogenase and malic enzyme, which are stimulated in the liver when glucose is fed. By contrast, the content of these enzymes in the liver decreases during starvation or in diabetes associated with insulin deficiency (122-124). In this way, the cell has large quantities of these enzymes needed for conversion of carbohydrate into fatty acid only when carbohydrate and ATP are available.

There is also a short-term control mechanism that acts on acetyl CoA carboxylase, the rate limiting enzyme in fatty acid synthesis. It involves modulation of the activity of acetyl CoA carboxylase. Citrate activates the enzyme by causing aggregation, while long chain acyl CoA inactivates it (125). Other enzymatic steps may also be important in the regulation of fatty acid synthesis (126). For example, pyruvate dehydrogenase has been implicated as a site of control (127-128).

It was found the intestine secretes triglycerides into mesenteric

lymph as VLDL sized particles. Studies by Cenedella et al. (129) demonstrated that rats fed orotic acid, known to block liver release of lipoproteins, developed a sustained entry of triglyceride into the plasma pool. The labelled triglyceride was thought to be of intestinal origin because of the time course involved in the appearance of TG in the plasma of orotic acid fed animals. This observation was consistent with the idea that triglycerides released from the liver gain immediate access to the plasma while those from the intestine must travel through the lymphatics. Orotic fed rats formed only ten percent as much circulating triglyceride as control animals. This, coupled with a considerable less rapid triglyceride turnover suggests that the intestines could produce ten percent of the plasma triglycerides presumably derived from liver.

The hepatic subcellular pathway of triglyceride secretion shares many common features with the intestinal pathway. In the liver, secretory vesicles near the cell surface fuse with the plasma membrane resulting in the discharge of VLDL into the Space of Disse. Recognition proteins probably exist in membranes of secretory vesicles and in the plasma membranes which result in fusion of the two. Reaven suggested that microtubule protein is essential in maintaining the structural integrity of Golgi membranes and associated structures (130), with the use of antimicrotubule agents leading to a disaggregation of microtubule protein structure. This disorganization of the Golgi results in impairment of VLDL (TG) secretion possibly because the membranes of the formed VLDL-containing vacuoles lack a critical component normally acquired through the Golgi complex. The liver, the major site



of fatty acid biosynthesis, then releases these triglyceride laden lipoproteins for subsequent deposition of triglyceride into the adipocyte. This synthetic mechanism is probably the main cause of diet induced hyperlipemia. Studies using radiolabeled triglyceride components have shown that this increased synthesis plays an important role in hyperlipemia (49, 57, 58).

#### D. THE HUMAN LIPOPROTEINS

##### I. Introduction

The plasma lipoproteins have become a focus of intense study in recent years. This has resulted in part from new technical advances in electrophoresis for the separation of lipoprotein classes (146). In 1967, Fredrickson published a comprehensive survey of the plasma lipoproteins and suggested a method for defining hyperlipoproteinemia on the basis of five phenotypes (147). The use of plasma lipoprotein phenotyping became widespread with clinical interest in these proteins increasing due to accumulating evidence which related elevated levels of plasma lipoproteins to premature coronary artery disease. It has become evident that the classification of different hyperlipemias is, in fact, based on changes in the subunit structure and distribution of the plasma lipoproteins.

##### II. Composition and Identification of Lipoproteins

###### a. Chylomicrons

Dietary triglycerides are hydrolyzed in the duodenum to free fatty acids and 2-monoglycerides. After absorption, triglycerides are resyn-

thesized and incorporated into the chylomicron particle in the intestinal wall (148-149). Lymph chylomicrons are heterogenous in size, ranging in diameter from 300 to 5000 $\text{\AA}$ . These particles mainly consist of triglyceride (70-90%) and there is a direct relationship between the size of the chylomicron and triglyceride content. Chylomicrons also contain a small fraction of phospholipid (8%) and cholesterol (5%). Phosphatidylcholine and sphingomyelin are the major phospholipids present (150). The protein content of chylomicron is small but variable. Kostner (151) found that human chylomicrons isolated from the thoracic duct contain all of the apoproteins of VLDL. The approximate protein composition of chylomicrons is 66 percent apo C, 22 percent apo B and 12 percent apo A. An interesting point of this study was that apo A and apo A<sub>1</sub> were present in approximately equal quantities in the chylomicron differing from the ratio of 3:1 found in HDL.

#### b. VLDL

In man, the very low density lipoprotein (VLDL) particle is the major transport vehicle for endogenously derived triglyceride (150). Streja and Kallai (152) found that VLDL molecules are very heterogenous with sizes ranging in a continuum of 280 $\text{\AA}$ -750 $\text{\AA}$ . It was also observed that larger lighter VLDL particles were metabolized more rapidly from the plasma compartment than smaller heavier particles. The size of the VLDL particle is related directly to the triglyceride content and indirectly to the phospholipid and protein content. Triglyceride is the most abundant lipid component (56 percent) of VLDL. The average VLDL lipid content is 19-21 percent phospholipid and 17 percent cholesterol.

In VLDL the esterified to unesterified cholesterol ratio is approximately 1. Phosphatidylcholine and sphingomyelin are the predominant phospholipids.

The protein composition of VLDL has been well characterized through the use of various isolation techniques. The percent composition of soluble and insoluble apoproteins in VLDL remains fairly constant regardless of sex and age of the individual. However, there are variations in concentrations of apoproteins in VLDL from one individual to another. Observed variations in the absolute concentrations of apolipoprotein in the VLDL subfraction from individual subjects probably reflects their nutritional and metabolic status. Lee and Alaupovic (151) found that an increasing plasma triglyceride level in the post-absorptive state is accompanied by higher levels of apo B and apo C. Esterification of fatty acids in the endoplasmic reticulum could stimulate apoprotein synthesis (154). Previous studies by Gustafson (153) found that the protein moiety is an integral component of the lipoprotein particle providing structural stability for the molecule. In the presence of detergent (e.g. sodium dodecyl sulfate) apo VLDL can be solubilized and fractionated into respective apoproteins. VLDL contains about 55 percent apo B and 45 percent apo A. Concentrations of apo A are immunologically detectable, but very low. AI and AII peptides accounted for less than one percent of the total protein. Other reports estimate the apo B concentration to comprise 35 percent of total VLDL apoprotein (154). Kane and Sata (155) found another apoprotein, apo E, rich in arginine, which represented approximately 20 percent of the total apoprotein content. Apo E was at first confused with apo B,

giving the relatively high value previously seen for apo B (155). These investigators also discovered a previously undetected entity of apo C having electrophoretic mobility compatible with an increment of one unit of charge over that of another apo C subgroup. These authors hypothesized that this moiety was a sialoprotein since neuraminidase treatment resulted in proteins migrating with decreased mobility (similar to typical apo C subspecies).

### c. Low Density Lipoproteins

In the U.S., the average normal fasting adult concentration of low density lipoprotein (LDL) is 400 mg/dl in males and 340 mg/dl in females. LDL contains approximately 75 percent lipid and 25 percent protein. Lipid composition by weight is 50 percent cholesterol esters, 30 percent phospholipids and 10 percent reesterfied cholesterol with 10 percent triglyceride. Phosphatidylcholine and sphingomyelin account for 65 percent and 25 percent of the total phospholipid respectively with linoleic acid as the major fatty acid in LDL (150). LDL particles appear almost spherical in size and are quite uniform in appearance when observed by electron microscopy with negative staining techniques (156). Eighty percent of LDL particles are between 210 and 250<sup>0</sup>Å in diameter with molecular weight ranges reported to vary between 2-35 X 10<sup>6</sup> daltons (159). Subfractions of LDL have been isolated using density gradient centrifugation between 1.019 and 1.063 g/L (154, 160). Although the molecular weight of the subfractions is different, each LDL particle contains a constant percentage of protein. Studies of the density subclasses of LDL (LDL<sub>1</sub> and LDL<sub>2</sub>) demonstrate that there is a constant

ratio of soluble (apo C, A, E) to insoluble (apo B) protein in both subclasses (154). However, marked differences in the apoprotein composition of these subclasses have been observed. In contrast to LDL<sub>2</sub>, the LDL<sub>1</sub> fraction contains roughly as much apo C (38 percent) as VLDL and LDL<sub>1</sub> may represent an integral part or continuation of VLDL metabolism. All three apo C subclasses were identified in LDL isolated in the 1.006-1.019 density ranges. The LDL<sub>2</sub> subclasses represent the only segment of the lipoprotein density spectrum consisting predominantly of apo B. The soluble apolipoproteins account for only 4-9 percent of the total protein content of LDL<sub>2</sub>. Apo B content of this subgroup is in agreement with the observed LDL apo B content estimated by Lees using radial immunodiffusion (161). Plasma LDL probably functions as a storage depot for cholesterol. Extrahepatic tissues can obtain LDL-cholesterol for membrane synthesis through a receptor mediated process. In metabolizing LDL for structural purposes, extrahepatic tissues constitute a major site of LDL catabolism.

#### d. HDL

High density lipoproteins (HDL) are relatively small aggregates of lipids and proteins with diameters ranging from 90 to 120<sup>o</sup>Å (156). Lipids account for about one-half of HDL by weight. Phospholipids (42-51 percent), cholesterol (32 percent) and triglyceride (10 percent) are the main components (150). Phosphatidylcholine constitutes 70-80 percent and sphingomyelin 12-14 percent of the total phospholipid. Phosphatidylserine and phosphatidylinositol are minor components. Linoleic acid is the predominant fatty acid of the esterified

cholesterol.

Protein moieties of HDL have been identified using DEAE-cellulose chromatography and consist of two major components (165). About 90 percent by weight of the protein in HDL is either apo AI or AII. AI accounts for 70 percent and AII about 20 percent of the total protein. Other peptide components, C and E protein subunits, have been isolated from (HDL<sub>2</sub>) subfractions but comprise minor amounts of the total HDL protein (166). The ratio of apo AI to apo AII varies within each HDL subfraction. These peptides are remarkable in that they can function as detergents capable of solubilizing lipids and cholesterol in large quantities. In fact, 1 g of HDL AI can solubilize up to 2.5 g of phospholipid in a disc-like micelle (167), structures analogous to the lecithin-cholesterol-bile salt micelles found in bile. However, bile salts act as detergents in fat digestion, whereas HDL proteins are the detergents in plasma (168).

A minor HDL component, HDL<sub>1</sub>, has been isolated between 1.050 and 1.063 g/L (HDL normally are between 1.063-1.21). HDL<sub>1</sub> has electrophoretic and immunochemical properties similar to normal HDL<sub>2</sub> (162). Several HDL subspecies have been identified by analytical and preparative gel electrofocusing (163-164), but their physiological significance is unknown. However, the human plasma HDL<sub>2</sub>/HDL<sub>3</sub> ratio is affected by several pathologic and physiologic processes.

#### e. Pre $\alpha$ Lipoprotein

A pre  $\alpha$  lipoprotein has been observed in human serum with electrophoretic mobility and staining characteristics in agarose gel similar to

albumin (160). Free fatty acids and a high content of the phospholipid lysolecithin are present in this fraction (170). The ability of albumin to bind both types of lipids lends support to the theory that albumin makes up a substantial part of the pre  $\alpha$  lipoprotein. The presence of other phospholipids and neutral lipids suggests that other apoproteins may be present in pre  $\alpha$  lipoproteins. Wille and associates (171) characterized the protein fractions utilizing immunological methods. They found that one single band was found on PAGE electrophoresis, but double diffusion experiments disclosed the presence of apo AI as well. The total amino acid composition is similar to albumin except for higher amounts of glycine, serine and glutamic acid. Use of mercaptoethanol to reduce disulfide bonds resulted in two bands on PAGE where one had been previously observed (172). One protein had a molecular weight comparable to albumin (67,000) and the other to apo AI (28,400). Albumin may attach peptides through disulfide bridges, particularly at position SH-34 cysteine (173). Since apo AI does not contain a cysteine residue, the apo AI molecule might be enclosed within crevices in the larger albumin molecule (174). A functional role has previously been ascribed to a pre  $\alpha$  lipoprotein in lysolecithin transport (180) and may function as an acceptor for the lysolecithin generated by the lecithin-cholesterol acyltransferase reaction in plasma (175).

#### f. Lipoprotein (A)

Lipoprotein A (LpA) exhibits the same properties as LDL, but is considered an additional lipoprotein class. It is isolated in the

1.050-1.120 g/L density range and has a molecular weight of about  $5 \times 10^6$  daltons. LpA exhibits pre- $\beta$  mobility on agarose gel electrophoresis and has a lipid composition similar to LDL, though its protein content is completely different (176). LpA, when delipidated, contains 65 percent apo B, 15 percent albumin and a unique apoprotein called apo Lp(A) (177). The occurrence of LpA in the general population may be as high as 75-85 percent with an average plasma concentration of 14 mg/100 ml. LpA concentration does not appear to correlate with age, sex, or lipid concentration, and the functional significance of LpA is not known (178-179).

#### E. THE APOPROTEINS

Until about ten years ago, lipoproteins were viewed as isolated families or species defined either by ultracentrifugation or by electrophoresis. This basis of classification provided little insight into causes of lipoprotein abnormalities or the role of the lipoproteins in lipid transport. Many processes involved in lipoprotein metabolism are regulated by enzymes whose activity is influenced by the apoproteins. It is generally accepted that there are at least seven different apoprotein classes which differ in their primary, secondary, and tertiary structure. These proteins also differ in their function and distribution throughout the lipoprotein spectrum. (Table 1).

##### a. Apo A

Chromatography of delipidated apo HDL yields two pure polypeptides designated apo AI and apo AII, the major apoproteins of HDL (181). Apo



AI is a single polypeptide chain of 245 amino acids and has a molecular weight of 28,331 daltons. The amino acid content determined by Baker indicated a protein composed primarily of cysteine, cystine, isoleucine and carbohydrate (182). Glutamine is the carboxyterminal amino acid and aspartic acid is the amino terminal group. Heterogeneity has been observed by Nestruck et al. who found four isoprotein subgroups of apo AI (183). They had identical molecular weights and common antigenicity with antisera raised against apo AI. Each form had a similar amino acid composition except for A IV which contained one isoleucine residue per mole. All forms of apo AI were LCAT activators except for AIV which inhibited the enzyme (183).

AI - AIII are considered equivalent polymorphic forms while AIV is considered a different form of unresolved heterogeneity. Apo AI has been isolated from a wide range of animal species, but all types have similar physiochemical properties (184). Results from many structural studies indicate lipid binding properties may be related to the degree of self-association of the proteins (185-188). Vitelli and Scanu (189) have found that the molecular weight of Apo AI can vary between 30,000 and 167,000 at protein concentration of 0.2-0.9 mg/ml due to a monomer-dimer-tetramer-octamer equilibrium. A minor apo AI component has been isolated by Weisgraber et al. that coelectrophoresed with apo AI on SDS polyacrylamide gel (190). It was immunogenically similar to apo I except that it contained two amino acids, cysteine and isoleucine, which are not found in normal apo AI. By virtue of its cysteine group, apo AI is capable of forming disulfide bridges.

## PROPERTIES OF LIPOPROTEINS

	Chylomicrons	VLDL	LDL	HDL <sub>2</sub>	HDL <sub>3</sub>
Major Constituents	triglycerides	triglycerides	cholesterol, protein, phospholipid	protein, phospholipid, cholesterol	
Apolipoproteins	B, A, C	B, C, E	B	A, C, D, E	
Size (Å)	> 1000	300-800	180-250	100	75
Density (g/ml)	< 0.95	0.95-1.006	1.006-1.063	1.063-1.125	1.125-1.21
Flotation (S <sub>f</sub> 1.063)	> 400	20-400	0-20	(F <sub>120</sub> <sup>0</sup> ) 3.5-9	0-3.5
Mobility	origin	pre-beta	beta	alpha-1	
Precipitation			↑?	↑	↑?      ↑

Table 1: Summary of the basic properties for the 5 specific lipoprotein classes listed.

Apo AII is the second most abundant apoprotein of human HDL. It consists of two identical polypeptide chains of 77 amino acids which are linked by a disulfide bridge at cysteine-6 (199). Histidine, arginine, tryptophan and carbohydrate are absent from apo AII. Stone and Reynolds (188) have calculated that apo AII has a dimeric molecular weight of 34,000. Gwynne et al. (193) on the other hand, reported the molecular weight of apo AII is dependent on concentration and obtained an equilibrium constant of  $2.8 \times 10^4 \text{M}^{-1}$  for monomer-dimer equilibrium.

Studies from other animals indicate the disulfide bridge is not crucial for apo II to fulfill a lipid carrier role (194-195). The interactions between A apoproteins and lipids have been extensively studied (196). Both apo AI and apo AII bind phosphatidylcholine to form protein-phospholipid complexes (197). When apo AII is added to a sonicated mixture of either lecithin or sphingomyelin, 79-90 percent of the apo AII can be isolated in the HDL density range. In contrast, only about ten percent of added apo AI is found in this range.

#### b. Apo B

Apo B is the least well characterized of the plasma apoproteins. Similar to integral membrane proteins, apo B is not readily soluble in aqueous solutions unless high concentrations of detergents are added (198-200). Results from solubilization studies have resulted in no general agreement concerning the number of subunits or the molecular weight of apo B. Reported weights range from 8000-275,000 daltons with the most commonly reported values between 25,000-30,000 daltons (201).

Apo B has been recently studied and found to have an amino acid composition similar to that of the other apoproteins (201). The concentration of apo B in normal human plasma is between 70-110 mg/dl and primarily found in LDL (204-205).

### c. Apo C

Fractionation of apo C into its component proteins can be achieved by ion exchange chromatography or isoelectric focusing (166). Apo CI is a single polypeptide chain of 57 amino acid residues (206). The protein contains about 55 percent  $\alpha$ -helix which is increased in the presence of phospholipid (207). Upon fractionating the protein in SDS urea gels, certain bands are produced through carbamylation of the  $\epsilon$ -amino groups of lysine with urea. This carbamylation converts the positively charged lysine to neutral homocitrulline and results in greater anodal migration on alkaline polyacrylamide gels (208). Once precautions are taken, these artifactual polymorphs disappeared.

Brown et al. (209) reported that all of the apo C's aggregate to form high molecular weight species in the absence of dissociating agents. In addition to binding and transporting lipid, apo C activates lipoprotein lipase and LCAT (210).

Human apo CII contains 79 amino acids and has a calculated molecular weight of 9110 daltons (211). In addition to binding lipid, apo CII is a potent activator of lipoprotein lipase from post-heparin plasma, adipose tissue and cow's milk (212-218). The work of Matsuoka et al. (70) demonstrates that apo CII acts on lipoprotein lipase by enhancing the interaction between lipoprotein lipase and triglyceride

within the surface monolayer of the lipoprotein particle (70). His work with apo CII deficient patients has provided much information concerning enzyme kinetics of the lipoprotein lipase-apo CII reaction. Phospholipids also appear to be necessary for optimal lipolysis. In the absence of lipid, apo CII forms a stable complex with purified milk lipoprotein lipase (214). Apo CII contains only 23 percent  $\alpha$ -helix and undergoes self-association at protein concentrations greater than 1 mg/ml (209).

Apo CIII, the most abundant apo C constituent, contains 79 amino acids, has a molecular weight of 8764 daltons and is a glycoprotein with an oligosaccharide attached to threonine at position 74. The oligosaccharide contains one residue of galactose and galactosamine and 0-3 residues of sialic acid (215). The complete amino acid sequence has been determined with amino terminal threonine and carboxy terminal alanine. Polymorphism of the protein on polyacrylamide gel electrophoresis is caused by differences in sialic acid content (215-216). Nestruck et al. (217) have shown that sialylation occurs in the Golgi apparatus of rat liver and the extent of sialylation increases with residence time of VLDL in the organelle. While the physiologic function of apo CIII is uncertain, Brown et al. (218) have found apo CIII is an inhibitor of lipoprotein lipase. This is due to binding of apo CIII to the lipase substrate with resulting prevention of enzyme substrate interactions. If apo CIII exceeds 2 percent of the weight of the lipase substrate, it reduces the hydrolysis of lipoproteins containing sufficient apo CII. Blood apo C concentrations are not stable and have been found to be influenced by diet and metabolic status. A

decrease in the ratio of apo CII/apo CIII has been observed in the of hypertriglyceridemia (69). In addition, high carbohydrate diets result in alterations in the metabolism of apo CII and apo CIII suggesting the secretion of both apoproteins is increased or their clearance is delayed (67).

#### d. Arginine Rich Apoprotein (Apo E)

The arginine rich protein was first isolated by Shore and Shore (166) from VLDL and HDL. In normal VLDL, apo E content is approximately equal to that of apo CI and apo CII and exceeded only by that of apo B and apo CIII (219). A molecular weight for apo E of 33,000 daltons was obtained by SDS-polyacrylamide gel electrophoresis. Approximately ten percent of the total amino acids are arginine. Lysine and alanine are reported to be the amino and carboxy terminal acids respectively (220). The arginine-rich protein has a high  $\alpha$ -helical content (221). It is present in several polymorphic forms which can be separated by either ion exchange chromatography or isoelectric focusing (222-225). In humans, a diet containing increased cholesterol causes elevations in plasma apo E levels, some of which are found in HDL. HDL containing apo E has been shown to have a greater affinity than LDL for the LDL receptor (226). Studies on patients with type III hyperlipoproteinemia have shown that plasma levels of total apo E are increased, but that a deficiency exists in one of the isoprotein subunits (227-223). The deficiency may be seen as a structural mutation of the isoprotein subunit causing a different migration on two dimensional electrophoretic gels. Studies by Havel and Chao (228) suggest

another function of apo E. In perfused rat livers, treated with ethynyl estradiol for five days, there was a stimulated uptake of lamellar complexes of apo E. Human lipoproteins with apo E, containing all of the major protein isoforms, were comparable to rat lipoprotein containing apo E, during estrogen stimulated uptake at the hepatic site. However, lipoproteins from patients with dysbetalipoproteinemia, Type III disease, were characterized by decreased hepatic uptake. Havel hypothesized that apo E is an essential component required for recognition by the hepatic receptor to remove remnant VLDL. Accumulation of remnant particles is often found in type III hyperlipoproteinemia. Further proof of the function of apo E was obtained by Weisgraber and Mahley (229) upon their discovery of a 46,000 MW complex in VLDL and HDL. This protein was reducible with mercaptoethanol or dithiothreitol into two chains indicating the presence of disulfide bonds. The two subunits were identified as apo E AII monomers by coelectrophoresis and immunochemical activity. It was found that conversion of the complex to the E apoprotein resulted in a markedly enhanced binding activity of the lipoprotein, containing the (apo E-AII), to receptors. The dimer is thought to block the recognition sites on apo E for the receptor. Elevated levels of this dimer complex could be responsible for decreased uptake of VLDL remnants and HDL by their receptors.

#### e. Apo D or Thin Line Apoprotein

A protein has been isolated from human HDL and has been referred to as thin line protein or apo D (223-231). The name, thin line

protein, is derived from a characteristic thin precipitin line formed near the antigen well with either anti-HDL or anti-whole serum. Apo D has been found in HDL<sub>3</sub>, LDL and VLDL (232). A preliminary estimate indicates this protein accounts for 1-2 percent of the total protein content of normal HDL (233). The protein is characterized by the presence of all common amino acids, including half-cystine. The carboxy terminal acid is serine, and carbohydrate analysis demonstrates apo D is a glycoprotein of 22,100 MW (234). Carbohydrate moieties present are glucose, mannose, galactose, glucosamine, and sialic acid (accounting for 18 percent of the dry weight of apo D). The lipid moiety contains cholesterol, cholesterol ester, triglyceride and phospholipid. The phospholipid content is characterized by a relatively high amount of lysolecithin and sphingomyelin and a relatively low amount of lecithin. Kostner (235) found that apo D activates LCAT and Olafsson and Gustafson (236) have suggested it may be a specific protein carrier for lysolecithin formed by the action of LCAT on HDL. Chajek and Fielding (237) demonstrated that human plasma contains a specific lipoprotein apoprotein that catalyzes the net transport of cholesteryl esters from HDL to VLDL and LDL. The purified protein is identical to that of apo D. It is possible that the normal catabolism of VLDL to LDL requires the presence of both HDL and plasma cholesteryl ester transport protein, apo D.

#### F. STRUCTURE OF PLASMA LIPOPROTEINS

Serum lipoproteins are referred to as soluble proteins to specify their solubility in aqueous media. Since they are fundamental in lipid



transport, a knowledge of their structural makeup is necessary to understand their function. Scanu (238) reviewed the methods of analysis used to ascertain membrane structure (238). Some of these methods include chemical and enzymatic studies, reassembly studies, ultracentrifugation, electron microscopy, Optical Rotatory Dispersion (ORD), Circular Dichroism (CD), IR, NMR, ESR, X-ray techniques and surface balance studies.

The ability of plasma apoproteins to interact to form soluble complexes with phospholipids cannot be explained by amino acid content or from direct inspection of primary structure. The overall amino acid structure is similar to other soluble plasma proteins. Segrest et al. (230) built space filling models for apoproteins whose sequences were known and found a common structural feature in the phospholipid binding region. Based on model-building studies and the assumption that an  $\alpha$ -helix is fundamental to the mechanism of phospholipid binding, he suggested that these regions, referred to as amphipathic helices, represented the binding sites of apo A and apo C for phospholipid. Such helices have both polar and non-polar sites each comprising 180° of the cylindrical surface. Such an arrangement facilitates simultaneous interaction of the protein with water insoluble lipids and with the external environment. Negatively charged amino acid residues (aspartic and glutamic acids) are clustered toward the middle of the polar face while positively charged basic side chains (lysine, arginine) are oriented toward the lateral edges of the interface between the polar and non-polar sides. This topographical arrangement of charged amino acids led to the suggestion that steric zwitterions are created and electro-

static attractions between the positively charged choline groups of the phospholipid with the glutamic acid or aspartic acid side chains and between the phosphate and the lysine or arginine residues could occur. Maleylation of apo AII abolishes the binding of phosphatidylcholine suggesting that lysine plays a role in the binding mechanism (240). If such ionic interactions do exist, they are probably less important than the hydrophobic interactions that are found.

Another possible function of these steric ion pairs is that they could produce an initial orientation of the apoprotein with respect to the phospholipid which favors the interaction of these species during the initial phase of binding. The steric zwitterions within the helix are composed of two amino acids which occur in a 1, 2 or 1, 4 relationship (235). These 1, 2 and 1, 4 ion pairs are not found in globular proteins which suggests they are of importance in lipoprotein phospholipid interactions (241). It has been suggested that these ion pairs could function to lock a section of the peptide backbone into an  $\alpha$ -helix through preferential hydration of the polar side of the helix. Sparrow et al. (242) have synthesized a series of model peptides which would be expected to have characteristics of amphipathic regions (242). The ability of these synthetic peptides to bind phospholipid was shown to depend on the degree of hydrophobicity of the non-polar surface of the helix. By making a substitution of Try-Trp for Ala-Ala, Sparrow found it was possible to transform a non-binding peptide into one which binds lipid more readily (242).

The mass of data on lipid and protein interactions have led to the development of three separate models for lipoprotein structure. The

lipid core model was proposed for VLDL and chylomicrons. The physiochemical problem of keeping a neutral lipid-rich particle in solution is overcome by putting triglycerides and cholesteryl esters into a central core while phospholipids and cholesterol are on the surface (243-245). Gustafson (246) has calculated that the quantity of protein, phospholipid, and unesterified cholesterol in VLDL is sufficient to cover the surface of the particle. Schumaker (247) has suggested that as triglycerides are removed from the particle by the action of lipoprotein lipase, there is a concomitant reduction in surface area affected through the removal of cholesterol by the action of LCAT.

Models of LDL structure are based on small angle X-ray scattering, nuclear magnetic resonance, and neutron scattering techniques (248-250). Mateu et al. (248) have concluded, from X-ray scattering, that there are outer and inner high electron-dense regions separated by an immediate low-electron-dense region (248). The LDL model suggested by this work has a phospholipid bilayer with cholesteryl esters, cholesterol and triglycerides separating an outer protein shell and an inner protein core. However, other investigators using neutron diffraction, demonstrated that the central core is predominantly occupied by hydrocarbon chains of lipid (250). Finer (249) has used NMR to probe the location of lipid and protein and has concluded that at least 30 percent of the choline residues are immobile and inaccessible to solvent. It has been suggested that LDL contains a trilayer structure that surrounds a central core of protein (249).

To date, the bulk of the experimental data resulted from analysis

of the HDL lipoproteins. While the data is diverse, several common features are shared by the different models proposed (251-253). The particle is micellar with the head groups of the phospholipids and the polar end of cholesterol oriented toward the surface. The apolar ends of these molecules are oriented toward the center with the cholesteryl esters sequestered on the inside of the particle. The major point of disagreement between suggested models is the relative location of the principal apoprotein components and how they interact with the phospholipids. Studies by Shen et al. (254) using compositional analysis have shown that a hydrophobic core of cholesterol ester and triglyceride is surrounded by a  $20\text{\AA}$  thick monolayer of cholesterol and phospholipid closely packed on the surface of the core. The curvature of the surface results in large gaps between the polar head groups of this monolayer. The unfolded protein fills these gaps and masks the polar head groups of free cholesterol. The essential features of this model are a sharply defined boundary between the hydrophobic core and the amphipathic layer surrounding it. This boundary is occupied exclusively by phospholipids and cholesterol and their tight packing does not allow for the penetration of any other component. The apoprotein is located in the same surface layer as the phospholipid head groups with these two components competing for the same space. Therefore, Scanu has speculated that an identical structure for all lipoproteins could be assumed regardless of size.

#### G. LIPOPROTEIN SYNTHESIS AND CATABOLISM

The liver and intestine are the major sites of synthesis of the

plasma lipoproteins. On a high fat diet, the bulk of triglyceride fatty acids are derived from chylomicrons synthesized in the intestine. Dietary carbohydrates may also be converted to fatty acids in both liver and adipose tissue.

#### a. Chylomicron Synthesis

Absorption of dietary fat occurs mainly in the upper small intestine. Tytgat (255) found that the major site of chylomicron synthesis in man is the jejunal mucosa. Electron micrographs indicate that the (SER) is the major cellular organelle involved in the transport of newly synthesized triglyceride and suggest that chylomicron synthesis is completed there (256-257). Synthesis of chylomicron lipoproteins presumably occurs in the ribosomes of the RER (257).

#### b. VLDL Synthesis

Both the liver and intestine incorporate radioactive amino acids into the protein moieties of VLDL (258). But no radioactivity was incorporated into apo C proteins when intestinal perfusion systems using labeled amino acids were employed. This suggests that apo C proteins are synthesized in the liver and their occurrence in lymph is due to a transfer from lipoproteins of hepatic origin. Intestinal synthesis of apo B has been observed in rats fed a diet containing orotic acid (259). The mesenteric lymph of these rats has mean normal levels of apo B, whereas the plasma contains virtually no apo B. Orotic acid-fed individuals accumulate hepatic fat as a result of their inability to mobilize lipid from the liver (260). The liver and intestine share many common

features in the synthesis and excretion of these particles. Secretion is influenced by the availability of non-esterified fatty acids of either endogenous or exogenous origin. The lipid composition of the particles is influenced by several factors. Heimburg and Wilcox (261) have shown that approximately 50 percent more VLDL triglyceride is secreted when isolated rat liver is perfused with oleic acid than with palmitic acid. VLDL secreted during palmitate infusion are more dense, contain a higher proportion of cholesterol to phospholipid and have a lower proportion of triglyceride as compared to rats fed oleic acid. During the early stages of VLDL synthesis, the Golgi apparatus of hepatocytes contain fat particles of 300-1000Å in diameter, which have physical and chemical properties similar to VLDL (262).

Other types of dietary manipulations can also effect the secretion of VLDL. Dolphian et al. (263) demonstrated that normal hepatic Golgi and secretory vesicles contain triglyceride-rich lipoproteins which have none or minimal amounts of apo CII and are deficient in apo E relative to serum VLDL. Hepatic fractions from hypercholesterolemic rats contained cholesteryl ester and apo E-rich triglyceride-depleted VLDL with size, immunodiffusion characteristics, and apo B to apo E ratios similar to hypercholesterolemic serum VLDL (263). He deduced that diet induced hypercholesterolemia resulted in lipoprotein secretory products which contribute to the plasma pool of abnormal cholesteryl ester and apo E-enriched lipoproteins. Witzum (66) observed that liver perfusion of rats on high carbohydrate diets resulted in increased triglyceride secretion. The VLDL isolated were larger, triglyceride-enriched, less dense than normal. Although total VLDL protein rose, the

amount of VLDL apo B decreased. Both apo C and apo E levels rose while apo CIII subfraction levels were altered. Apo CIII<sub>1</sub> increased, while apo CIII<sub>2</sub> decreased. This suggests the amount of time spent in the Golgi was decreased because of the small amount of protein sialylation (reflected in decreased apo CIII<sub>2</sub> levels).

Finally, Zannis and Breslow (264) demonstrated that patients with Type III hyperlipidemia (Broad Beta Disease) had non-characteristic apo E isoprotein patterns. They demonstrated a post-translational modification of apo E by showing that treatment of VLDL with neuraminidase prior to electrophoresis resulted in a disappearance of the non-characteristic band. This indicated that the group of faulty proteins occurred by sialation of apo E.

### c. Chylomicron Catabolism

Chylomicron catabolism has been found to occur rapidly with a half life of less than one hour. Initially, this catabolism involves triglyceride hydrolysis catalyzed by lipoprotein lipase followed by the removal of a triglyceride poor remnant by the liver. Phospholipids are hydrolyzed by both lipoprotein lipase and lecithin cholesteryl acyl transferase. The role of the apoprotein components in chylomicron metabolism has been reported by Havel (271) to consist of net transfer of apo CII proteins from HDL to chylomicrons and back to HDL. Other apo C proteins are transferred at the same time. The presence of apo CII on the surface of the chylomicron could promote hydrolysis by stabilizing triglyceride emulsions. The apo C proteins very likely modulate the catabolism of triglyceride-rich chylomicrons through this cycling

mechanism involving HDL. The chylomicron particle that remains after hydrolysis forms the remnant particle which is removed by the liver (272). This observation is based on studies of the catabolism of lipoproteins from hepatectomized rats. Radiolabeled chylomicrons were injected into these animals and remnant particles enriched with phospholipids and cholesteryl esters were isolated from plasma (273). Cholesterol taken up by the liver is converted to bile and released from the liver.

#### d. VLDL Catabolism

The degradation pathway of VLDL is similar to that of the chylomicrons. It is believed that chylomicrons and VLDL share a common catabolic pathway and their triglycerides are hydrolyzed at extra-hepatic tissues by the same lipoprotein lipase system (267). Labeling studies with the apo C component of VLDL have shown that these apoproteins rapidly appear in HDL fractions after the addition of LPL (274). In addition to the radioactivity found in the HDL fraction, transfer of radioactively labeled proteins was observed in the 1.006-1.019 density range and then in the low density lipoprotein range. This would indicate a precursor-product relationship between VLDL and LDL. Eisenberg et al. (275) demonstrated that lipolysis induces transfers of apo CII and apo CIII from VLDL to HDL. This transfer was proportional to the extent of triglyceride hydrolysis and similar for the two apoproteins. Their results suggested that there is no preferential removal of CII or CIII from VLDL particles. The redistribution of apo C between VLDL and HDL during lipolysis may be caused by deletion of core VLDL consti-



tuents, surface VLDL constituents and changes in mass ratios between the two lipoprotein classes. Glangeaud et al. (276) demonstrated that the presence of HDL is not always an obligatory requirement for the lipolysis of VLDL to occur. Their observations showed that in the absence of albumin or serum, extremely minimal lipolysis occurred and apo C was not released. However, with albumin present, lipolysis occurred with release of apo C protein. HDL, though it acted as a pool for apo C, was not necessary for lipolysis, and in the absence of HDL the apo C proteins were isolated in several density regions with different lipid composition. Eisenberg (277) had demonstrated this same phenomenon when he found that surplus surface constituents, phospholipids, apo C, and cholesterol were freed from the VLDL to form a surface fragment particle. In a system devoid of plasma or lipoprotein, he observed a decrease in the VLDL surface area that was independent of any acceptor protein. The remaining VLDL remnants had the same chemical and physical properties as those which underwent lipolysis.

During the catabolism of VLDL, the loss of surface components converts the lipoprotein into a progressively smaller particle designated as the intermediate density lipoprotein. VLDL remnants are taken up more rapidly than larger VLDL particles by cells in culture (278). This partial hydrolysis of the triglyceride-rich lipoprotein appears to be necessary for efficient cellular uptake. The LDL receptor recognizes two apoproteins, apo B and apo E, both of which are contained in the VLDL remnant (279). The enhanced reactivity of remnants with cells may be due to several factors: 1) As larger lipoproteins are converted to smaller remnants, steric hindrance to their interaction with receptors

is reduced. 2) The selective retention of apo B and apo E could concentrate apo B and E over lipoprotein surfaces. 3) The conformations of the binding sites of apoproteins could be altered as the surface area of the lipoprotein is reduced. Binding sites could also be unmasked as a consequence of lipid and protein removal (280), a possibility recently investigated by Shelburne and Windler (281-282). They studied the effect of different apo C proteins on liver removal of triglyceride emulsions incubated with apo E and found that emulsions containing both CI and CII proteins did not effect the increased hepatic uptake of either the synthetic emulsion or chylomicron produced apo E remnants. However, the CIII protein had a pronounced inhibitory effect on hepatic uptake which could be influenced by the state of apoprotein sialation. It is possible that this glycoprotein is similar to other sialated glycoproteins that demonstrate a negative correlation between neuramminic acid content and hepatic receptor affinity (283). The observation that at least two apoproteins control hepatic uptake provides a mechanism for denying the lipoproteins hepatic access until the appropriate peripheral metabolism has occurred. If CIII were not present, an ineffectual recycling to the liver might result.

The second step in VLDL metabolism is remnant or intermediate uptake by the parenchymal liver cells (284). Formation of low density lipoproteins has been postulated to follow the uptake of IDL by the liver with a subsequent release of apo B-cholesterol ester-rich particles. Man, who has the highest LDL concentration, is the only species in which almost all VLDL apo B is recovered in LDL (285). Deckelbaum et al. (286) have shown that in humans, a direct conversion

of VLDL to LDL can occur solely by the action of extrahepatic lipase. These particles have many features in common, but are not identical to normally formed plasma LDL. This pathway could function as a major non-hepatic VLDL catabolic route. Recently, data from the labs of Janus and Nicoll (287) have demonstrated that in some patients, not all VLDL is converted to LDL, but that some lipoprotein is degraded directly. In normal controls, VLDL-B derived LDL-B production was found to be virtually identical, suggesting that all LDL-B is produced from VLDL. However, in hyperlipemic individuals, the mean conversion of VLDL to LDL was only 45 percent, suggesting a direct catabolism of VLDL not previously observed (287). Since IDL has been shown to be as potent as LDL in suppressing receptor mediated HMG CoA, it is not unreasonable to assume that binding and degradation of IDL to cells might form an integral part of this observed effect (288) (Figure 1).

#### e. Lipoprotein Lipase

A description of the catabolic events that occur when chylomicrons or VLDL are broken down would be incomplete without an understanding of the lipoprotein lipase (LPL) enzyme system. Recent studies have shown that the lipase activity released by heparin is actually a combination of lipoprotein lipase, extra-hepatic triglyceride lipase, hepatic triglyceride lipase, monoglyceride hydrolases, and phospholipases (265). There are at least two triglyceride lipases related to post-heparin lipolytic activity which differ in substrate specificity and other physiochemical properties (265). Lipoprotein lipase (LPL) has a pH optimum of 8.0, requires a serum cofactor, and is inhibited by prota-

mine sulfate. The lipoprotein lipase component of post heparin lipase activity (PHLA) is a composite of enzymes from adipose tissue, skeletal muscle, heart, mammary gland, lungs, and aorta (266). These enzymes are assumed to be on the capillary surface since their release occurs almost immediately following heparin infusion.

In 1955, Korn and Quigley (267) described a serum factor which activated lipoprotein lipase. This protein activator, found in VLDL and chylomicrons is CII (212-218). Recent studies have shown that apo CII is the major cofactor in human serum for adipose tissue, skeletal muscle, and purified plasma LPL. Apo CII stimulates product formation and has been found to increase the catalytic rate of LPL. Jackson and Matsuoka (268) have found that there is a direct relationship between the amount of lipoprotein lipase bound to VLDL or chylomicrons, and the amount of apo CII present. By adding  $I^{125}$  lipoprotein lipase and HDL to VLDL they were able to show that as apo CII was transferred to HDL the amount of lipase bound to VLDL diminished proportionately. Other apoproteins activate lipoprotein lipase. Ekman and Krauss (269-270) demonstrated that apo AII activated LPL but apo E, apo CI, apo AI, and apo CIII inhibited LPL. These investigators hypothesized that inhibition of the lipolytic processes by high concentrations of these apoproteins in certain disease states may be a contributory factor in the development of hypertriglyceridemia since the proteins decrease substrate availability to the enzyme active site.

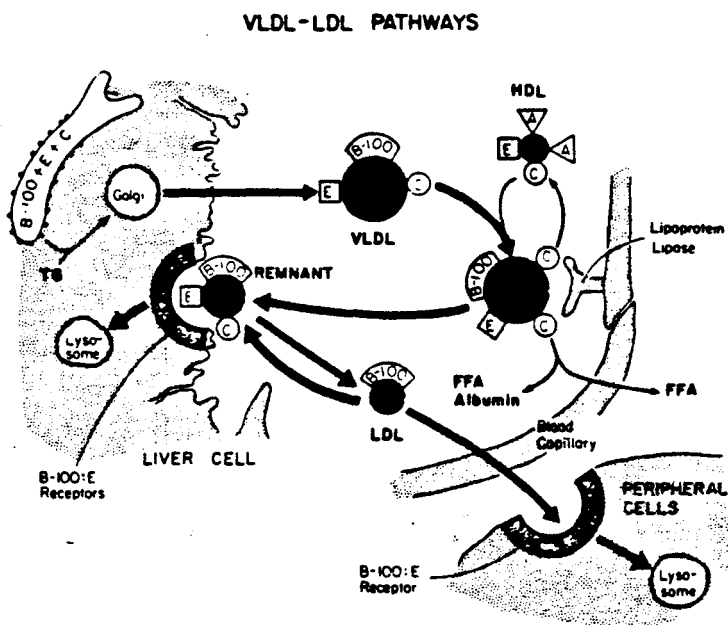


Figure 1: Schematic representation of VLDL-LDL catabolic pathways. The VLDL produced in the liver is partially catabolized in the periphery at the lipoprotein lipase site releasing triglyceride and FFA into the blood and adipocytes. Apo C protein is also lost from the VLDL and is scavenged in the plasma by circulating HDL which acts as a pool for apo C. After loss of the apo C, partial surface destabilization of the VLDL, now called a remnant, occurs. These remnants are returned to the liver. Apoprotein E found on the surface of the remnant is a recognition marker for the liver cell receptor which binds the remnant and removes it from the circulation. The remaining triglyceride is released to the hepatocyte and a molecule rich in cholesterol and containing primarily apoprotein B is released into the plasma pool.

#### f. LDL-Synthesis and Catabolism

Synthesis of LDL presumably occurs after hepatic lipolysis of remnant VLDL. Direct synthesis may also occur in the liver. As the size of the VLDL particle diminishes, it becomes an intermediate density lipoprotein. Excess surface material, mostly phospholipid and cholesterol, are transferred to HDL. HDL particles interact with LCAT, (lecithin cholesterol acyltransferase) which esterifies the excess cholesterol with fatty acids from the 2-position of lecithin. The synthesized cholesteryl ester is transferred back to IDL, apparently through apo D. The IDL is then absorbed in the liver sinusoids where most of the remaining triglyceride is lost and a pure LDL is released into the circulation.

In humans, LDL is the principal lipoprotein carrier for cholesterol. Approximately three-fourths of the cholesterol is esterified and half of the cholesterol esters contain oleic acid. Low density lipoprotein receptors were first discovered by Goldstein (289) using cultured human fibroblasts. The initial event in cellular LDL metabolism involves the binding of LDL to this receptor. This binding exhibits saturation kinetics, high affinity and specificity. In addition, the process is stimulated by the presence of calcium and severely inhibited by pronase. LDL binding involves ionic interaction between the protein component and the surface receptor which appears to be a glycoprotein (290-291). In order to achieve the physiological effect, the LDL that is bound to the receptor enters the cells by endocytosis. A low affinity uptake can also occur and may represent a non-specific endocytosis since uptake is proportional to the lipoprotein concentra-

tion in the system, is not affected by pronase, and shows no apparent saturation (291). The degradative process appears to be similar regardless if the LDL is bound to a specific high affinity receptor or low affinity receptor (291). Goldstein (289) hypothesized that LDL is catabolized through the formation of endocytic vesicles called endosomes, which fuse intracellularly with lysosomes. The protein component of LDL is then catabolized to amino acids and the cholesteryl ester component of LDL is hydrolyzed by a lysosomal acid lipase. Intracellular accumulation of unesterified cholesterol regulates the activities of two microsomal enzymes. A reduction in cholesterol synthesis results from modulation of HMG CoA reductase (3-hydroxy-e-methylglutaryl coenzyme A reductase) and an increase in cholesterol esterification results from activation of acyl CoA: cholesteryl acyltransferase (292). The net effect of the LDL receptor-mediated process is to transfer free and esterified cholesterol from LDL to the cell altering the fatty acid composition of cholesteryl esters from polyunsaturated to a more saturated form (293).

The activity of the LDL receptor is regulated by a feedback mechanism. Brown et al. (290) observed that incubation of fibroblast monolayers with LDL progressively reduces the ability of the cells to bind  $I^{125}$  labeled LDL due to a reduction in the number of receptors synthesized (290). More than 90 percent of the cholesterol produced in the body comes from the liver or the intestine, even though all non-hepatic tissues need cholesterol and possess the enzymatic capacity to synthesize it (294). Brown (295) proposed that cholesterol synthesis is suppressed, in vivo, in non-hepatic tissues because these tissues pre-

ferentially take up and utilize LDL cholesterol through the LDL high affinity receptor.

Human fibroblasts produce a maximum of 20,000-50,000 receptors per cell. Brown (296) characterized the receptor which requires a divalent cation (either calcium or manganese), is sensitive to different proteases, but resistant to glycosidases. The receptor recognizes both apo B and apo E, but not AI or AII apoproteins. The receptor is extremely efficient with high amounts of LDL internalization occurring within 15 minutes due to receptors located in coated pits (297). These represent specialized regions of the cell surface that are indented and coated on the cytoplasmic surface with a fuzzy surface material. The coated pits take up only two percent of the cells surface but contain 50 to 80 percent of the receptors. Pearse (298) observed that the fuzzy coat is composed of a protein of 180,000 MW called clathrin. The LDL receptor is synthesized in membrane bound polyribosomes, glycosylated in the Golgi apparatus and inserted into the plasma membrane at random sites (296). The genetic data suggests that the receptor has two active sites (296). One, the binding site for LDL, must be on the external membrane surface, and the second, the internalization site, allows the receptor to be recognized as a component of the coated pits. Receptors that contain a functional internalization site migrate laterally and cluster in the coated pits. Brown (296) postulated that this clustering occurs as a result of the specific interaction of the internalization site of the receptor with the coat protein clathrin. The receptors escape destruction as LDL endosomes attach to the liposomes and are likely recycled to the cell surface



where they again cluster in the coated pit regions (299).

Several lines of evidence indicate that the hepatic uptake of chylomicron and VLDL remnants is mediated by a receptor that resembles the extrahepatic LDL receptor and recognizes apo E (300). Both receptors promote the uptake of lipoproteins by endocytosis, are subject to metabolic regulation and bind apo E-containing lipoproteins with higher affinity than LDL (300). But extrahepatic receptors take up LDL, in vivo, whereas hepatic receptors take up chylomicron remnants. This may stem from the large size of the remnant particles as compared to LDL. Remnants can cross the endothelium of extrahepatic tissues slowly, if at all. The rapid hepatic uptake of remnants implies that these particles cross the endothelium that lines the hepatic sinusoids either by bulk flow through the fenestrated sinusoidal epithelium, which permits passage of particles up to 1000A<sup>o</sup> in diameter or by a specific transendothelial transport (301).

Kovanen and Bilheimer (302) have recently discovered that the liver can also manufacture LDL receptors in response to pharmacological interventions with colestipol (a bile acid sequestrant) and mevinoxin (a cholesterol synthesis inhibitor). Colestipol is a bile acid-binding resin that produces an increased requirement for cholesterol for conversion to bile salts. Mevinoxin is a specific inhibitor of 3 HMG CoA reductase, the rate limiting enzyme in cholesterol synthesis. These two drugs can act synergistically raising the hepatic demand for cholesterol. These investigators observed that membranes prepared from livers of normal animals treated with this drug combination showed an increase in the number of lipoprotein binding sites. The resulting

combination of decreased cholesterol synthesis and increased cholesterol removal led to a 70 percent drop in plasma LDL levels. This study raises the possibility that mevinolin and colestipol may have a useful effect in lowering human plasma LDL-cholesterol levels therapeutically (303).

Chait and Bierman (304) have also discovered that insulin, which stimulates cholesterol synthesis by enhancing HMG CoA reductase activity, also enhances the degradation of LDL by increasing LDL receptors. This could have the effect of depositing cholesterol in cells that are actively synthesizing this compound causing an overabundance and accumulation of cholesterol in these cells.

Another route of removal of LDL from the plasma is by the action of macrophages and other scavenger cells (305-306). These cells become so swollen with cholesterol droplets that they are called foam cells. The mechanism by which the macrophages take up LDL differs in three important respects from the high affinity receptor system (86). These are 1) the cellular antigens that activate each pathway; 2) the specificity of the surface binding sites; and 3) the fate of cholesterol liberated from the degraded lipoprotein. The macrophages internalize only modified lipoproteins and possess at least 3 binding sites for these proteins. The first is an acetyl LDL binding site which recognizes an epsilon amino group of lysine that is acetylated on the surface of the LDL. Other receptors recognize and incorporate maleylated LDL and malondialdehyde LDL into the macrophage (86).

The cholesterol liberated from cellular receptor-mediated uptake of LDL regulates receptor concentration and de novo cellular synthesis

of cholesterol so that overaccumulation will not occur. The macrophage scavenger pathway shows no such regulation. About half the cholesterol that enters the macrophage through this pathway is excreted in the presence of a substance capable of binding the cholesterol. Red blood cells, albumin, and in particular, HDL are effective in this process. The other half is re-esterified and stored as cholesteryl ester droplets which can result in excessive accumulation.

#### g. HDL Synthesis and Catabolism

HDL precursors appear to originate from two sources: direct secretion by the liver or intestine of discoidal HDL into plasma or lymph; and from the surface components of VLDL and chylomicrons. Studies in the rat have shown that discoidal HDL is produced in perfused rat liver and the small intestine (307-308).

During the transformation of chylomicrons and VLDL to their remnant form, triglyceride, soluble apoproteins (A and C), and phospholipids are rapidly transferred to the HDL fractions. In man, 90 percent of labeled apo A peptides from chylomicrons and VLDL are found localized in HDL one hour after intravenous injection (309). The action of lipoprotein lipase on VLDL and chylomicrons has been documented. The shrinkage of the lipid core probably causes a redundancy of the polar surface constituents. The increase in lateral pressure on the surface causes the monolayer to fold into bilayers that protrude from the cell surface. Some of the bilayers may be dissolved as discoidal micelles in association with the detergent A or C apoproteins present on the VLDL surface. Since there are not enough A or C apoproteins to

convert all the bilayers to discs, most of the fragments probably leave the surface as larger sheets that can seal into phospholipid vesicles (310). Subsequently, these vesicles are converted into spherical HDL by their interaction with circulating HDL and LCAT. Apo AI, released from the HDL pool, leads to the formation of soluble phospholipid apoprotein complexes (311). During the transformation of precursor particles into HDL, a large influx of cholesterol occurs. The driving force for this influx is the relative deficiency of cholesterol in the VLDL derived precursor of HDL which results in a concentration gradient for the movement of cholesterol into HDL.

Plasma HDL half-life values are reported to range from 3.3 to 5.8 days (312-313). HDL catabolism is enhanced in nephrotic patients and in hypertriglyceridemic subjects, especially on high carbohydrate diets (312-314). Studies in animals indicate that the liver and kidney lysosomes play an important role in HDL catabolism (315-316). More recent research is consistent with the concept that extrahepatic tissues may play a predominant role in HDL catabolism. Normal human fibroblasts have been shown to take up, internalize, and degrade HDL in vitro (317). Another possible mechanism for HDL catabolism was suggested by Tall and Small (310). Since apo AI is loosely integrated into the HDL molecule, plasma HDL acts as a pool of AI for VLDL and chylomicron formation. As a consequence of HDL losing some of this apo AI emulsifier, the spherical HDL particle becomes thermodynamically unstable and can fuse with another lipoprotein. Since remnants are avidly taken up by the liver, their fusion with unstable HDL could provide

another route for HDL catabolism to the liver.

#### H. Role in Clinical Disorders

Increased plasma lipoprotein concentrations may be seen as either a primary manifestation associated with a genetic defect or a secondary hyperlipoproteinemia due to a primary disease state.

The classification of the hyperlipoproteinemias proposed by Fredrickson and Lees (318) in 1964 is based on plasma concentrations of the various lipoprotein types (Table 2). In patients suspected of having hyperlipoproteinemia, analysis of fasting triglycerides or cholesterol usually gives a reasonable estimate of their lipoprotein phenotype. Type I is a primary hyperchylomicronemia; this is followed by Type V, which is mixed familial lipoproteinemia with elevated VLDL and chylomicrons. Types IIA and IIB usually show a predominant elevation in LDL with Type IIB indicating an increase in VLDL as well. Type III or broad beta disease dysbetalipoproteinemia is characterized by VLDL and LDL lipoproteins which electrophoretically comigrate. Finally, Type IV hyperprebetalipoproteinemia is characterized by an elevation of the VLDL lipoprotein.

Abetalipoproteinemia is an uncommon disease in which levels of apo B are either reduced or non-existent (319-320). The patients appear normal at birth, but at some time in infancy develop foul-smelling diarrhea due to malabsorption of fat. The defect involves a critical factor in the synthesis or secretion of the lipoproteins that contain apo B. Thus, undetectable levels of chylomicrons, LDL, and VLDL are usually observed in this disease.

Table. 5. Disorders of Lipoprotein Metabolism Characterized by Elevations in Lipoprotein Classes

Chylomicrons Type I	Chylomicrons + VLDL Type V	VLDL Type IV	VLDL Type III	VLDL + LDL Type IIb	LDL Type IIa	HDL
<u>Increased Levels</u>						
Lipoprotein lipase deficiency	Familial hypertriglyceridemia (severe)	Familial hypertriglyceridemia	ApoE <sub>2</sub> homozygosity	Familial hypercholesterolemia (LDL receptor abnormalities)	Familial hypercholesterolemia (LDL receptor abnormalities)	Hyperalpha-lipoproteinemia
	ApoC-II deficiency		ApoE deficiency	LCAT deficiency	Betasitosterolemia	
	Familial combined hyperlipidemia	Familial combined hyperlipidemia	ApoE <sub>1</sub> phenotype	Familial combined hyperlipidemia	Familial combined hyperlipidemia	
	ApoE <sub>4</sub> variant combined ApoC-II and Lipoprotein Lipase Deficiency			Hepatic lipase deficiency	Hyperapobeta-lipoproteinemia	

Table 2: Description of Fredrickson's Classifications of Hyperlipemia. The disease stages and primary cause of the elevated plasma lipid are represented.

Familial dysbetalipoproteinemia or broad beta disease is characterized by the accumulation, in the density range of VLDL and LDL, of an unusual class of lipoproteins with reduced electrophoretic mobility relative to normal lipoprotein groups. Chemical studies show that this B-VLDL differs from normal VLDL in having more cholesterol esters and fewer triglycerides in their core (219-223). Studies have shown that the particles have a disproportionate amount of apo E with a missing or altered apo EIII (223, 227-228). Schneider et al. (321) demonstrated that apo E isolated from the plasma of these patients is markedly defective in binding to LDL receptors.

It is believed the VLDL from dysbetalipoproteinemic individuals is normally metabolized in the periphery by extrahepatic lipoprotein lipase. The lipoproteins lose triglyceride and apo C with remnant-like particles remaining, containing more apo E and apo B, that are not cleared by the liver because of the mutant apo EIII. Thus an elevated concentration of VLDL is present in the plasma pool of these patients.

Familial hypercholesterolemia is one of the best defined clinical disorders of lipoprotein metabolism. The disorder is characterized by elevated concentrations of LDL-cholesterol and deposition of cholesterol in specific sites to form subcutaneous lipid deposits called xanthomas (322). The disease is inherited as an autosomal dominant and is expressed phenotypically as a deficiency in the LDL receptor that regulates the uptake and degradation of LDL (323). The mean total cholesterol in these affected individuals is twice normal. Although the concentration of LDL is increased in the plasma of patients with familial hypercholesterolemia, the lipoprotein is of essentially normal compo-

sition and structure (324).

The primary hypertriglyceridemias are manifested in two forms, familial endogenous hypertriglyceridemia and familial multiple lipoprotein-type hyperlipidemia. Familial endogenous hypertriglyceridemia is a disorder inherited as an autosomal dominant in which heterozygotes manifest an isolated elevation in plasma VLDL (325-326). In addition to hypertriglyceridemia, the affected individuals have one or more of the following metabolic disturbances: adult obesity, insulin resistance coupled with fasting hyperinsulinemia, glucose intolerance and hypertension (326). Most patients demonstrate an elevated VLDL concentration in excess of 1000 mg/dl with larger particles of chylomicron-like characteristics observed. These patients are carbohydrate sensitive and will develop fasting chylomicronemia by increasing carbohydrate intake (327).

Familial multiple lipoprotein-type hyperlipidemia (familial combined hyperlipidemia) has been recently studied. In affected individuals, elevations in VLDL, LDL or both, could occur, making diagnosis of the disease difficult. Patients with familial multiple lipoprotein-type hyperlipemia exhibit elevated LDL, but this disorder is genetically distinct from endogenous familial hypercholesterolemia and hypertriglyceridemia. First, the plasma lipid level, while significantly increased, tends to be lower than in other disorders (326, 328). Second, the pattern of lipid abnormalities is unique. About one-third of the affected relatives have isolated hypercholesterolemia, about one-third have hypertriglyceridemia and one-third have both. Third, in contrast to the findings of familial hypercholesterolemia, plasma cholesterol



concentrations in children with the combined disease are normal (326). In patients with primary endogenous hypertriglyceridemia, VLDL are larger, contain more core triglycerides and cholesteryl esters and fewer surface components. Particles of comparable size from multiple lipoprotein type hypertriglyceridemic individuals usually contain more cholesterol esters and fewer triglycerides in the lipoprotein core (331). VLDL size is a combined function of synthetic, secretory and catabolic rates (330). The differences in VLDL between the two groups may reflect a longer plasma half-life of these lipoproteins and a greater transfer of cholesteryl ester to VLDL from HDL in the patients with mixed hyperlipoproteinemia. VLDL from these patients also tend to have more apo CI than apo CII protein (155).

There are numerous secondary disorders of lipid transport, but the major category in which some of the selected patients for this dissertation study can be grouped, is diabetic hyperlipemia associated with insulin resistance. As the key anabolic hormone, insulin has a major role in the transport and utilization of fatty acids and glucose. All states of insulin deficiency and/or resistance are accompanied by hyperlipemia, mainly involving triglyceride-rich lipoproteins. Insulin deficiency is commonly seen in type I (insulin dependent) diabetes, whereas insulin resistance is normally found in type II (non-insulin dependent) diabetes. The pathogenesis of these forms of diabetes are characterized by a deficiency in the action of insulin in peripheral tissues causing a mobilization of fat from adipose tissue. Although much of the fat is utilized by the liver for energy, an appreciable fraction is esterified to form triglycerides (332). Initially, the

increased production of triglyceride is accompanied by augmented VLDL excretion. After a few days however, a reduction of hepatic VLDL secretion is observed along with a reduction in lipoprotein lipase activity (333-334). Fatty acids not taken up in the periphery are recycled back to the liver, leading to a futile cycle in which the liver eventually becomes engorged with fat. Reports on VLDL transport in poorly controlled diabetics suggest that FFA and VLDL secretions are often increased along with ineffective VLDL catabolic mechanisms (335). The abnormalities of lipid transport in obesity and diabetes may combine to increase hepatic secretions of VLDL, reduce removal, or both. Many questions still remain unanswered regarding the effect of diet and carbohydrate intake on triglyceride concentrations. Low fat diets, which are inherently high in carbohydrate, have been used effectively in the management of diabetes. However, these diets still remain to be evaluated for their effects on diabetic hypertriglyceridemia.

## I. INSULIN & GLUCAGON: ROLE IN HYPERTRIGLYCERIDEMIA

### a. Introduction

Mammals have developed elaborate homeostatic requisites in the regulation of energy supply and consumption. They must maintain blood glucose levels within a very narrow range and have an optimal storage level of glycogen for emergency fuel needs with the capability to convert any excess carbohydrate calories into fat. Mammals also must maintain an optimal supply of protein for both enzyme production and structural development. They must also have the ability to preferentially use fat for energy during times of caloric need. All of these meta-

bolic guidelines are accomplished by the dynamic equilibrium of two well-studied hormones, insulin and glucagon.

Insulin, with a molecular weight of 5734 daltons is manufactured in the cells of the islets of Langerhans in the pancreas. Circulating insulin serves to coordinate fuel storage and fuel mobilization into and out of various depots according to the needs of the organism. High insulin levels reflect a non-fasting state. Insulin's main effects occur in peripheral tissue where it exerts many metabolic effects on responsive cells, altering membrane characteristics such as glucose permeability, amino acid transport and potassium flux. Insulin modulates RNA, protein, and fat synthesis intracellularly and lowers cyclic AMP levels in target tissues. Insulin also inhibits lipolysis in adipose tissue and probably proteolysis in muscle. Its versatility is undoubtedly due to the initiation of second messengers, which in turn alter enzymes involved in the aforementioned mechanisms.

Glucagon is an antagonist to insulin. It is synthesized in the alpha cells in the islets of the Langerhans of the pancreas and has a molecular weight of 3485 daltons. Constant availability of glucose for the brain and other organs depends on glucagon mediated maintenance of hepatic production of glucose at whatever rate is required to prevent hypoglycemia. In periods of starvation, glucagon stimulates the production of ketones which substitute for glucose as cerebral fuel. Such glucagon mediated catabolism (the production of fuels from the macromolecules, glycogen, protein and fat) is modulated by the anti-catabolic effect of insulin. Both the rate and direction (anabolic vs. catabolic) of fuel fluxes are therefore governed by the relative con-

centrations of these two antagonistic hormones.

#### b. Role in Diabetic Hypertriglyceridemia

Since insulin plays a critical role in both the production of triglyceride rich lipoproteins and their removal from plasma, it is not surprising that alterations in insulin secretion can lead to hypertriglyceridemia. At one extreme, severe insulin deficiency associated with untreated symptomatic Type I insulin dependent diabetes mellitus, leads to impairment of tissue lipoprotein lipase (LPL) activity and consequently impaired clearing of circulating triglycerides (443). The insulin deficient state is also associated with accelerated mobilization of free fatty acids (FFA) from adipose tissue which adds to the hyperlipemic condition (444).

Endogenous hypertriglyceridemia can also occur when Type II non-insulin dependent diabetic patients ingest high carbohydrate diets. This hypertriglyceridemia is proportional to the postprandial hyperinsulinemia produced by the diet (445). The underlying abnormality in most Type II diabetic patients with endogenous hypertriglyceridemia is resistance to insulin mediated glucose uptake (446). In an effort to maintain glucose homeostasis these insulin resistant patients secrete increased amounts of insulin. The compensatory hyperinsulinemia in turn acts upon the liver to accelerate hepatic triglyceride synthesis and secretion resulting in elevated plasma triglyceride levels. Thus, the central role in the control of triglyceride metabolism may be insulin concentration. Evidence that insulin can directly promote hepatic VLDL-TG production was observed by Salans et al. (447) who

demonstrated in liver slices that insulin increases incorporation of carbon atoms from  $^{14}\text{C}$ -glucose into hepatic total lipid and triglyceride. Using perfused liver slices, Letarte and Fraser (448) showed insulin promotes the incorporation of  $^{14}\text{C}$ -glucose into fatty acid moieties of hepatic lipid and increases their subsequent release into the media. Other in vitro studies show considerable evidence that hyperinsulinemia in the diabetic could influence VLDL-TG levels by promoting hepatic TG synthesis and secretion (449-450).

Glucagon is closely linked with insulin secretion, not only because of its antagonistic properties, but because the alpha cell belongs to a family of sensitive cells in which insulin is necessary for permitting glucose entry into the cell resulting in inhibition of glucagon release. The hypersuppressibility of plasma glucagon after oral glucose, reported by Hatfield (451) in hyperinsulinemic patients, contrasted against the lack of suppressibility of the lean insulin deficient diabetic subject supports the concept that insulin, when present, may permit glucose to inhibit glucagon secretion. In type II diabetics with hypertriglyceridemia, abnormalities in glucagon secretion exist despite the persistence of insulin. In these patients, a hyper-response to arginine or protein infusion accompanied by decreased suppressibility by glucose are two of the major malfunctions (452). A possible reduction in the sensitivity of cells to insulin could be possible. Glucagon dysfunction is most probably linked exclusively to the hyperinsulinemia caused by insulin resistance in these patients which seems to be the leading causative factors in hypertriglyceridemia.

## J. THE PLATELET

### a. Introduction

Platelets are approximately two  $\mu\text{m}$  in diameter and normally number 200,000 to 400,000 per cubic millimeter of human blood. They are produced in the megakaryocytes of bone marrow by the coalescence of cytoplasmic membranes formed by invaginations of the megakaryocyte surface (339). Under normal circumstances, platelets circulate in the blood for ten days as smooth, disc-shaped cells that are non-adherent to each other and to vascular endothelium. When the endothelium is ruptured, platelets can adhere to a variety of substances such as collagen, basement membranes and microfibrils associated with elastin. Adhesion initiates a process during which substances found in platelet secretory granules such as adenosine diphosphate are extruded (340-342). The term primary or first phase aggregation refers to the direct aggregation of platelets by exogenous ADP and other substances such as epinephrine, serotonin, thrombin and ristocetin. Aggregation mediated through the release of endogenous ADP from platelets is sometimes referred to as second phase aggregation.

### b. Structural Properties

The platelet is found to have a trilaminar plasma membrane similar to that of other cells covered with an amorphous coat 100-200<sup>o</sup>Å thick (339). Many of the plasma proteins that are absorbed to the platelet surface (for example, fibrinogen and IgM) are probably located within this amorphous coat. Platelet specific proteins appear to be arranged asymmetrically within the membrane with only a few of

the membrane glycoproteins exposed extracellularly (343). The platelet surface contains adenylyl cyclase and various glycosyl transferases which may influence adhesion and aggregation (344,345). Another distinguishing feature of the platelet membrane is its invagination at many points to form a network of channels that burrow through the platelet cytoplasm. This system enlarges the surface area of the platelets, serves in the uptake of plasma-borne substances and allows for the extrusion of granule-bound secretory products during the release reaction.

Two types of granules have been identified in platelets. The major form is the alpha granule which is of moderate electron density and contains enzymes such as acid hydrolases and cathepsins characteristically associated with liposomes (346). The second type of granule is found to contain calcium, serotonin, ATP and ADP complexed in high molecular weight aggregates (347, 348).

Platelets also contain a circumferential band of hollow, cylindrical structures similar in appearance to microtubules observed in many other cells (349). The walls of these microtubules contain protofilaments approximately  $35\text{\AA}$  in diameter and are composed of tubulin (350, 352). White (351) has suggested the theory that microtubules provide a cytoskeleton which orients contractile activity in the platelet.

### c. Role in Coagulation

Platelets can adhere to a variety of substances present in the vessel wall and in the perivascular tissue (354). Injury to the endo-

thelial cells causes the exposure of subendothelial tissues such as collagen and basement membrane to platelets. Platelets adhere to collagen, become degranulated, and recruit other platelets to form a superimposed platelet thrombus. Basement membrane and non-collagenous substances appear to be much less effective than collagen in inducing platelet release reactions or platelet aggregation (355). These observations suggest that collagen is the most platelet-reactive material present in the vessel wall and that the adhesion of platelets to collagen may be a critically important step to arrest bleeding (356).

Once adhesion has occurred, release of ADP and thromboxane triggers the formation of platelet aggregates. The release reaction is the secretory process whereby substances are packaged and extruded from the cell. Studies by Nachman and Ferris (360) suggest that ADP may bind a specific receptor on the platelet membrane. Present evidence suggests that the binding protein may be related to actomyosin and the surface actomyosin may mediate ADP-induced aggregation. In addition to ADP, substances released from platelets include serotonin, a product that can neutralize heparin, acid hydrolases, and other factors that modify vascular permeability (349, 362).

When platelets are stimulated by release inducers, arachidonic acid, probably made available by hydrolysis of membrane phospholipids, is converted by cyclo-oxygenase to a labile cyclic endoperoxide that is a precursor of prostaglandin  $E_2$  and  $F_2 \alpha$  (364). The endoperoxide can directly induce the release reaction resulting in second phase aggregation (365).



#### d. The Platelet and Diabetes

Many reports have emphasized the abnormalities in platelet adhesion and aggregation that occur in patients with diabetes mellitus (88-91, 374, 375, 380). An increased tendency for thrombosis and a higher incidence of occlusive vascular disease in patients with diabetes mellitus is generally recognized. Abnormal factors in diabetic plasma could alter the platelet response and cause hyperaggregation (Figure 2). Kwaan et al. (376) discovered that normal platelet-rich plasma, mixed with small amounts of diabetic platelet poor plasma, resulted in normal platelets aggregating as if they were from diabetic subjects. Colwell (93) postulated that von Willebrand's factor may be the cause of the increased aggregation. He also found increased levels of cyclo-oxygenase which converts arachidonic acid to prostaglandin  $E_2$  and suggested the increased activity of the enzyme plus excess arachidonic acid could cause platelet hypersensitivity. Halushka (374) found that prostaglandin endoperoxidases and one of their byproducts, thromboxane  $A_2$  could be responsible for initiating the platelet release reaction as well as aggregation. He observed that platelets obtained from diabetic subjects synthesized much greater amounts of PGE-like material than those of control subjects when (1 $\mu$ M) ADP was given in vitro. Increased PGE synthesis seen in diabetic platelet-rich plasma may reflect increased activity of the prostaglandin synthetase system.

It has been demonstrated that impaired glucose or insulin metabolism could lead to an increased activity of the prostaglandin synthetase system. Tannebaum (378) demonstrated that increased glucose

concentrations and blood osmolality can increase malondialdehyde formation, a product related to the thromboxane pathway. Recently, Halushka (379) found that platelets from diabetic subjects synthesize much greater amounts of thromboxane  $A_2$  and that malondialdehyde was formed in equimolar quantities with thromboxane  $A_2$ . He also found that hyperglycemia may be associated with enhanced platelet aggregation and thromboxane  $A_2$  synthesis.

#### e. Hyperlipemia and the Platelet

The effect of long chain saturated fatty acids on platelet aggregation was studied by Haslam in 1964 (381). He found that palmitate, stearate, arachidate, behenate, and lignocerate at a concentration of 0.1mM caused rapid platelet aggregation in the presence of 1.1mM calcium chloride. Haslam believed that fatty acids functioned by releasing ADP through direct damage to the plasma membrane or by activation of an absorbed protease (381). Previous investigations had shown that injection of unbound long chain fatty acids in dogs was followed by generalized thrombosis and death (382, 383). Since most fatty acids travel in the plasma either bound to lipoproteins or to albumins, Hack et al. (384) evaluated the effect of albumin-bound fatty acids on thrombus formation and found the platelet aggregation and thrombus was increased, but the hypercoagulability was not as great as with unbound fatty acid injection. In a later paper, the same group went one step further and stated that thrombus does not result from the effect of bound fatty acids until the concentration is such that the two tight binding sites on albumin are no longer available (385). If fatty acid levels climb

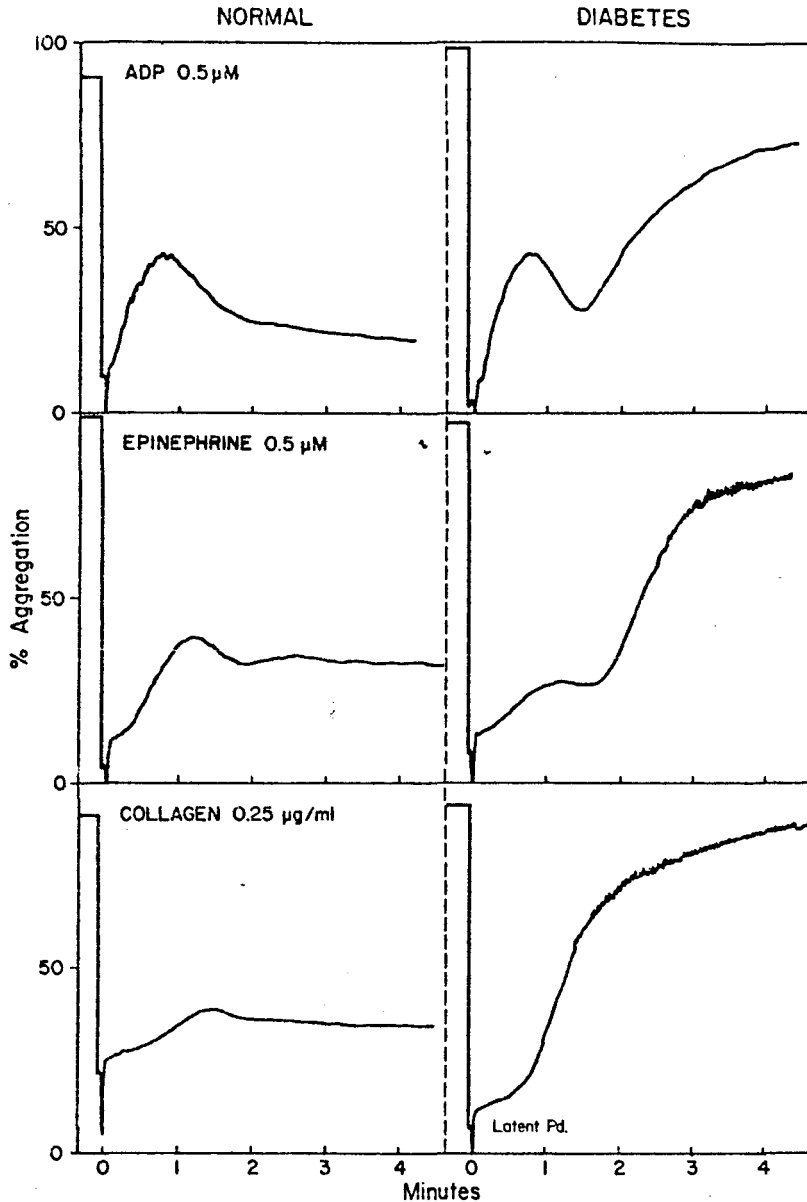


Figure 2: Graphical representation of platelet aggregation response to different aggregating agents. Both normal and diabetic patients are represented. These curves were generated from a chronolog platelet aggregometer, Model #330. Note the second phase response of the diabetic platelet is more exaggerated than the normal platelet response.

above a threshold value of 1150 to 1200  $\mu$ Eq/liter, as can be found in certain disease states, than the unbound fatty acids could cause thrombogenic complications. Renaud et al. (386) studied the effects of different types of fat on platelet aggregation. Butter and stearic acid are highly thrombogenic, but corn oil and linoleic acid appear to inhibit the potentially thrombogenic effect of cholesterol and butter. Oleic acid, despite its effect on inducing hypercholesterolemia, did not induce hypercoaguability or increased susceptibility of platelets to aggregation.

Dietary manipulations and their effects on platelet aggregation have been investigated (387). Thrombin-induced platelet aggregation was much greater in platelet-rich plasma (PRP) prepared from blood taken from rats fed stearic acid than the (PRP) from rats fed oleic acid. This study was the first to clearly demonstrate alterations in platelet function related to dietary fat. Evidence that dietary saturated fat produces changes in platelet function suggests alterations of the fatty acids in platelet membranes may be involved. Similar studies were conducted in humans with comparable results indicating decreased aggregatability of platelets in subjects receiving diets low in saturated fat as compared to those who consumed diets high in such fat levels (94).

Shattil and Cooper (388) studied platelet membrane microviscosity with the fluorescent probe, 1, 6 diphenyl, 1, 3, 5-hexatriene (DPH) under conditions where the platelet membrane had larger amounts of cholesterol and fat (in patients with type IIB hyperlipidemia). Platelets with high membrane cholesterol showed increased microvis-

cosity over platelets that had low membrane cholesterol. Cholesterol decreases molecular motion of the hydrocarbon chains within the hydrophobic core of the lipid bilayer and increases the degree of order in the platelet membranes leading to increased membrane viscosity. Thus, changes in membrane viscosity could be assumed to cause increased platelet aggregation.

Since platelets acquire cholesterol by an exchange or fusion fashion, the lipoproteins that carry fat and cholesterol are thought to be important in membrane changes observed in the platelet. Farbiszewski et al. (389) investigated the effect of alpha, beta and chylomicron lipoproteins on platelet aggregation, finding that LDL had a distinct effect on platelet adhesiveness. Chylomicrons also affect platelet aggregation, but only at elevated levels. It seems the lipoprotein interacts with certain receptor sites on the platelet surface. This was demonstrated in experiments in which lipoproteins altered platelet electrophoretic mobility (98). Platelets may incorporate total low density lipoproteins or components into their membranes altering surface characteristics when they circulate for long periods of time in hypercholesterolemic plasma. Shattil (390) demonstrated this exact phenomenon in platelets from type IIA individuals, in which he found an increase in the C/PL ratio caused by cholesterol enrichment of the membrane. A good correlation was found to exist between the magnitude of free cholesterol in the platelet membrane and the amount of LDL in the plasma. Receptors for LDL were isolated from platelets quite recently by Aviram (391). The LDL interaction with platelets is similar to the LDL interaction with fibroblasts.

Using chemical modification, Aviram et al. (392) concluded that arginine and lysine, but not tyrosine and histidine, are important amino acids for the binding of LDL to platelets.

An interesting observation on the effect of increased cholesterol and lipid incorporation into the membrane of platelets was reported by Sinha and Shattil (398). They investigated the membrane enzyme adenylyl cyclase and the production of CAMP in normal platelets and those of hypercholesterolemic individuals. Since CAMP is a potent inhibitor of platelet aggregation, a decrease in CAMP concentration would cause increased platelet aggregation. These studies demonstrated that increased membrane cholesterol in platelets is associated with decreased stimulation of adenylyl cyclase and decreased amounts of CAMP. These investigators concluded that phospholipids were found to be involved in adenylyl cyclase activity and therefore cholesterol might inhibit the phospholipid-adenylyl cyclase interaction by competing directly with the enzyme. Alternatively, cholesterol could restrict phospholipid mobility and impair transmembrane events necessary for stimulation of the enzyme (398). Finally, Zahavi et al. (99) demonstrated enhanced in vivo platelet release reactions and MDA formation in patients with hyperlipemia. The mechanism of the enhanced release reaction and MDA formation could be related to the elevated serum lipids observed.

### 3. PROPOSED INVESTIGATION AND SPECIFIC OBJECTIVES

The relevance of diet in the production of metabolic disease states such as hyperlipemia and atherosclerosis is of prime importance in the diabetic population. Knowledge of the specific effects of the disaccharide sucrose on lipid production is contradictory, if not absent in many respects. Therefore, investigations must be undertaken to determine the effects of sucrose on lipid production in diabetic patients prone to hypertriglyceridemia. Since many studies used normals or hyperlipemic individuals who were obese, the patient population must be well defined as diabetic with specific attention given to the metabolic state of the individual upon entry into the study. In addition, most reports dealing with the effects of sucrose on a given population used artificial diets, either composed of liquid nutrients or extremely high in carbohydrate proportion. In this study, the diets are composed of natural nutrients which more closely mimic the intake pattern observed in these diabetic individuals.

The experimental work will involve the characterization of the specific effects of sucrose on blood lipid concentrations over an extended period of time. This will include individual measurements of these lipids, along with the hormones insulin and glucagon, which are important in lipid production and catabolism. Specific correlations will be made as to the concentrations of both hormone and lipid as they relate to each other during ingestion of the study diets. To ascertain the metabolic effect of high sucrose, high carbohydrate diets on diabetic homeostasis, blood glucose and urinary glucose output will also be monitored during the entire study.

In an effort to determine if dietary manipulation results in hyperlipemia, due to increased lipid synthesis, a part of the study will attempt to characterize the triglyceride production and removal rates of these individuals. The most commonly used technique, triglyceride turnover, labels the endogenous pool of triglyceride with radioactive precursor (either labeled glycerol or free fatty acids). Assuming that at equilibrium, synthesis equals removal, the rate of loss of the label from the pool is used to calculate the synthetic rate of triglyceride.

Another line of approach will involve the assumption that high sucrose, high carbohydrate diets could alter apoprotein composition of VLDL in sensitive individuals and thus affect the removal of the lipoprotein from the plasma pool. Using SDS-polyacrylamide gel electrophoresis and isoelectric focusing, I hope to separate the different apoproteins that comprise the VLDL and determine if changes occur in these proteins when different diets are administered. Total lipoprotein electrophoresis will also be done in an attempt to monitor all lipoprotein fractions and determine their changes in relation to VLDL. It appears likely that hyperlipemia could be caused by a combination of events in which increased synthesis could be coupled with decreased peripheral removal. The studies with apoproteins of VLDL will provide the opportunity to compare their concentrations in relation to triglyceride levels over a long term ingestion of a study diet. It is hoped that some correlation will be found between individual apoproteins and triglyceride concentrations.

In a parallel study, the relationship of sucrose ingestion to



hyperlipemia will involve study of the effects of this diet on platelet aggregation. Since sucrose may cause hyperlipemia, and considering it has been demonstrated that hyperlipemia causes increased platelet aggregation, a natural link is developed between sucrose consumption and platelet aggregation. I hope to demonstrate changes in platelet aggregation during sucrose ingestion by using sensitivity studies of platelets to different concentrations of aggregating agents. The turbidometric method of platelet aggregation will be used to measure platelet sensitivity. The percent of primary and secondary aggregation will be compared from control to study diets to determine the change in platelet response to different amounts of sucrose consumption.

Finally, since three dietary levels of sucrose will be given to the patients, I hope to ascertain the amount of dietary sucrose needed to elicit these metabolic changes. It could be possible that a critical level of sucrose may have to be reached in order to metabolically alter an individual's metabolism toward hyperlipemia. Perhaps, low levels of sucrose may be well tolerated by the patient population in question.

From these different studies, it is hoped that the objectives of this work will gain some insight into the physiological complications involved in the hyperlipemic response when different levels of sucrose are ingested in these sensitive patients.

## 4. MATERIALS AND METHODS

### A. Description of Patients Used in this Study

Patients for this study were recruited from in-patient subacute and chronic care facilities, as well as outpatient clinics at Hines V.A. Hospital, Hines, Illinois. All observations and studies were done on hospitalized patients in the Special Diagnostic and Therapeutic Unit (SDTU) at Hines. Informed consent was obtained from these patients prior to their entry into the study. Participation was strictly voluntary and patients were informed that they could withdraw from the study at any time. Subjects for this study were overt adult onset male diabetics, 30 to 70 years old, who did not require insulin or oral hypoglycemic agents and had fasting hypertriglyceridemia (exceeding 1.5 g/L or higher). Patients with type V hyperlipemia of the Fredrickson's classification were excluded as well as those whose weight exceeded 20 percent of ideal body weight.

### I. Patient Type

#### a. Description of Procedure to Evaluate Patients

Patients were incorporated into the study only if the previous criteria were satisfied. The patients were judged as diabetic by the NIH criteria for the classification of diabetes, frankly overt hyperglycemia (394). The patients selected for the study had to have persistent fasting blood sugars of at least 140 mg/dl a minimum of 3 times prior to admission. These diabetics has to be insulin independent, not ill and non-ketotic (type II diabetics). The study patients had to be

within 20 percent of normal body weight as classified by the method developed by Abaira et al. (395). Ideal Body Weight (IBW) was determined by the following: height (without shoes) 5 ft. = 105 + 5 lbs. for each additional inch of height. All patients were weighed in issued hospital clothing. Patients with complicating diagnoses that could independently affect weight or diet (such as congestive heart failure, cirrhosis of the liver, or renal insufficiency) were not admitted into the study. Finally, patients who were receiving any drugs known to affect blood lipid levels (such as clofibrate, cholestyramine, nicotinic acid, or glucose sulfonylureas) were not allowed in the study.

Before patients entered the study, blood samples were drawn for triglycerides, blood glucose, electrolytes, BUN, creatinine, liver enzymes and total protein analysis. Any patient with triglyceride levels of less than 150 mg/dl at the initiation of the study was released. Also, patients who had abnormal SMA-12 values, (excluding glucose) before the study began were dropped from the study if the values did not return to normal after hospitalization.

Finally, a lipoprotein electrophoresis was done to determine the patient's hyperlipemic classification. Fredrickson's guidelines were used indicating all of the patients to be type IV hyperlipemic individuals. Type I and V individuals were dropped from the study because the interest in this study was limited to VLDL's and endogenously derived triglycerides.

#### b. Informed Consent

Written informed consent was obtained from patients prior to study

initiation. All procedures and protocols were explained to patients before admission and patient compliance was monitored by the SDTU technician and dietitian. The study was sanctioned by the Research Committee at Hines V.A. Hospital. Human studies approval at Hines and IRB board approval at Foster G. McGaw Hospital have been obtained for the project (along with the written consent). The project has also received approval by the Department of Nuclear Medicine at Hines, with all Food and Drug Administration guidelines followed from the U.S. Department of Health, Education and Welfare's report on "Clinical Testing for Safe and Effective Drugs" (296-297). Participation was on a voluntary basis with certain procedures exempted upon patients request.

### c. Care of Patients

Subjects were followed under the usual inpatient clinical care scheme and seen by medical residents spending a 3 month rotation on the ward. Each patient was seen by a clinical dietitian twice weekly and instructed on compliance procedures for the study. The residents reported directly to Dr. M. Emanuele who was one of the physicians responsible for and in charge of patient care. Any manipulations or drug therapy prescribed for the patients were first assessed to determine any potential effect on the planned experiments. When patients entered the study, all diabetic medication was halted. The only medications allowed were Tylenol or sleeping pills (Dalmane R.). Though ambulatory, the patients were restricted to the SDTU and only allowed occasional off-ward activities. Direct supervision of all patients and clinical responsibility was assumed by Dr. C. Abraira. Once patients were in-

corporated into the study, they were hospitalized a minimum of six weeks to a maximum of eight weeks. During this time, they were under strict supervision by the SDTU technician and the author so that no breach in protocol occurred. Any meals which were not eaten or other changes in study protocol were noted and results from that day's testing were discarded for that patient.

#### d. Dietary Description and Study Protocol

One week prior to entry into the hospital all patients were given dietary instructions for an individual weight maintenance diet similar to the regular diet at Hines V.A. Hospital consisting of 50% carbohydrate, 35% fat and 15% protein. One hundred and fifty grams of carbohydrate must be sucrose or other definable disaccharides. Once the patients enter the hospital, they are given a diet designated as the control diet (intermediate sucrose content) which is isocaloric with the outpatient diet except the sucrose intake is recalculated to be 120g per day. The 120g approximation is based on the average intake of sucrose in the American population (11). After two weeks consumption of the control diet, the patients are randomized to a high sucrose diet (HSD) approximately 225g or low sucrose diet (LSD), approximately 3g daily. All of the nutrients were composed of natural food stuffs and were made from normal trays given to the rest of the patient population to approximate a mixed American diet. Dietary intake was divided into three meals per day, with 20% of the calories consumed during breakfast, 40% during lunch and 40% during dinner. No bedtime snack was given. Fiber was kept constant at 20g per day along with sodium at 150 mmol

(approximately 4g per day) (461). Each diet was individualized according to the size and weight of the patient. There were approximately 30 different nutrients which could be consumed, several of which were singled out to be kept as constant as possible (10% variation). Calories, protein, fat, carbohydrate, sodium, fiber and sucrose content remained constant in the individual diets. The dietary pattern from one diet to another remained the same, with each patient receiving vegetables, meat, potatoes, and salads for lunch and dinner, with eggs and high fiber cereals consumed at the breakfast meal. Sucrose content was altered by adding or subtracting sweet jellies, different juices and different types of sucrose containing soft drinks. Vitamins, though not specifically monitored, were assumed to be constant because of the repetition of the diet and substitution of similar foods with varying sucrose content during the study (137). Patients were ambulatory, but were confined to the metabolic unit during the six-week period. Study compliance was excellent with the dietary trays prepared and checked in the metabolic kitchens of Dr. H. Spencer. Intake was monitored by the metabolic dietitian who checked the trays to ensure complete nutrient consumption.

The study was six weeks in duration, the first two weeks comprising the control period, with the subsequent four weeks comprising the study diet period. The study is novel, in that each patient acts as his own control, with most experimental results compared against those previously determined while the patient was consuming the control diet. Since we were interested in the mechanism by which sucrose induced hyperlipemia occurs, we wanted to study these patients before dietary

adaptation to sucrose content began. A four-week study period was chosen because the effect of sucrose had been observed to maximize and remain relatively constant after this time (453). A total of 30 patients were studied, 17 consuming the high sucrose diet with 13 consuming diets low in sucrose.

## B. Description of Methodology for Basic Clinical Tests

The different procedures used to ascertain the blood levels of glucose, triglyceride, cholesterol and HDL-cholesterol were basic techniques routinely used in hospitals. Routine quality control procedures were performed with these assays since some of the values were also used in patient monitoring during the study. Blood and urine glucose values were measured in the Special Diagnostic and Therapeutic Laboratory by myself and the technician in charge. All other blood chemistries were done in the diabetes research laboratories using manual methods.

### I. Serum Glucose

#### a. Specimen Collection

Samples were drawn for serum glucose three times weekly, both fasting and three hours postprandially. A small amount of whole blood was placed in a 500 ul microfuge tube and allowed to clot. It was spun at high speed for three minutes on the Beckman Microfuge, Model #152 (Beckman Instruments, Palo Alto, California). The serum was collected using a Pasteur pipette and immediately assayed for glucose.

### b. Procedure for Glucose Determination

The Y.S.I (Yellow Springs Instrument) glucose analyzer, Model 23A (Yellow Springs Instrument, Yellow Springs, Ohio) uses an oxidase enzyme hydrogen peroxide sensor. This technique allows direct readout of glucose concentrations and no sample modifications are required.

Twenty-five (25) microliters of serum is needed per sample (all samples were run in duplicate). The principle involves the conversion of glucose and oxygen to gluconic acid and hydrogen peroxide.

The  $H_2O_2$ , generated in proportion to glucose concentration is oxidized at the platinum anode. The subsequent oxidation at the anode and reduction at the silver electrode creates a current that is proportional to the amount of  $H_2O_2$  which diffused in. This is directly calculated as a glucose reading on the instrument utilizing an analog/digital converter (414).

### c. Calculation of Results

Precision of the assay was determined using Monitrol I and Monitrol II Standards (Dade Scientific Co., Miami, Florida). Percent coefficients of variation were calculated as mean divided by standard deviation times 100. Interassay variation of glucose measured at concentration level of 104 mg/dl and 230 mg/dl were 0.96% and 1.3% respectively.

## II. Urine Glucose Measurement

### a. Specimen Collection for 24-hour Urine

Patients were instructed to collect 24-hour urines in receptacles



provided by the SDTU. At the beginning of the collection period (usually when the patient awakens) the bladder was emptied, the specimen discarded, and the time noted. All urine specimens passed thereafter were collected for a 24-hour period with the bladder being emptied at the same time the following day and this urine specimen being added to the collection. To prevent loss of glucose via bacterial glycolysis, the jugs contained 5 ml of glacial acetic acid. All urine collection bottles were refrigerated during collection.

#### b. Procedure for Determination

The urine samples were collected from the refrigerators every morning and the volumes were measured and recorded. An aliquot was sent to the clinical labs at Hines for creatinine measurement. Another aliquot was used to determine the urinary glucose concentration with the sample procedure and analyzer utilized for serum glucose assays.

#### c. Calculation of Results

Glucose concentrations outside the instrumental range of linearity were appropriately diluted with total 24-hour glucose excretion in the urine calculated by multiplying the urinary concentration of glucose by the total volume of the daily urinary output. Coefficient of variation (CV) determinations for the assay were similar to those determined for the serum controls. Fisher Urichem Level I with a spiked glucose at 100 mg/dl was used for control purposes. CV's for the urine were comparable with plasma having a value of 1.7%.

### III. Triglyceride Determination

#### a. Specimen Collection

Patients were instructed to fast for 12 hours before blood was drawn for fasting triglyceride levels. They were allowed to drink water or take prescribed medications. The patients sat quietly for 10 minutes before blood was collected (generally obtained from the antecubital or another convenient arm vein). A tourniquet was used, but released before drawing samples because an artifactual increase in plasma lipid concentration can occur with prolonged interruption of venous blood flow (398, 399). The blood was allowed to stand at room temperature for 30 minutes, a time in which the blood should completely clot. The sample was centrifuged at 2000 RPM for 15 minutes at 5°C. The serum was immediately transferred to an ice bath and kept at 2-4°C for subsequent analyses. Duplicate samples were drawn, and if gross hemolysis did occur, the sample was discarded.

#### b. Specimen Storage

Samples for triglyceride measurement were immediately transferred to a clear 12 X 75 mm Falcon polystyrene tube and capped. The sample could be stored at 4°C for a few days or for longer periods at -20°C. Samples were removed from the freezer and allowed to thaw at room temperature prior to analysis. Since certain serum components tend to concentrate at the bottom of the storage vial when the sample is frozen and water from the plasma may condense on the inner walls and cap of the vial during storage, the vial was mixed by gentle inversion before unsealing until a homogenous sample was observed.

### c. Assay Protocol

Triglycerides were assayed in the laboratory by the manual method of Biggs et al. (400). In this extraction technique, serum is mixed with water, sulfuric acid, isopropanol and heptane. Any hemolysis that occurred was negated by this extraction procedure. The resulting two-layer system is allowed to separate with triglycerides entering the heptane phase once the sulfuric acid loosens the lipid-lipoprotein bonds. Saponification of an aliquot of the heptane (upper) layer with a solution of sodium methoxide converts triglycerides to glycerol and free fatty acids. On addition of sodium periodate solution containing acetic acid and ammonium acetate, glycerol is converted to formaldehyde and formic acid (the Malaprade Reaction). On treatment of the formaldehyde with acetylacetone, a yellow cyclic 3, 5-diacetyl-1, 4-dihydroxypyridine compound is formed (the Hantzsch Reaction) which can be read at 410 nm on a Gilford spectrophotometer, Model #240 (Gilford Instruments, Oberlin, Ohio).

### c. Calculation of Results

A calibration curve is prepared to check the linearity of the instrument and is performed every 6 months. The assay is linear over a range of 0-500 mg/dl. Patient samples higher than these values must be diluted with saline, reassayed and final results multiplied by the appropriate dilution factor. Triglyceride concentrations may be determined by comparing the absorbance of the unknown with that of the standard. Interassay variation at a triglyceride level of 508 mg/dl = 3.9%.

#### IV. HDL-Cholesterol Determination

##### a. Specimen Collection

Blood was drawn from the antecubital vein and serum specimens were assayed both fasting and postprandially for HDL-cholesterol.

##### b. Specimen Storage

The serum can be stored at room temperature up to three days or at 2-8°C up to six days. Refrigerated or frozen specimens which have completely thawed require a 24-30 hour period to fully resolubilize the lipoproteins at room temperature. Specimen results determined before the required equilibration period may be artificially low.

##### c. Assay Protocol

Many different methods have been used to analyze cholesterol and HDL-cholesterol (402-402). The kit used for cholesterol determinations is the Pierce Rapid Stat Kit (product #44100, 44102, Pierce Chemical Division, Lancer Chemical, Rockford, Illinois) which utilizes cholesterol oxidase and esterase with a peroxidase/phenol/4-aminoantipyrine system. The method involves selective precipitation of LDL and VLDL by a magnesium/phosphotungstate precipitant reagent (403). The HDL-cholesterol fraction present in the supernatant is then analyzed with the enzymatic cholesterol reagent. The enzymatic reagent breaks all cholesterol ester bonds via cholesterol esterase and the total free cholesterol is oxidized in the presence of  $O_2$  by cholesterol oxidase to give cholest-4-ene-3-one and  $H_2O_2$ . The peroxide and phenol react with 4-aminoantipyrine via peroxidase enzyme to give a quinoneimine dye,

which is colored pink and read at 510 nm on a spectrophotometer.

#### d. Calculation of Results

The procedure for HDL-cholesterol is linear over a concentration range of 0-150 mg/dl. HDL-cholesterol concentration is calculated by comparing the absorbance of the unknown to that of the standard and multiplying by the known concentration of the standard. Since the original sample was diluted with the precipitant reagent, a factor of 2 is needed to correct for the dilution.

Lipemic specimens on occasion may give a supernatant that is turbid after addition of precipitating reagent and centrifugation. If an additional centrifugation at 12,000 X g (5000 RPM) does not clear the supernatant, the specimen is diluted 1:1 with saline and the precipitation step is repeated. The final result is multiplied by 2 to obtain total serum HDL cholesterol. Interassay variability was 6.3% at an HDL-cholesterol level of 30 mg/dl. Calibration curves for HDL-cholesterol were performed every six months to check spectrophotometer response and calibration.

### V. Cholesterol (total) Determination

#### a. Specimen Collection

Collection procedures for total cholesterol were exactly the same as those for HDL-cholesterol. Serum collected from patients was used for the determination of total cholesterol.

### b. Specimen Storage

Specimens for cholesterol determinations were stable up to six days at 2-8°C or 2 days at 20-25°C. Storage for longer periods of time require freezing at -5°C. Samples to be assayed should be thawed at room temperature and serum mixed by inversion until homogenous. A re-solubilizing period for total cholesterol is not needed and samples may be assayed after complete thawing and mixing.

### c. Assay Protocol

The identical chemical procedure for HDL-cholesterol determinations were also used for total cholesterol measurements. Sample volumes were changed, however, and no precipitation step was performed.

### d. Calculation of Results

The range of linearity for this assay was from 0-500 mg/dl. Again, the absorbance of the standard was compared to that of the unknown patient sample. A ratio was obtained, the value of which was multiplied by the known value of the standard. Since no dilutions were made except in cases where total cholesterol was greater than 500 mg/dl, a dilution factor was not used. A standard curve was again determined for this assay to measure the linearity at differing cholesterol concentrations. Interassay variation at a level of 200 mg/dl cholesterol = 5.9%.

## C. The Development and Use of the Radioimmunoassay for Insulin and Glucagon

## I. Introduction

Radioimmunoassay, introduced by Yalow and Berson for the measurement of insulin concentrations (404), is based on competition for an antibody between a known amount of radioactively labeled antigen and its unlabeled counterpart in the unknown. The amount of bound radioactive antigen is inversely proportional to the concentration of this same antigen in the patient's sample. Different methods have been used to separate the bound insulin from the unbound moiety (405-406). The two antibody system described by Morgan et al. (407) was used because of its greater sensitivity and specificity. In summary, the two step reaction is as follows: 1) insulin forms a soluble complex with its specific antibody usually raised in guinea pigs (Ains-GP); 2) this soluble complex is precipitated by an antibody to guinea pig serum which is obtained from a goat. The percent of radioactivity in the precipitate is inversely proportional to the concentration of unlabeled insulin in the reaction mixture. Temperature ranges between 0-10°C caused relatively little change in antibody-antigen binding. At 37°C however, the binding of the complex is much less (407).

In order to determine the amount of unknown in the sample, the amount of competitive inhibition observed in the unknown is compared to that obtained in separate solutions in which known amounts of purified antigen are added. A standard curve plotting the known concentrations of antigen against the ratio of bound to unbound antigen can thus be obtained (Figure 3). This curve is used to ascertain the level of unknown antigen in the sample by comparing its ratio of bound to unbound with that of the standard curve.

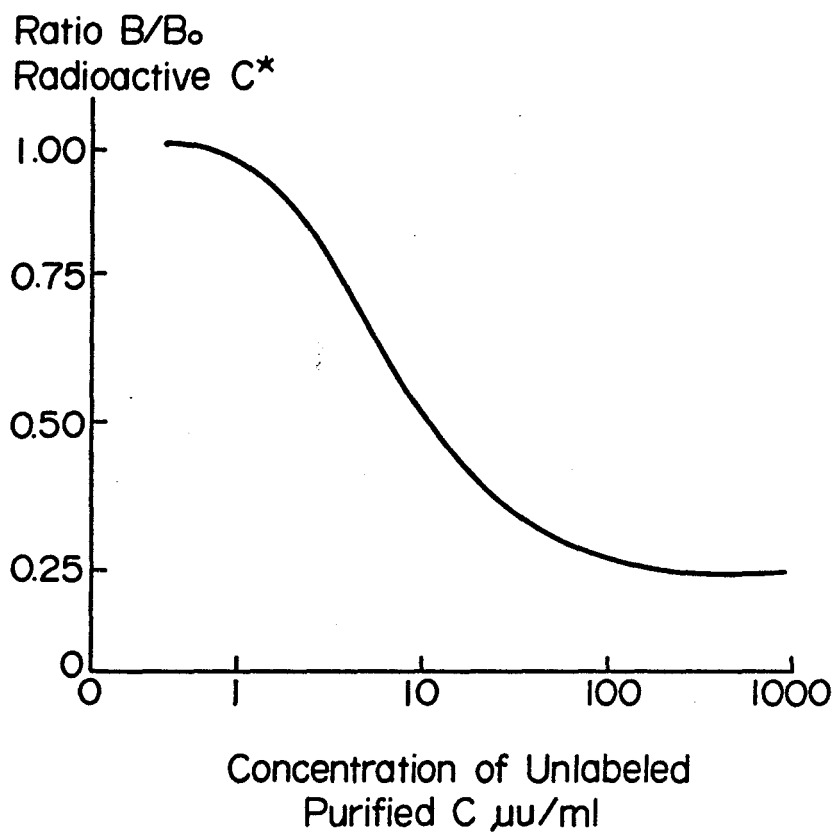


Figure 3: Standard calibration curve for radioimmunoassay.  $B/B_0$  represents the ratio of bound to unbound antigen present.  $C^*$  represents cold unlabeled antigen.



In the double antibody system, the first antibody which is directed against the antigen to be measured is added at a level that enhances competition between labeled and unlabeled antigens for binding sites. The second antibody (anti-antibody) is added in excess to ensure complete immunoprecipitation of the first antibody antigen complex.

## II. Insulin RIA

### a. Preparation of Insulin Standards

Stock purified insulin was obtained from Eli Lilly and Company (Indianapolis, IN) at a concentration of 21.7  $\mu$ /mg protein/ml. Appropriate dilutions of the stock insulin must be made to obtain concentration ranges which would be normally found in patients. A 1:1000 dilution of stock insulin was first made (stable for 10 days at 2-8°C).

50  $\mu$ l of this solution was diluted to 2 cc with V/A buffer (final concentration of standard was 542  $\mu$ U/mg/ml). Serial dilutions were done to this standard with gelatin buffer 0.1%. Once standard dilutions were prepared they were pipetted into the assay tubes in triplicate, with one set of NSB tubes per standard curve.

### b. Assay Protocol

The performance of this specific assay was dependent on antibody concentration, amount of labeled antigen added and incubation times which all influence the specificity and reactivity of the assay. Binding studies were done with varying concentrations of insulin antibody to obtain the correct antibody dilution to perform the assay. A 1:16,000

dilution of first antibody was found to produce the greatest reactivity. A subsequent study was performed to ascertain the amount of antibody that could be added to obtain the greatest reactivity between insulin and insulin antibody (Figure 4). Insulin excess will use all antibody added and give the most accurate binding results. A volume of 100  $\mu$ l of the 1:16,000 dilution of first antibody was determined to be the best. The second antibody is a goat-antiguinea pig antibody (Pel-Freeze, Rogers, Arkansas) specific for the  $\alpha$  globulins of the guinea pig first antibody. A 1:12 dilution of this second antibody was found to give the optimum degree of precipitation of insulin.

#### c. Data Reduction-Insulin Radioimmunoassay

All radioactivity was counted on a Micromedic System 2/200 gamma counter (Micromedic Systems, Philadelphia, PA). Assay data reduction was performed using a Hewlett-Packard Clinical Laboratory Radioassay Computer (programmed by cassette tape) (Hewlett Packard, Loveland, CO). This program evaluates the standard curves by weighted and unweighted least square regressions using the log dose vs. % bound, logit of % bound and probit of % bound (Figure 5). These data reduction transformations are automatically analyzed for best fit and the curve plus all pertinent data are printed via automatic printout. All samples were done in triplicate with each sample having an (NSB) tube run.

Spiked insulin buffer samples (100  $\mu$ U/ml and 50  $\mu$ U/ml) were used as internal controls on 20 insulin assay runs. CV% of 10% or less was deemed acceptable. At levels of 103  $\mu$ U/ml and 49  $\mu$ U/ml, coefficients of variation were 9.7% and 10.2%, respectively.

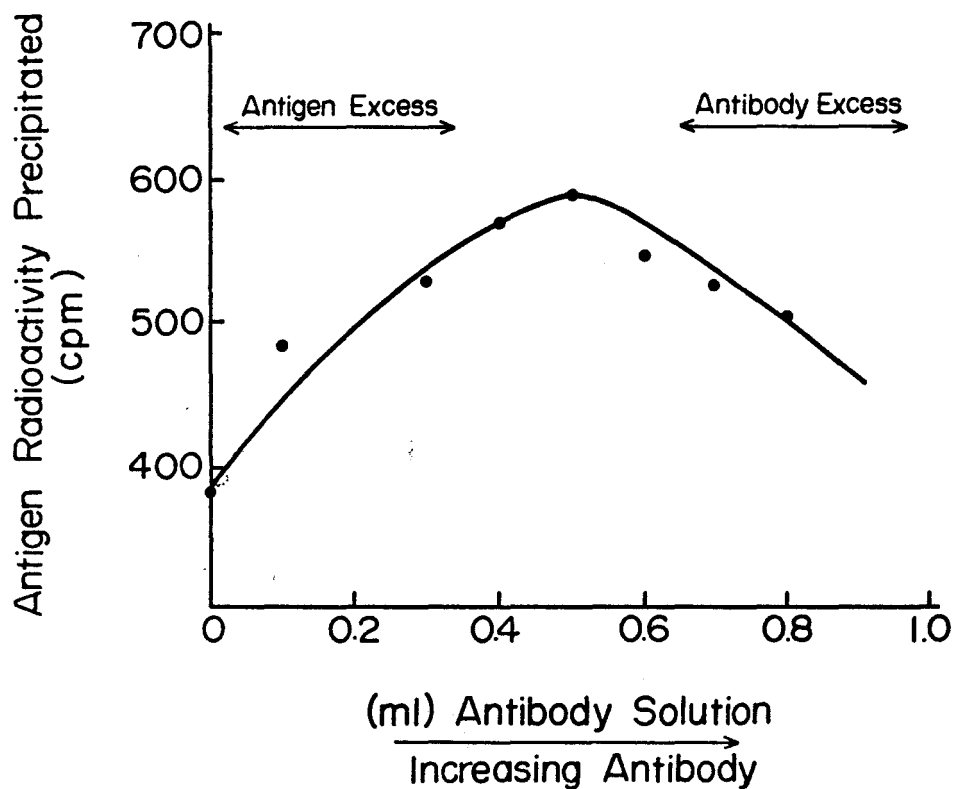


Figure 4: Precipitin curve for monospecific system, one antigen and corresponding antibody. This binding study was performed to ascertain the amount of insulin antibody that should be added to obtain the greatest reactivity between insulin and antibody.

ASSAY INSULIN,  
TECHNOLOGIST SCOTT

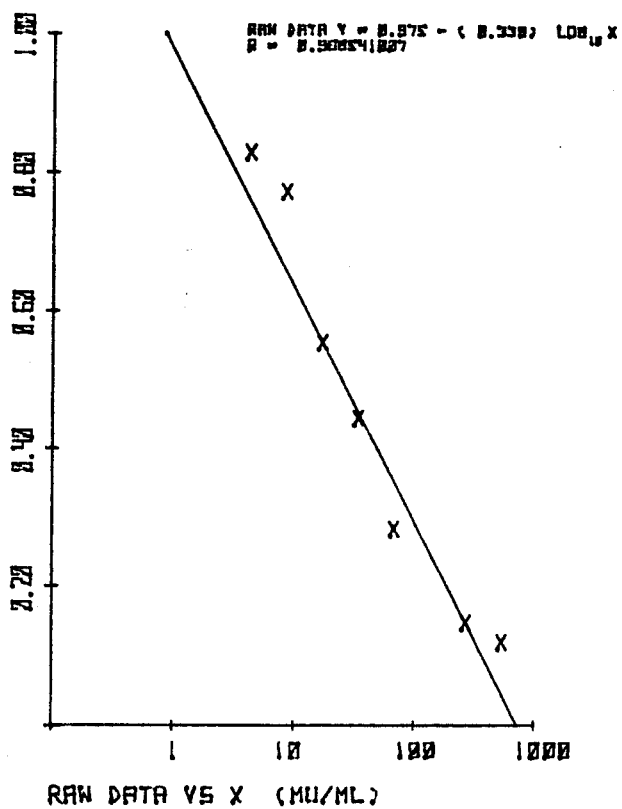
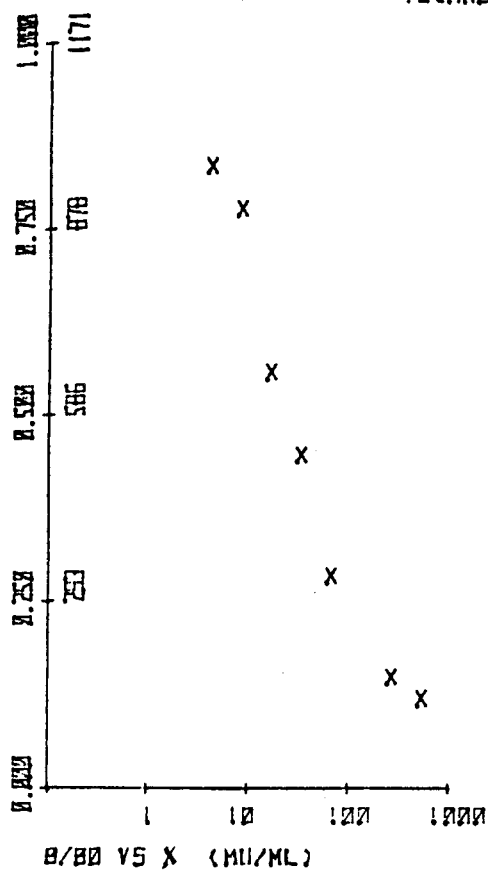


Figure 5: Insulin standard curve plotted as B/Bo vs. ( U/ml). B and Bo denote bound and unbound antigen respectively.

Figure 6: Insulin standard curve plotted as raw data vs. ( U/ml).

### III. Glucagon (RIA)

#### a. Preparation of Glucagon Standard Curves

Stock purified glucagon was obtained from Lilly and Company (Indianapolis, IN) at a standard concentration of 1 mg/ml. Appropriate dilutions of stock glucagon must be made in order to obtain physiological concentration ranges approximating patient's samples. 40 of stock solution was diluted to 4 cc (1:100 dilution) to obtain 10,000 pg/ml concentration.

Once standards were prepared (0-10,000 pg/ml), they were immediately used for assay. Standards were pipetted in triplicate with one set of NSB tubes.

#### b. Assay Protocol

For the glucagon assay, binding studies similar to the insulin assay were performed with different antibody dilutions. Anti-glucagon guinea pig antibody, lot number (OJA-G2) obtained from Dr. A. Lawrence's lab was used for the study. Final dilutions of first antibody which gave best binding results were 1:17,500.

10 ul (OJA-G2) was diluted to 17.5 ml with glucagon buffer. 5 ul/cc of normal guinea pig sera (NGPS) was added as a carrier. All assays were incubated at 4°C with assay tests run in 12 X 75 culture tubes.

#### c. Data Reduction-Glucagon Radioimmunoassay

Radioimmunoassay data were treated similarly for the glucagon assays as for insulin determinations. Weighted regression and best

fit analysis for the standard curves were performed (Figure 7). All unknown samples were determined by comparison to the standard curve. Normal ranges for human serum glucagon are 0-300 pg/ml. Internal control was monitored by spiked glucagon buffer. At levels 1011 and 495 pg/ml, coefficients of variation were 13.4% and 11.3% respectively.

#### IV. Specimen Collection Procedures

Fasting (14 hour fast) and postprandial, insulin (3 hours) samples and fasting glucagon samples were collected. Patients were asked to sit quietly for 10 minutes. Blood was then drawn from the antecubital vein and placed directly into tubes containing 0.3 ml of the aprotinin trasylol which inhibits protease breakdown of insulin and glucagon (Mobay Chemical Corp. FBA Pharmaceuticals, New York, NY). These samples were immediately placed in ice baths and after 45 minutes the blood was spun in a refrigerated centrifuge at 3000 RPM. In the rare instances when gross sample hemolysis occurred, the sample was discarded. After centrifugation, the serum was aspirated with Pasteur pipettes and placed in 12 X 75 cm polystyrene tubes with snap caps and frozen at -20°C. Frozen samples were thawed at room temperature and inverted several times to provide a homogenous sample for assay.

#### D. Triglyceride Turnover Methodology

##### I. Introduction

The use of  $^3\text{H}$ -glycerol in the labeling and measurement of triglyceride transport in the plasma pool is well-documented (53-59, 408-411). The method first developed by Farquhar (412) was used to directly

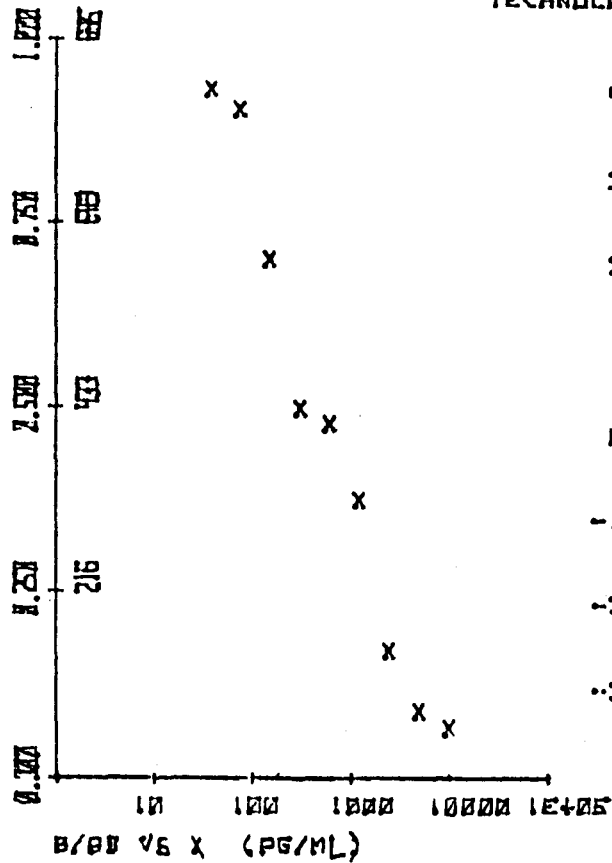


Figure 7: Glucagon standard curve plotted as B/Bo vs (pg/ml).

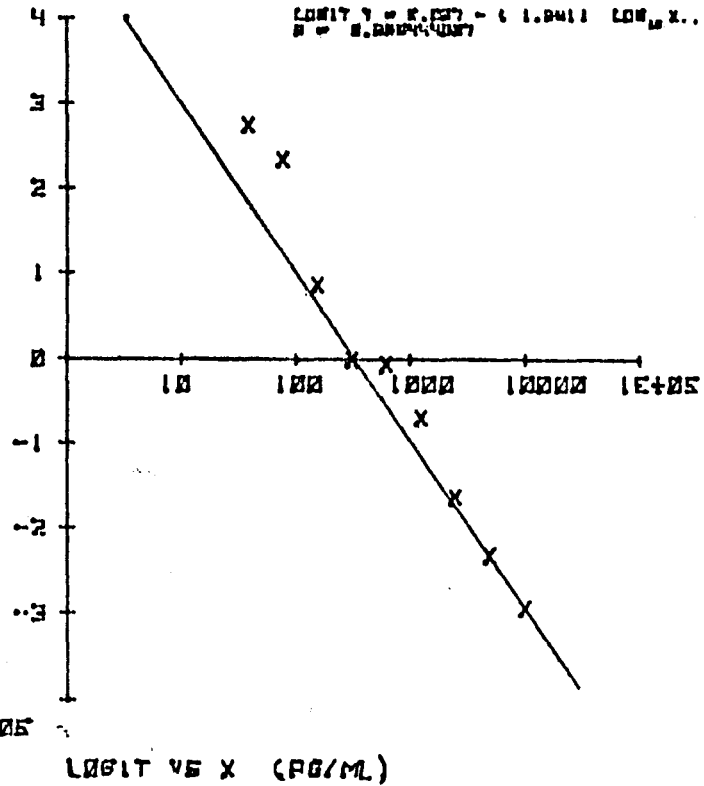


Figure 8: Glucagon standard curve plotted as logit vs. (pg/ml).

assess the input and removal rates of human plasma triglycerides. Using glycerol tritiated on carbon number two instead of a fatty acid carbon is preferable since glycerol recycling by the body is minimal. The procedure is based on the principle that plasma concentrations of any solute will change as a function of its removal from or release into the plasma. After radioactive glycerol is injected into the patient, the appearance and disappearance of radioactivity from the plasma pool is monitored from the monoexponential portion of the curve. A K value or decay constant is then calculated. Biphasic decay curves have been observed suggesting either two triglyceride pools, one rapidly removed from the plasma and one removed slowly possibly due to recycling, or one single pool in which stepwise removal of triglyceride is slowed as the case in remnant VLDL formation (413).

## II. Preparation of Labeled Glycerol for Injection

### a. Purification and Dilution of Label

$^2\text{H}$  glycerol in ethanol, 1 mCi/ml, was obtained from Amersham Searle (Arlington Heights, IL). The stock solution was diluted with saline to an approximate concentration of 10 uCi/ml. This working solution was lyophilized to dryness to remove radioactive water and then resolubilized with saline. No loss in radioactivity was observed.

All glassware and instruments were autoclaved for 15 minutes at  $120^\circ\text{C}$  and a sterile hood was used to prepare the radioactive label for injection. Pharmacological purity was insured by pouring the samples through a Nalgene membrane filter with a 22 um pore size. Once the sample was filter sterilized it was poured into autoclaved pharmaceu-



tical serum vials and capped. An aliquot of this solution was given to the Chief Research Microbiologist for culturing of bacterial growth. No evidence of bacterial growth after seven days culturing was considered proof of sample sterility. Standard procedures for radioactive handling and spillage were utilized. For added safety, sterilized samples for injection were refiltered upon injection by the use of a Swinex syringe filter (Millipore Corp., Bedford, Mass.).

#### b. Calculation of Total Body Dosage by Tritium Injection

Approximately 50 uCi per patient of  $2^3\text{H}$ -glycerol was injected for each turnover procedure. FDA policy stipulates that calculated radiation absorbed dosages be estimated and total body dose estimates be obtained. Amersham Searle Data indicates the maximum body burden for tritium ( $^3\text{H}$ ) to be 1 mCi per dose with an effective biological half life of 12 days. Safe dosages for any drug according to FDA rulings have to be less than 3 RADS. Maximum penetratable range for tritium in tissue is  $6 \times 10^{-3}$  mm. Dosage rate was calculated from the MIRD index for dosimetry (MIRD pamphlet #1 and #10) (396-397).

### III. Specimen Collection Procedures

#### a. Injection of Label

A 20 gauge polyethylene catheter was inserted into the patient's left antecubital vein with extension tubing leading into a 3 way flow stopcock. A slow intravenous saline drip was initiated and blood flow from the stopcock was checked. In the right antecubital vein, a butter-

fly catheter was implanted momentarily and the radioactive bolus was injected. During the interim course of the study the patient was fasted. Water was taken ad libitum and a small evening meal (broth and dietetic lemonade) was consumed. The total turnover procedure is 24 hours in duration with the catheter in place for eight hours. Patients were continually monitored throughout the period with P.M. blood collections obtained by the medical staff.

#### b. Specimen Collection and Processing

During the 24 hour period, specimens were drawn from patients using both the indwelling catheter and venupuncture methods. Specimens collected during the first eight hours were drawn by the indwelling catheter and stopcock. After this time, the I.V. system was removed and the remaining samples were collected by venupuncture. Seventeen samples (12 ml each) were drawn from the patients at different times during the twenty-four hour period.

The blood was allowed to clot at room temperature (approximately 45 minutes) and then centrifuged at  $1000 \times g/15 \text{ min}$  (2000 RPM) to separate red cells from serum. The serum was collected without a preservative and stored at  $4^{\circ}\text{C}$ . At the end of the procedure, all samples were frozen in polystyrene vials at  $-28^{\circ}\text{C}$ . Two turnovers were performed on each patient during the study, one at the end of the control diet and one after two weeks on the study diet.

### IV. Assay Protocol

#### a. Isolation and Purification of Radioactive Triglyceride Sample

A chloroform-methanol extraction procedure was used in the separation and purification of lipids. The non-polar triglyceride was solubilized in chloroform and the polar lipids which were solubilized in the methanol layer were discarded. Once the lipids were isolated and washed, they were concentrated and resolubilized in a fluor scintillant used for counting. P.C.S. (phase combining system), (Amersham Searle, Arlington Heights, IL), the scintillation fluor used, is a complete xylene surfactant which contains all ingredients necessary for counting organic and inorganic samples. The P.C.S. fluor and samples were poured into Kima glass scintillation vials. The vials were cooled and allowed to dark adapt. They were then counted along with a blank and tritium standard for 20 minutes on a Packard Tricarb liquid scintillation counter, Model 3390 (Packard Instruments, Downers Grove, IL), with an appropriate external standard to correct for quenching.

#### b. Scintillation Counting Procedures

The external standard method for quench correction employs Compton electrons derived from the interaction of  $\gamma$ -rays with the counting glass vial and the scintillation solution. Different color intensity blanks were run with the external standard in place for a period of time. The changes in counts from the non-colored blank as compared to the different intensity colored blanks were used to determine the counting efficiency of the colored samples.

### VI. Calculation of Results

#### a. Fractional Turnover Rate

Quench corrected counts were obtained from all patient samples. Radioactive time curves were plotted on semilogarithmic scale paper with plasma activity plotted against time. A Hewlett Packard Model 7830A computer was used to obtain the slope and intercept from linear regression analysis of the monoexponential portion of the decay curve. The line was extrapolated back to zero and total injected dose of tritiated glycerol was determined. Ascertaining the time when half the total number of counts has disappeared ( $T_{1/2}$ ) allowed calculation of  $k$  by the following equation:

$$K = \frac{0.693}{T_{1/2}} \quad \text{where } 0.693 = \ln 2$$

#### b. Total Turnover Calculations

The fractional turnover rate ( $k$ ) of the total plasma triglycerides was used to calculate the turnover rate ( $V$ ) which equals the removal rate as mg/kg/hr and was calculated from the following formula:

$$V = k \left( \frac{1}{\text{hr}} \right) \times S(\text{mg/dl}) \times 0.45 \left( \frac{\text{dl}}{\text{kg}} \right) \times b \quad (57)$$

$s$  = the triglyceride concentration in mg/dl

0.45 = indicates a mean plasma volume of 0.45 expressed as deciliters/kg

$b$  = a body weight correction factor. Since plasma volume is less in excess adipose tissue, all turnovers must be weight corrected.

$$b = \frac{4500 + a \times c}{100 + c} \quad 45$$

$a$  = plasma volume/kg adipose

c = excess of relative body weight (RBW) (RBW %-100)

### c. Kinetic Determinations and Statistical Calculations

Enzyme kinetic data was calculated by plotting triglyceride concentration/turnover ( $[S] / V$ ) versus triglyceride concentration ( $[S]$ ). This is comparable to a linear regression analysis performed on turnover plots after control diets and study diets were consumed. In this type of plot the point at which the lines intercept the X-axis gives the value for  $K_m$  with  $V_{max}$  estimated as  $1/\text{slope}$ .

The relationship between radioglyceride determined triglyceride turnover and triglyceride concentration was determined by plotting one against the other. The lines of best fit were determined by linear regression analysis. Again control values were compared to study diet values (high and low sucrose diets).

Correlation coefficients were calculated to determine if any relationship exists between fractional turnover and plasma TG levels after the control diet and after two weeks on the study diet.

## E. Total Lipoprotein Electrophoresis

### I. Introduction

The principles of lipoprotein electrophoresis are based upon the fact that a charged particle placed in an electric field will migrate toward one of the electric poles of the field (419). After electrophoresis, proteins are visualized by specific chemical detection. Protein levels can be interpreted qualitatively (by visual inspection) or quantitatively (subjected to densitometric scanning).

## II. Specimen Collection and Storage

Blood samples were drawn for lipoprotein electrophoresis by techniques similar to those for triglyceride and cholesterol measurement. Lipoproteins are unstable and easily disturbed by sheering forces or hemolysis. Therefore, samples for lipoprotein analysis should be collected in EDTA tubes with enough anticoagulant to produce a final concentration of 1-1.5 mg/ml (the EDTA also protects against lipoprotein oxidation). The collection tubes were filled to capacity (6 ml) and immediately immersed in an ice bath. The specimen was next placed in a refrigerated centrifuge and spun at 1500 x g (3000 RPM). The plasma was transferred to a clean storage vial using a Pasteur pipette. Lipoproteins will not tolerate freezing or long term storage in solution without undergoing changes that are reflected in alterations in their ultracentrifugal and electrophoretic characteristics (415-417). Lipoprotein concentrations are therefore most accurately measured in fresh samples as soon as possible. If samples were stored for longer periods of time (7 days), the plasma fraction was kept in the dark at 4°C.

## III. Agarose Gel Method

### a. Importance in Lipoprotein Measurement

The agarose gel system was used to detect lipoproteins by means of charge and size separation. A sample densitometric scan of a few of the basic lipoprotein types representing patients from our study is seen (Figure 9).

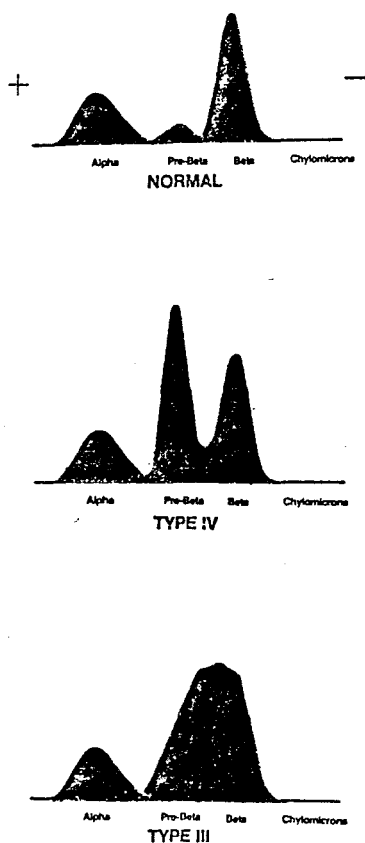


Figure 9: Graphical representation of lipoprotein patterns obtained in normal, type IV and type III hyperlipemic individuals. In type III individuals, the pre- $\beta$  band is again elevated, and migrates similarly to the  $\beta$ -lipoprotein band; causing the broad  $\beta$  band observed.

#### b. Assay Protocol

The agarose gel electrophoresis system used was the Beckman Microzone system (419). In this system, electrophoresis must be performed after rehydration of the gel. Excessive equilibration (longer than 45 minutes) was avoided since this may result in high current levels and joule heat which distorts the appearance of the patterns.

#### c. Staining Procedure

After fixing and drying the gel, it was stained with Sudan Black B in a flat tray of appropriate size for 5-10 minutes. If overstaining of the gel occurred it was destained by placing it in the gel/fixative solution until the background coloration disappeared (usually 10-20 minutes).

#### d. Densitometric Scanning and Data Interpretation

All gels were scanned with a Gelman Computing Densitometer, Model #ACD 18 (Gelman Instruments, Ann Arbor, Michigan). All gels were scanned at a wavelength of 500 nm and the area under each peak integrated to determine the percent of total lipoprotein present.

#### Data Interpretation

Densitometric scans were compared to the standard electrophoretic patterns of the Fredrickson classification of hyperlipoproteinemia and patients were classified accordingly. Normal values for percentages of the different lipoprotein fractions have been established and these were used in objective classifications (420).



## F. VLDL Apoprotein Analysis by SDS-polyacrylamide Gel Electrophoresis and Isoelectric Focusing

### I. Introduction

The reliability and resolution capabilities of SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis and isoelectric focusing have made these techniques invaluable in lipoprotein determinations (421). The measurement of apoprotein species from different lipoproteins, by these high resolution techniques, have enabled quantitation of individual apoprotein subunits (63, 64, 66-72, 422-426). These gel systems were used to isolate individual apoproteins in sufficient quantity and purity.

### II. Sample Collection and Storage

Sample collection procedures were identical to those for triglyceride and lipoprotein determinations except that blood was placed in tubes containing sodium citrate. These samples were placed in an ice bath for 10 minutes and plasma was separated in a refrigerated centrifuge at 1000 x g/15 min (2000 RPM). The plasma was removed carefully with Pasteur pipettes and placed in polystyrene storage vials. One mg/ml of EDTA was added to prevent oxidative breakdown of some of the protein constituents. Samples were refrigerated at 4°C and analyzed within one week of collection.

### III. Isolation of VLDL Apoprotein

#### a. Ultracentrifugation of Total Plasma for VLDL

The method of Havel et al. (431), based on flotation ultracentri-

fugation, was used to provide VLDL lipoprotein for quantitative analysis of apoprotein subfractions. Density adjustments were made with solutions containing NaCl and KBr up to a density of 1.006 (gm/ml).

The plasma mixture was gently poured into 5/8 x 3 polyallomer ultracentrifugation tubes (total volume 13 ml) which were filled to capacity, sealed with flat gasket caps and placed in a Ti-50 fixed angle rotor. Samples were centrifuged at 105,000 x g (40,000 RPM) for 20-22 hours at 10-15°C in a Beckman Spinco Ultracentrifuge, Model #L2-65B (Beckman Instruments, Palo Alto, CA) (Figure 10).

Following the centrifugation, lipoproteins of less than the solvent density of 1.006 were concentrated at the top in a layer approximately 1/2 inch thick. Beneath this layer, occupying 1/2 of the length of the tube, a clear zone was observed. Concentrated at the bottom, the remaining serum was stratified below the clear zone. The lipid layer at the top and approximately 2/3 of the clear zone were carefully collected by aspiration with a Pasteur pipette. The collected fraction was then purified and delipidated.

#### IV. Purification Procedure

##### a. Dialysis and Delipidation of VLDL

Separated VLDL lipoproteins were placed in cellulose dialysis membrane tubing 1-1/8 mm inflated diameter. The sample solutions were dialyzed against a bath containing 0.05 M tris buffer, 0.05% EDTA, and 4.0 M NaCl, pH 8.6 for 48 hours at 4°C.

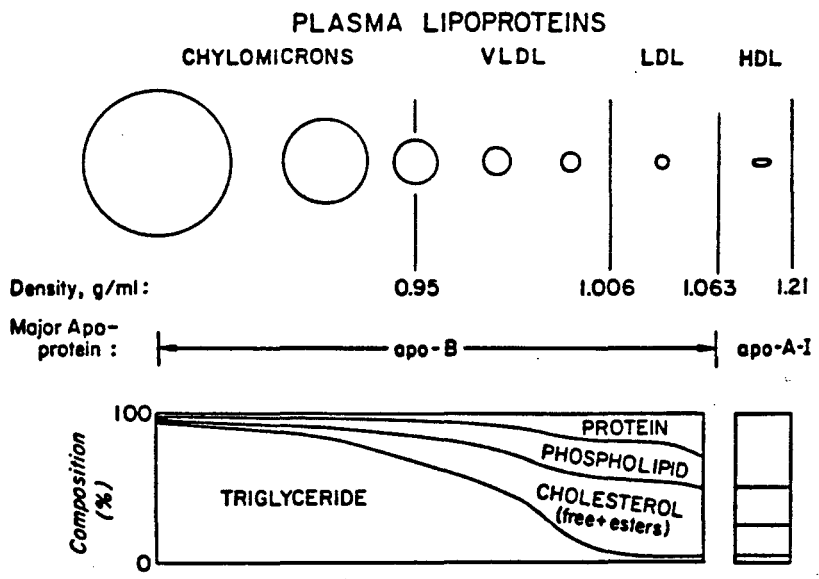


Figure 10: Schematic representation of plasma lipoproteins separated by size and lipid composition. The largest lipoproteins are the least dense and contain the most triglyceride. The smallest lipoproteins are the most dense and contain the largest amount of lipid as cholesterol. Apo B is found in all lipoprotein classes with the exception of HDL.

The dialyzed VLDL solution was then poured into a serum bottle and quick frozen in an acetone dry ice bath. Samples were then freeze dried on a Virtis lyophilizer for approximately eight hours. The freeze dried samples were removed and immediately delipidated with 10 mls of chloroform: methanol (2/1, V/V). Lipid-free protein was washed twice with 10 ml volumes of chloroform/methanol and placed in 15 ml conical glass tubes and centrifuged at 1000 x g for 10 minutes (2000 RPM) at 5°C. Protein samples (pelleted after centrifugation) were dried under a stream of nitrogen for three hours.

#### b. Solubilization Procedure for Isolated Apoprotein

Delipidated apoprotein was solubilized in a series of steps that involve reduction with mercaptoethanol and dialysis against dithiothreitol. For 24 hours at room temperature, the apoprotein pellet was suspended in 1.0 ml of a solution consisting of 2.5% sodium dodecyl sulfate, 1.0% Na<sub>2</sub>CO<sub>3</sub> and 10% B-mercaptoethanol, pH 8.5. After this time, partial solubilization of protein occurred. The protein suspension was gently poured from the conical tubes into 1-1/8 mm dialysis tubing where it was dialyzed for 48 hours at room temperature against 200 ml of 0.1% SDS, 1.6 M urea, 0.05% dithiothreitol and 0.01 M tris-HCl, pH 8.6. Solubilized protein was removed from the dialysis bath and poured into 12 X 75 mm glass culture tubes. One gram of sucrose was added to each tube and the sucrose was allowed to dissolve in the apoprotein solution to increase the density.

#### c. Protein Analysis of Sample

To determine the protein concentration of the solution, a Lowry protein assay modified to assay SDS solubilized proteins was performed (432). High backgrounds developed upon addition of folin-phenol reagent during color development and it was determined that the interference was due to residual mercaptoethanol which was not dialyzed out of solution. A method was employed which is considerably less susceptible to interference. This is the dye binding technique developed by Bradford et al. (433). In this assay, the absorbance of an acidic solution of Coomassie Brilliant Blue G250 (BioRad Laboratories, Richmond, CA) shifts from 465 nm to 595 nm when binding of the dye by protein occurs. Thus Coomassie Dye can be used for quantitative protein determinations by measurement of optical absorbance at 595 nm.

An aliquot of mercaptoethanol buffer, dialyzed with the patient's samples was used as a reagent blank with bovine serum albumin, treated similarly to the patient's samples used as a standard. The protein-dye mixtures were incubated for 60 minutes at room temperature. Absorbance at 595 nm was quantitated on a Gilford Spectrophotometer, Model #240 (Gilford Instruments, Oberlin, Ohio).

#### d. Calculation of Protein Concentrations

All spectrophotometric measurements were done with the absorbance of the instrument set to zero with the reagent blank. The concentration of the unknown was determined by comparison of the absorbance of the unknown with a known standard or read against a standard curve (Figure 11).

## Standard Curve Protein Assay

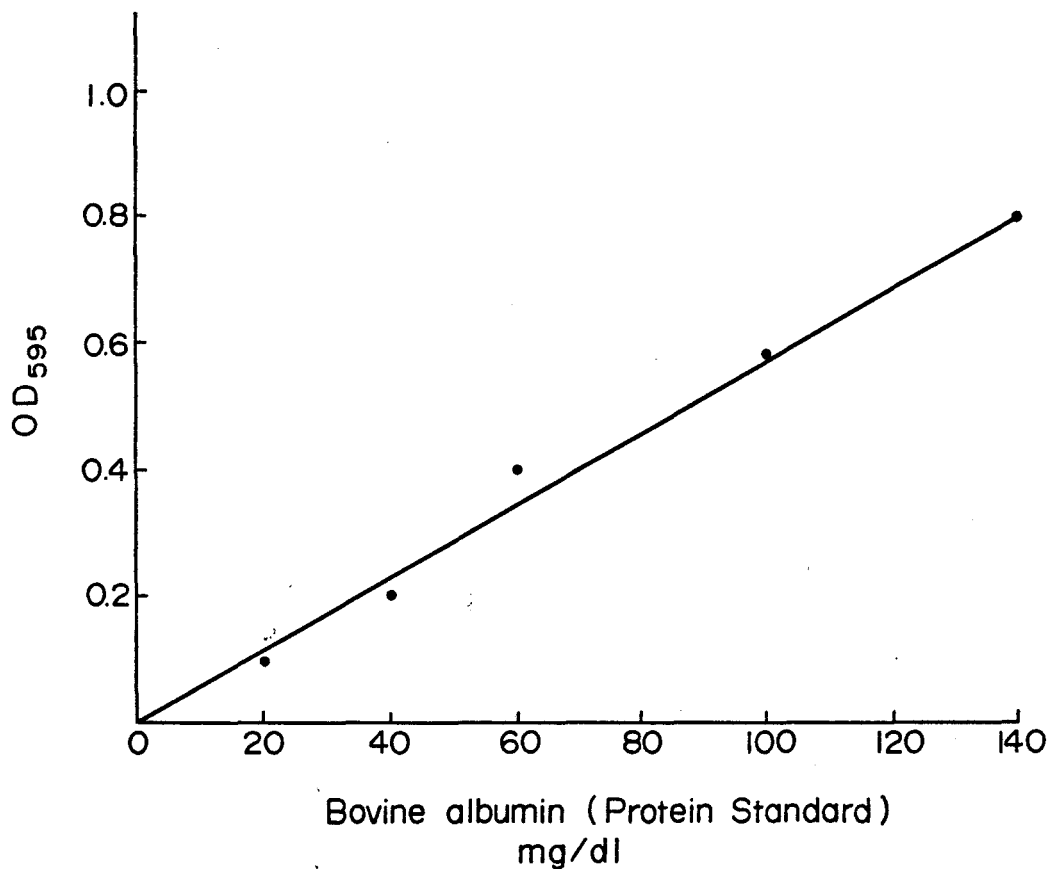


Figure 11: Standard curve determined from the dye binding technique of Bradford et al. (433). This method is used to determine the amount of unknown VLDL lipoprotein collected by ultracentrifugation of human plasma samples.

## v. SDS - Polyacrylamide Gel Electrophoresis

### a. Explanation and Brief Summary of the Principles

The methodology for disc electrophoresis and its application to human serum proteins was introduced by Davis et al. (434). Shapiro, et al. (435) reported that the separation of proteins by polyacrylamide electrophoresis in the presence of the anionic detergent sodium dodecyl sulfate is dependent on the molecular weights of their polypeptide chains. Since the dodecyl sulfate ions negate any charge related migration, the proteins separate strictly on the basis of their diameters and thus, their molecular weight. Thus, with this system, the pore size is tailored to the dimension of the molecule to be separated by varying polyacrylamide gel concentration (437).

### b. Protocol

#### Preparation for Electrophoresis

The electrophoretic unit used was a Canalco Model #12, (Canalco Instruments, Rockville, Maryland) electrophoresis cell, designed for disc gel separations. Both stacking and separating gels were used in the determination of VLDL apoproteins. The separating gel was a 10% SDS gel and the stacking gel was 2.5%. The dense sucrose-protein sample was added to the top of the gel with a Hamilton syringe. The addition occurs in the cold room at 5°C with the gel tubes in the electrophoretic cell. The protein solutions were layered on the gel through the upper tank buffer (approximately 150 ug of protein per gel).

Solution Used to Prepare Gels were Modified from Existing ProceduresSeparating Gel

10% gel pH 8.6

Solution A

0.12 M tris-HCl buffer, pH 8.6

0.23% V/V TMED (N,N,N<sup>1</sup>,N<sup>1</sup>-tetramethylethylene diamine)

0.02 M sodium dodecyl sulfate solubilized with 8 M Urea

Solution B

40% W/V acrylamide

1.1% N, N<sup>1</sup>-methylenebisacrylamide solubilized and diluted to  
100 ml with 8 M Urea

Solution C

0.01 M (NH<sub>4</sub>)<sub>2</sub> S<sub>2</sub>O<sub>8</sub> (ammonium persulfate) in 8 M Urea

Gel solutions were mixed in the following ratio:

4 ml (A) + 8 ml (B) + 4 ml (8M Urea) + 16 ml (C)

Stacking Gel Solutions

2.5% gel pH 6.6

Solution D

0.7 M Tris-HCl buffer pH 6.6 - 6.8

1N HCl

TMED



8 M Urea

Solution E

5% W/V acrylamide solution

2.5% NN methylene bisacrylamide

8 M Urea

Solution F

$1 \times 10^{-4}$  M riboflavin

8 M Urea

Solution G

40% sucrose in 8 M Urea

The solutions for the stacking gel were mixed in the following proportions:

1 ml (D) = 2 ml (E) + 1 ml (F) + 4 ml (G)

Photoflo 600 Solution

0.17% V/V Photoflo 600 (Eastman Kodak Co., Rochester, N.Y.)

Upper Tray Cathode Buffer pH 9.0

0.04 M Tris

0.05 M glycine

1N HCl

8 M Urea

Lower Tray Anode Buffer pH 8.1

0.12 M Tris

1N HCl

Bromphenol Tracking Dye10 mg dissolved in 1000 ml H<sub>2</sub>OElectrophoresis

Samples were electrophoresed for 8 hours at approximately 3mA per tube at 5°C. 1.5 hours after the marker dye migrates off the gel, the flow of current was halted and gel tubes were removed from the cell. Since the apoproteins migrate much slower than the tracking dye, the 1.5 additional hours were found to be the time of maximum migration of the apoproteins. The gels were recovered for protein staining by placing them in an ice bath. After five minutes, they were removed and gently rimmed free from the gel tube with a stream of water.

c. Staining Procedures

The recovered gels were placed in 12 X 75 mm tubes and were stained with a solution of 1% Amidoschwarz 10B in 7.5% acetic acid for 16 hours at room temperature. Gels were removed from the stain and excess dye was removed by repeated washings of 7.5% acetic acid (48 hours is the usual destaining time). The gels were scanned densitometrically and peak integration was used to obtain a quantitative measurement of the constituent apoproteins.

## VI. Isoelectric Focusing

### a. Explanation and Brief Summary of Principle

The technique of isoelectric focusing has been applied to the analysis of many proteins, including human apolipoproteins (423, 426). Proteins of similar molecular weight but different charge will also separate in this system enabling the identification of isoprotein species.

### b. Assay Protocol

The protein preparations were electrophoresed in the electrophoretic apparatus as described IV, b, 1. However, the system was equipped with a larger lower tank to accommodate gels which were polymerized in tubes 12 cm long with an I.D. of 5 mm, 7.5% polyacrylamide gels, with added ampholytes, were poured into the tubes to a depth of approximately 11 cm.

## Gel Solutions

### Acrylamide Solution

37.5% acrylamide solution

0.8% NN<sup>1</sup> methylenebisacrylamide

Ampholine carrier ampholytes were obtained from commercial preparations by LKB Instruments, Inc., (Rockville, MD). The ampholyte solution consists of different aliphatic polyamino-polycarboxylic acids. The pH range chosen for this assay was pH 4-6.

Ammonium Persulfate Solution

1.07% ammonium persulfate

Upper and lower chamber buffers are prepared as follows:

Upper Chamber (cathode buffer) pH 13

0.02 M NaOH

Distilled H<sub>2</sub>O

Lower Chamber Buffer (anode buffer) pH 1

0.01 M phosphoric acid

Distilled H<sub>2</sub>O

The cell unit containing the gels was placed in a cold room at 5°C and the gels were prefocused at 1mA/tube for 30 minutes to establish the pH gradient. Electrophoresis was terminated and protein samples were directly applied to the buffer gel interface with a Hamilton or tuberculin syringe (400 ug of protein in 500-700 ul volume).

c. Electrophoresis

Proteins were electrophoresed at 400 volts until the current stabilizes at around 1-1.2 mA for 12 gels (12-15 hours). One gel in each run was used as a blank for pH gradient determinations. Gels were removed after electrophoresis by rimming the tube with water. The gels containing the separated protein were fixed and stained, as described previously. The blank gel was sliced with a razor blade into 5 mm segments. These segments were transferred to small test tubes, 1 ml of

deionized water was added, the tubes were capped and stored at 4°C. Subsequently, the pH of the 1 ml solution was measured at room temperature with a Model 26 Radiometer pH meter (Radiometer, Copenhagen, NV, Denmark).

#### d. Staining Procedure

A method developed by Malik and Berrie (438) utilizes Coomassie Brilliant Blue, but eliminates the need for fixing, deampholyting and destaining. This method avoids intense background staining and has resolution capacities comparable to other procedures using Amidoschwarz and Coomasie Brilliant Blue. After focusing, the gels were removed and placed directly in the stain fixative (1% stain solution). Protein bands appear within 15 minutes and intensify with longer staining periods (overstaining has not been encountered with treatment times as long as 48 hours). Gels were typically stained for 12 hours and were transferred to distilled H<sub>2</sub>O (a suitable storage media). A short immersion in 0.2% H<sub>2</sub>SO<sub>4</sub> will decolorize unwanted background, if desired, but prolonged exposure to H<sub>2</sub>SO<sub>4</sub> will eventually remove all stain.

Stained and fixed gels were photographed and then placed in scanning tubes. The Gelman densitometer used previously to scan and integrate the protein peaks of the SDS polyacrylamide gels was also used to determine the protein content of the isoelectrically focused gels. All apoproteins were identified by their isoelectric point which was compared with tables of known standard apoproteins (Table 3). Protein peaks were expressed as percent of total apoprotein present.

Multiple samples were collected for apoprotein determination during the control and study diet periods. Therefore, any changes induced by the diet would conceivably be detected since each patient can serve as his own control. Changes in apoprotein composition determined from the values obtained while consuming an isocaloric control diet could thus easily be identified.

### G. Platelet Aggregation Analysis

Platelet aggregation is a term used to denote the adherence of one platelet to another. A variety of agents to which platelets may be exposed in vitro cause them to change shape, aggregate and release the constituents of their storage granules. Some of these constituents are collagen, thrombin, ADP, epinephrine, serotonin and vasopressin.

#### a. Sample Collection and Preparation for Platelet Aggregation

By sterile venupuncture of the antecubital vein, 9 ml of whole blood was drawn into a vacutainer containing 1 ml of 3.8% sodium citrate. The blood was drawn after an overnight fast with the patient seated and rested 15 minutes before drawing. The blood was collected in 17 X 100 mm polypropylene tubes and spun and assayed within two hours.

The blood was centrifuged at room temperature at 150 X g/15 minutes (800 RPM). The upper plasma layer was syphoned off (approximately 2 ml) and transferred to polypropylene tubes with plastic transfer pipettes. This was designated platelet-rich plasma (PRP). The remaining plasma was centrifuged at 2000 X g/15 minutes (2100 RPM). The

TABLE 3

TABLE Classification of apolipoproteins of HDL and VLDL

Isoelectric Designation	Protein Designation <sup>a</sup>	Estimated Mol. Wt.	Sephadex Fraction
>6.00	C-I	7,000	HS-3 (VS-3)
5.82	A-I	27,000	HS-2
5.75			
5.65			
5.55			
5.46 <sup>b</sup>			
5.41	ARP and A-IV	35,000	HS-1 (VS-2)
5.39			
5.36	A-II C-II C-III-0 C-III-1? C-III-2? C-III-3 C-III-4?	46,000	HS-1
5.31			
4.83			
4.74			
4.67			
4.61			
4.57			
4.50			
4.43	11,000		

Isoelectric points of all apolipoproteins found in HDL and VLDL lipoproteins. Apo B is considered insoluble because of its large size and is not represented. ARP is synonymous with apoprotein E, the arginine rich protein. Data represented from reference 430.

remaining plasma was pipetted off the red cells and this is designated platelet poor plasma (PPP). These two plasma preparations were used in the platelet aggregation assay.

#### b. Description of Aggregometer

The platelet aggregometer measures changes in light transmission scattering through platelet-rich plasma that is maintained at 37°C and constantly stirred at 1000 RPM (440-442). The instrument used was a Chrono-log Aggregometer, Model #330 with a Linear Instrument Chart Recorder #702. (Chrono-log Corp., Havertown, PA). The instrument operates on the principle that platelets in a stirred solution will adhere to one another and fall out of solution when an aggregant is added. The platelet-rich plasma situated in a small cuvette is slightly turbid due to the presence of platelets in suspension. When aggregating agents were added, the turbidity decreases because of the clumping action of the platelets. When a light beam is passed through the cuvette containing platelet-rich plasma, the amount of light transmitted will increase as turbidity decreases. This quantity was recorded on a linear strip chart recorder and the resultant curve was a record of the rate and amount of platelet aggregation.

#### c. Assay Procedure

Platelet solutions were counted and (PRP) concentrations were adjusted by dilution with (PPP). 2.5 ml of the working (PRP) was made with a final concentration of platelets set at  $200,000 \pm 20,000$  platelets/ml. For each separate patient, a platelet poor blank was made



by taking 0.45 ml of (PPP) and adding 0.05 ml of saline to it. This blank was used to adjust the baseline of the instrument to zero. Triplicate tubes of patient platelet-rich plasma (PRP) were made with 0.45 ml of the working solution. The cuvettes were allowed to warm up to temperature (37°C) and then 0.05 ml of aggregating agent was added (adrenaline chloride, final concentration of epinephrine-hydrochloride in saline was 0.1 mg/ml, Parke-Davis, Grand Rapids, MI).

#### d. Sensitivity Studies

Platelet sensitivity to the aggregating agent was monitored when serially diluted epinephrine mixtures were added to the (PRP). The concentration of aggregating mixture at which both first and principally second phase aggregation was lost is considered to be the threshold level of epinephrine needed to elicit an aggregation. The solutions used range from a concentration of 50  $\mu$ m to 0.1  $\mu$ m. None of the patients from the study demonstrated an aggregation response with concentrations of less than 0.1  $\mu$ M.

The maximum change of OD =  $OD_f - OD_i$ .  $OD_f$  was measured at the maximum point of aggregation and  $OD_i$  was measured at the initial point, before the aggregant was added. The values were expressed as percent aggregation using the (PRP) working solution as 0% and the (PPP) as 100%. Aggregation curves obtained were compared between the control and study diet periods. The changes in aggregation response or sensitivity of the platelets from control diets were recorded.

## H. Statistical Analysis of Data

Except in experiments where statistical evaluations of data are listed, results were analyzed by the paired or nonpaired two tailed t test. In essence t is the ratio of the difference between two means to the standard deviation of the difference. Since there is no justifiable basis for selection of pairs of subjects which can be expected to behave or respond more nearly alike than any other two, the unpaired t test for significance treats the individuals from each sucrose group as independent samples from a common population. The t value is based on the number of degrees of freedom (the number of samples or subjects involved minus two). Since the unpaired t allows each series to be summed independently to obtain two sample means, 2 restrictions to the number of degrees of freedom are usually imposed. The t value is used to find the significance level or probability level that the two sets of data are the same. In this study, a 5% significance level ( $p < .05$ ) or below is considered significant. Thus, if t were found to have a P value of  $< .01$  this would indicate that the two sets of data have a less than 1% chance of being the same. Paired t analysis was used to compare values from the same patient when consuming the control diet or study diet.

## 5. RESULTS

### A. Description of Patient Population at Time of Entry Into Study

All patients matriculated into the Sucrose Study were adult onset diabetics who were hypertriglyceridemic and non-insulin dependent. Pertinent patient information is given in Table 4, including age and weights, along with fasting serum glucose, triglyceride, HDL-cholesterol and cholesterol ranges and means. TC/HDL-C, a ratio used in assessing susceptibility to atherosclerosis and coronary heart disease, was also determined indicating the majority of patients to be in a range consistent with their age group. All patients were type IV hyperlipemic individuals. Fasting serum chemistries for all test subjects were indicative of hyperlipemic adult onset diabetes. Fasting mean values observed were a serum glucose level of 198 mg/dl, a serum triglyceride level of 300 mg/dl and a serum cholesterol level of 207 mg/dl. A total of thirty patients participated in the study for a six week time period.

### B. Results of Chemical Determinations During Study Diet Period

Serum glucose levels were obtained fasting and postprandially along with a 24 hour urine glucose determination. Daily weights were also obtained from the patient population during the study. The results are depicted in Figures 12-15.

#### a. Fasting and Postprandial Serum Glucose

Fasting and postprandial serum glucose levels were monitored on

TABLE 4  
DESCRIPTION OF PATIENT POOL USED FOR SUCROSE STUDY

Number of Subjects	Subject Age	Subject Weight	Fasting Serum Glucose	Fasting Serum Trigs	Fasting Serum HDL-C	Fasting Serum Cholesterol	TC/HDL-C	Lipoprotein Pattern
30	Range 39-74	Range 144-282	Range 96-308	Range 150-752	Range 14-46	Range 142-310	Range 3.9-12.5	Type IV elevated pre B
	Mean 60	Mean 202	Mean 198	Mean 300	Mean 35	Mean 207	Mean 6.4	

all patients during the control and study diet periods. The data in Figure 12 demonstrates that fasting blood sugars in both study groups fell from control values when patients consumed either the high or the low sucrose diets. However, a more significant decrease ( $p < 0.02$ ) in serum glucose was observed in patients consuming high sucrose diets. The mean control values for this group were approximately 195 mg/dl with study diet mean fasting glucoses as low as 160 mg/dl. Decreases of this magnitude were not observed in the low sucrose study group.

The mean postprandial serum glucose levels showed no significant changes from the control values. Again, a slight decrease in mean postprandial serum glucose was observed in the high sucrose study individuals. An interesting observation is noted concerning the patients consuming the low sucrose diet. Their mean serum postprandial glucose showed a small increase from the control values until the fourth week, at which time glucose levels were comparable to those observed during the control diet period.

The data in Figure 13 demonstrates the direct relationship between fasting and postprandial serum glucose levels while consuming the different study diets. No significant differences were observed between fasting and postprandial mean serum glucose levels at any time point for either diet.

#### b. 24 Hour Urine Glucose Measurements

24 hour urine glucose measurements were obtained on all patients during the study and Figure 14 summarizes the pertinent data. Since many of the patients had markedly different levels of glucose excretion

## Fasting and Post Prandial Serum Glucose

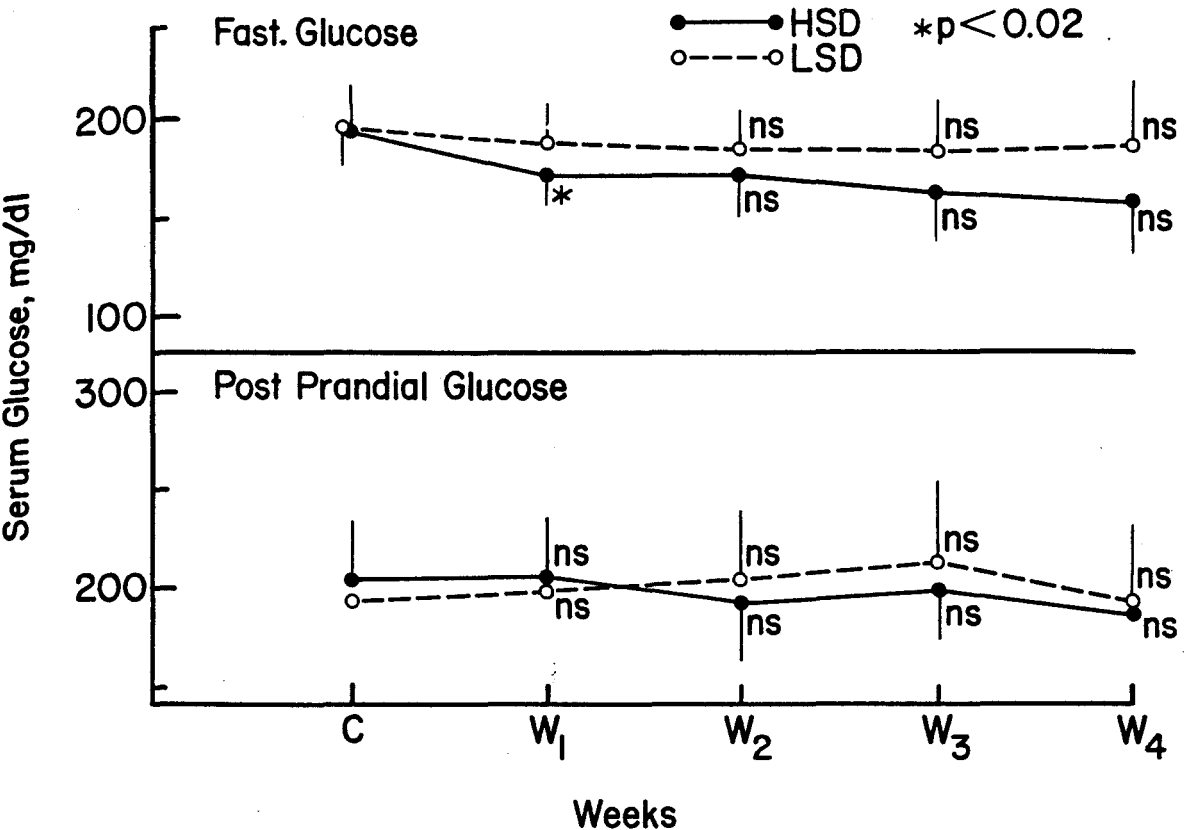
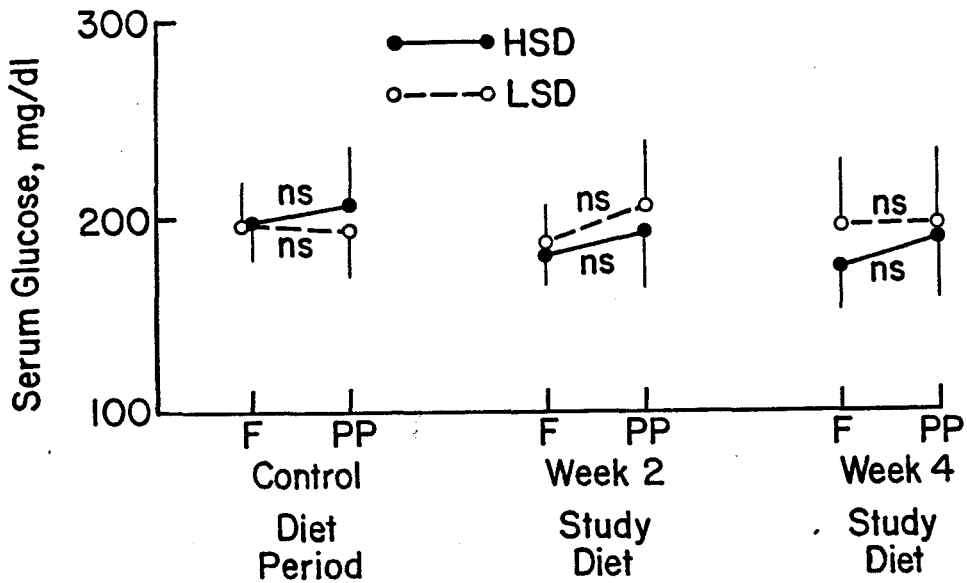


Figure 12: Fasting and postprandial serum glucose levels averaged from 17 patients consuming high sucrose diets and 13 patients consuming low sucrose diets. C represents a control period in which patients consumed an intermediate diet consisting of 120 g of sucrose per day for 2 weeks. The value represents the last 2 measurements from this period. The W<sub>1</sub>-W<sub>4</sub> values represent the weekly periods of high and low sucrose dietary consumption after the control period. All weekly values are compared to the control period values for that particular group. Three values obtained per week with the mean and S.E.M. of all patients per group per week are represented by each point.

### Fasting and Post Prandial Serum Glucose



**Figure 13:** Direct relationship between fasting and postprandial serum glucose is demonstrated. Control period fasting (F) and postprandial (PP) values along with  $W_2$  and  $W_4$  of the study diet periods are represented. Each point is the mean and S.E.M. of weekly glucose values obtained per group. The difference between fasting and postprandial serum glucose is measured for significance.

into the urine, the average of the results from each group did not reflect the changes that occurred. In order to obtain the best representation of the spread in urine glucose excretion, the patient population was divided into two groups, those excreting in excess of 15 g of glucose daily (>15g) and those excreting less than 15 g of glucose daily (<15g). Results were expressed as the change from the mean urinary glucose excretion during the last 5 days of the control diet to the particular week of the study. Urine glucose excretion showed little change from control values regardless of the study diet in individuals who excreted low amounts of glucose in their urine, in contrast to the substantial changes observed in those who excreted high amounts of glucose in their urine. When patients began consumption of the low sucrose diet an immediate drop in urine glucose from the control values was observed. Patients consuming the high sucrose diet showed little change from control values during the first two weeks on the study diet. Both groups, however, showed significant decreases in 24 hour urine glucose output after 4 weeks. By this time, high sucrose study patients decreased their urinary glucose output an average of 10 grams/day ( $p < 0.01$ ) and low sucrose study patients lowered their urinary glucose output by approximately 25 grams/day ( $p = 0.05$ ).

### c. Average Weekly Weights

An important aspect for this study was the assurance that the different diets administered were kept relatively isocaloric and the patients metabolically stable. Large decreases or increases in weight can not be tolerated because of the metabolic consequences of weight



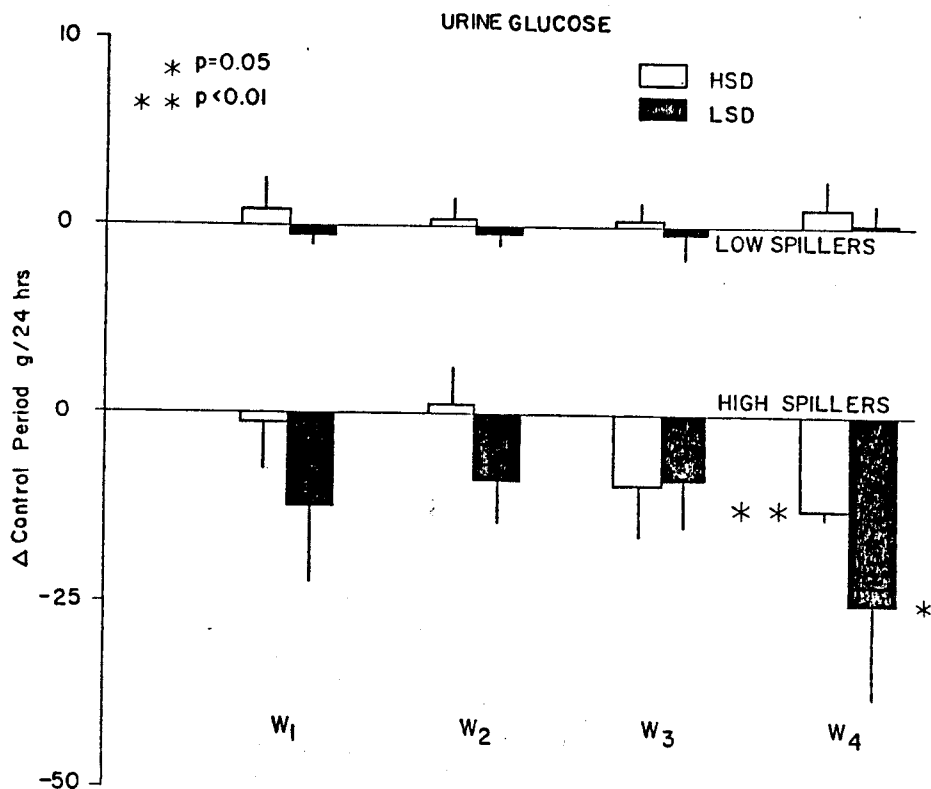


Figure 14: 24 hour urine glucose levels obtained for both high and low sucrose diet study groups. Two subgroups consist of those excreting more than or less than 15 grams of glucose per 24 hours. All values are expressed as changes from the control period values which are averaged for the last 5 days before the study diet is initiated. All figures represent means and S.E.M. for all patients sampled per group per week.

changes on hormonal levels and fatty acid metabolism. Figure 15 is a graphical representation of patient weight change from the control period during the administration of the two study diets. A weight change of  $\pm 5\%$  was considered tolerable for the study. As observed, the mean weight change from control values was insignificant for both study groups.

### C. Lipid Levels During High and Low Sucrose Diets Compared Against Control Diet (120 gm Sucrose Level).

Fasting and non-fasting lipid levels were obtained on all of the patients studied. Triglycerides, HDL-cholesterol and serum cholesterol were measured three times weekly in all test subjects. Results were averaged and in all cases were depicted as alterations from the concentration obtained during the control period. These results are summarized in Figures 16-21.

#### a. Serum Fasting and Non-Fasting Triglyceride Levels

Blood for fasting serum triglycerides was usually drawn following a 12 hour fasting period. The results are shown in Figure 16. Patients switched to a high sucrose diet showed a large and rapid increase in their fasting triglyceride concentration from control diet levels. Within the first two weeks of consuming the high sucrose diet, triglyceride concentration in these patients averaged 65 mg/dl higher than their control period values ( $p < 0.01$ ). This increase reached approximately 70 gm/dl by the third week ( $p < 0.01$ ). However, patients on the low sucrose diet demonstrated minor fluctuations in triglyceride

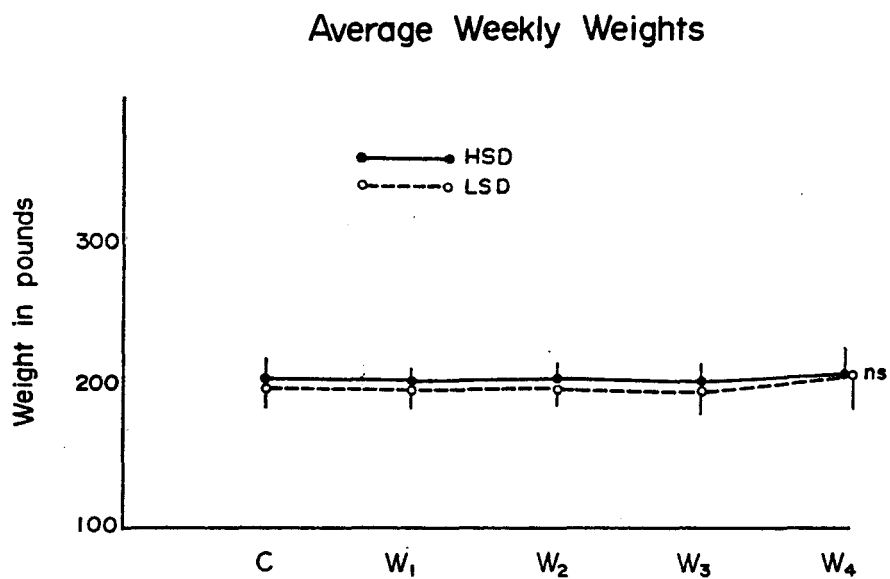


Figure 15: Patient weights were taken daily, averaged weekly per patient and combined by study group. Each point represents the mean and S.E.M. for all patients per group per time sequence in diet.

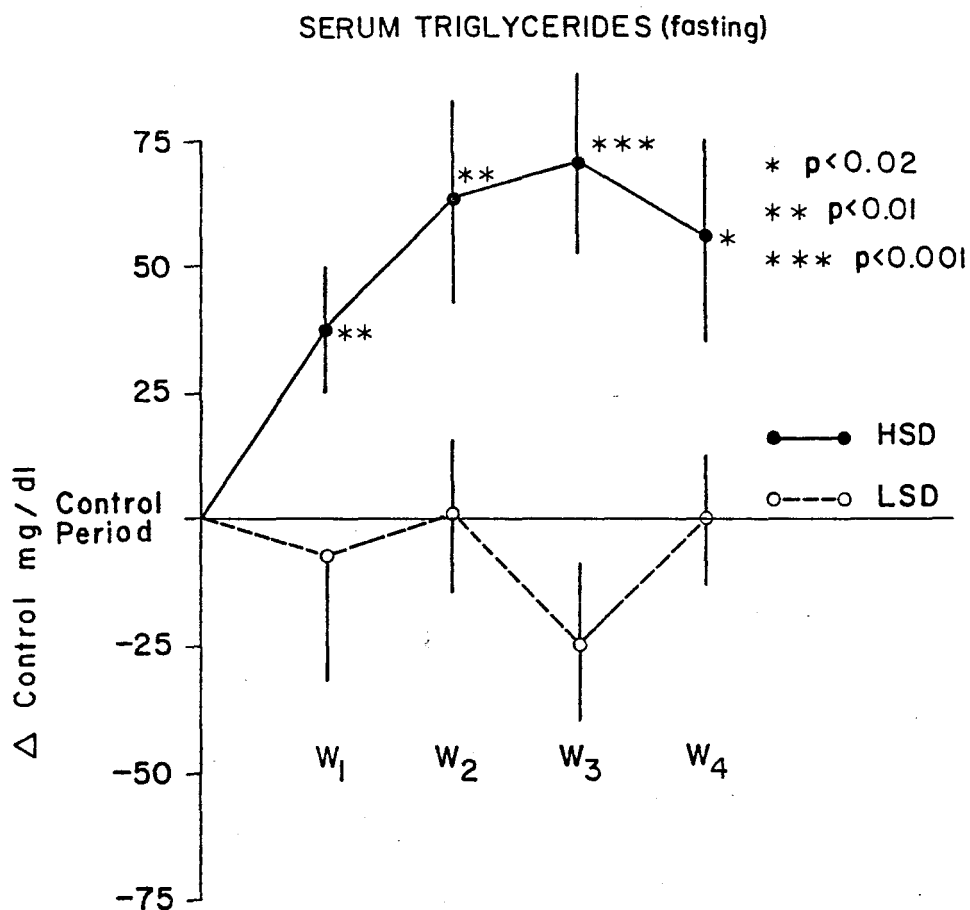


Figure 16: Fasting serum triglycerides measured from both patient populations. All values are compared to the control period value which was designated as the average of the last 3 measurements during the control (intermediate) diet. Each point represents the mean and S.E.M. for all patients per group measured during that time sequence. Significance is calculated as the difference from the values obtained during the control period. Each patient value per week is an average of 3 values obtained during that time period of the study.

concentration. The displacement from control diet triglyceride values was neither large nor sustained in this group.

The results delineated in Figure 17 represent the change in non-fasting triglycerides from control period levels. Both groups of study patients increased their non-fasting triglyceride concentrations by the fourth week. However, the patient group consuming the low sucrose diet showed the largest jump in triglyceride concentration with an average increase of 75 mg/dl over control values by the fourth week ( $p < 0.01$ ). This can be compared to a 20 mg/dl increase over control values in patients consuming high sucrose diets. The rise in postprandial triglyceride concentration during low sucrose consumption is almost instantaneous with diet change and may reflect the type of diet administered. This diet contained higher (10%) proportions of fat compared with the control diet.

#### b. Serum Fasting and Non-Fasting HDL-Cholesterol Levels

Figures 18 and 19 represent HDL-cholesterol levels during both fasting and non-fasting periods respectively. It can be observed in Figure 18 that the mean increase in HDL-cholesterol levels is 5 mg/dl over control diet values with consumption of low sucrose diets ( $p = 0.05$ ). High sucrose diet group patients showed a slight decrease in HDL-cholesterol levels during the first two weeks which was not significant ( $p = 0.198$ ).

Figure 19 illustrates HDL-cholesterol levels obtained during non-fasting periods. These values were relatively unchanged from control values when either study diet was consumed. Non-fasting HDL-cholesterol

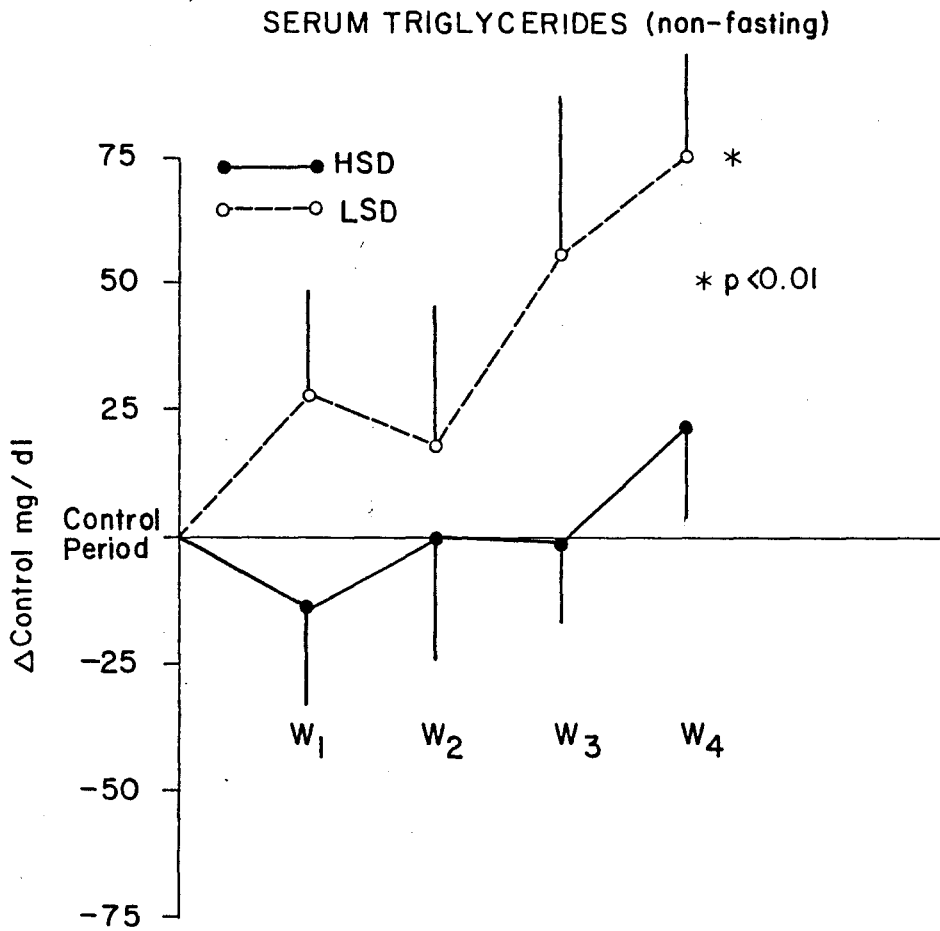


Figure 17: Non-fasting serum triglycerides measured from both patient populations. The description of the graph and the relative relationships are the same as described in Figure 17.

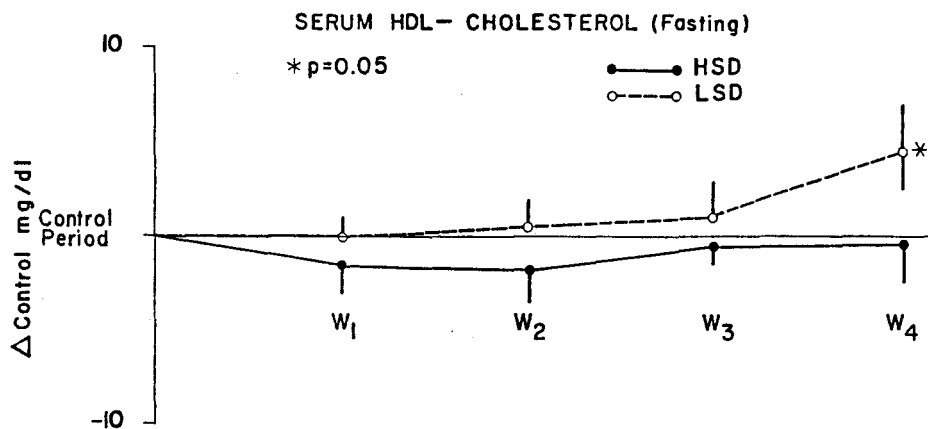


Figure 18: Fasting serum HDL-cholesterol changes from the control period values are observed for both groups of patients studied. The points represent the mean and S.E.M. values for all patients per group measured during each particular time sequence. All values are expressed as mean change from the control period point which is the mean of the last 3 days averaged during the control period. Significance is expressed as the difference from the control point.

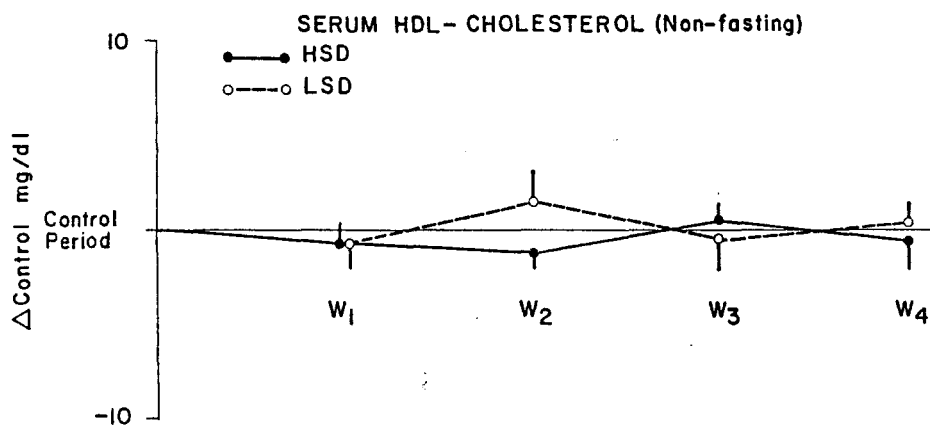


Figure 19: Non-fasting serum HDL-cholesterol changes from the control period values are observed for both groups of patients studied (high and low sucrose). The description of the graphical relationships is identical to and found in Figure 19.



levels were found to average 5 mg/dl lower than the fasting values observed.

### c. Serum Fasting and Non-Fasting Cholesterol Values

The data presented in Figure 20 shows the effect of diet on fasting serum cholesterol levels. A mean decrease of 17 mg/dl in cholesterol concentration is observed during the second week of the study period with consumption of high sucrose diets ( $p = 0.05$ ). Patients who were given the low sucrose diet after the control diet showed no significant changes in fasting cholesterol concentrations, although a slight increase in serum cholesterol concentrations from the control period can be observed during weeks 2 and 3.

Figure 21 illustrates the changes in non-fasting serum cholesterol levels from control values. Again, patients consuming high sucrose diets showed a significant decrease of approximately 10-15 mg/dl in serum cholesterol concentrations ( $p = 0.05$ ) relative to serum values when consuming the intermediate control diet. Patients consuming the low sucrose diet after the control diet showed no significant change during the first two weeks of study diet consumption. However, during weeks 3 and 4 a slight decrease in non-fasting cholesterol was observed from control values.

### D. Effect of (120 gm) Intermediate (control) Diet on Patient

#### Population Subgroups

Administration of 120 grams of sucrose per day to patients prone to hyperlipemia could cause their lipids to increase. The 120 g/day

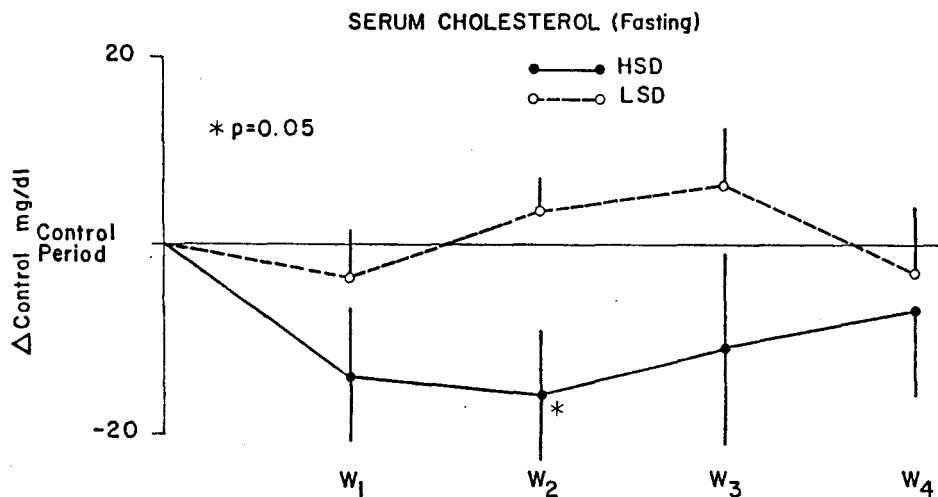


Figure 20: Fasting serum cholesterol changes from the control period values are observed for both groups of patients studied. Each point represents the mean and S.E.M. for all patients per group measured during each time sequence. Significance is expressed in the difference from the control period value.

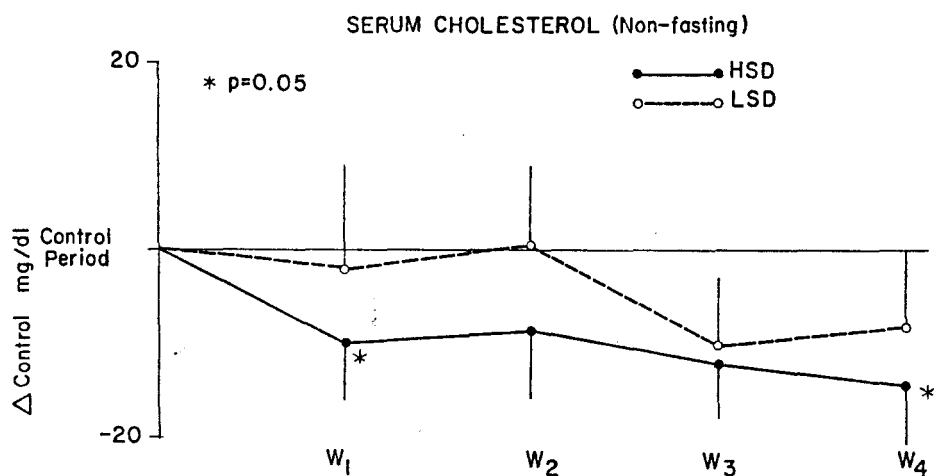


Figure 21: Non-fasting serum cholesterol levels expressed as changes from the control value are observed for both groups of patients. The description of the graphical relationships and points is identical to Figure 21.

value is representative of the average daily sucrose consumption in the American diet (11). In order to determine the effects of the control or intermediate diet on the patient population, two studies were done. All patients who were incorporated into the study were monitored during the control period. Fasting and postprandial blood sugars and triglyceride levels were used as measures of the dietary effect of 120 gm of sucrose on all patients. Patient values were monitored at day 0 (entrance into the study) and at day 10 of the control diet. Also, six additional patients who were incorporated into the study were kept on the control diet for two weeks and then maintained for an additional 4 weeks on the same intermediate or control diet as before. The results of these two studies are presented in Table 5 and Figure 22.

a. Blood Glucose and Triglyceride Values Compared During the Control Period

Table 5 shows the changes that occur in triglyceride and blood sugar levels in fasting and postprandial specimens from day 0 and day 10 of the control period. Fasting glucose decreased from admission values by an average of 18 mg/dl while postprandial serum glucose decreased by an average of 10 mg/dl. Fasting triglycerides remained fairly constant with a mean decrease of only 20 mg/dl from the admission values in all patients studied. Postprandial triglycerides showed a similar mean drop of 30 mg/dl from admission values.

TABLE 5

EFFECTS OF CONTROL DIET (120GM SUCROSE)  
ON PLASMA GLUCOSE AND TRIGLYCERIDE LEVELS  
(ALL SUBJECTS)

DAY 0 (ADMISSION)		DAY 10
FBS	211 $\pm$ 16	193 $\pm$ 14
4PM BS	218 $\pm$ 19	208 $\pm$ 19
FASTING TG	290 $\pm$ 31	270 $\pm$ 21
4PM TG	378 $\pm$ 53	358 $\pm$ 26

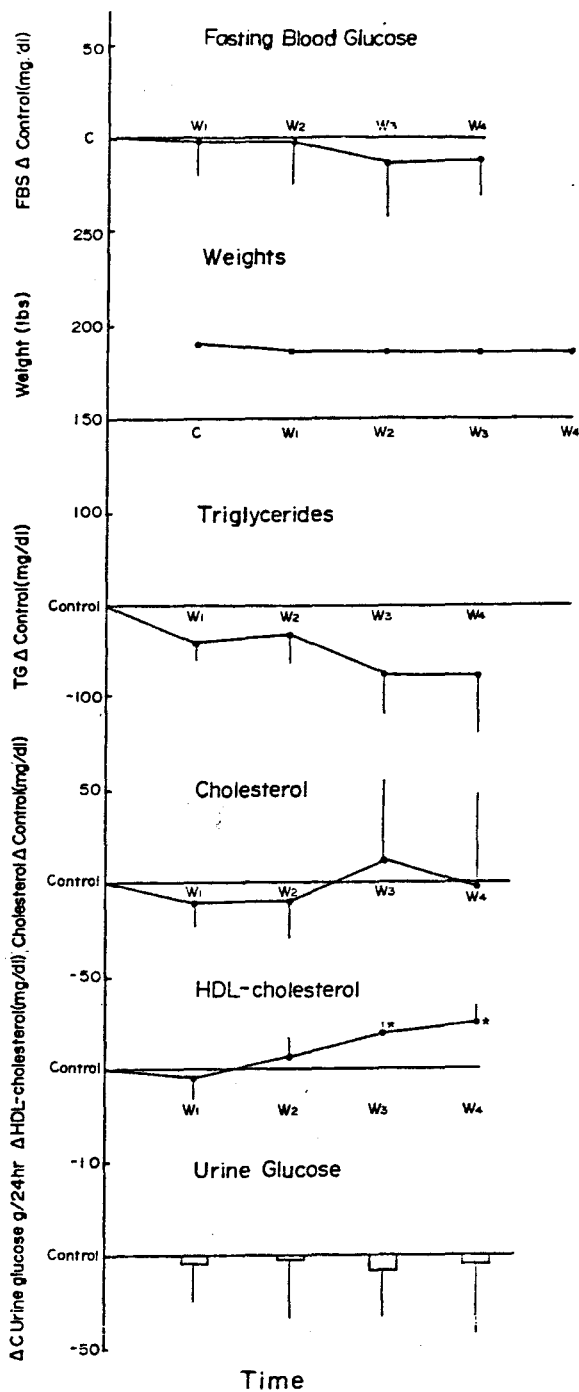
The effects of the control diet given to all subjects before they were randomly assigned to the study diets. All values are expressed as mean and S.E.M. for all patients (36) studied. Day 0 represents their fasting admission blood values and day 10 represents that number of days consuming the control diet.

b. Fasting Blood Glucose, Weights, Triglyceride, Cholesterol, HDL-Cholesterol and Urine Glucose Results Monitored in Control Subgroup

Results of basic chemistries obtained from a subgroup of patients on the control diet for four weeks are illustrated in Figure 22. All routines and protocols were identical to the study diets except no dietary changes were incurred after the two week control interval. Average weekly weights for this subgroup showed relatively little change from the control period weights. This demonstrates that weight loss and gain is not a contributing factor for the changes observed while consuming the control sucrose diet.

Fasting serum triglycerides for the control subgroup decreased when patients were administered the 120 gm sucrose diet for an additional 4 weeks after the initial 2 weeks. These results parallel those of the low sucrose subgroup which showed no change or a decrease in triglyceride values compared to control period values. Although a decrease was noted in the triglyceride concentration from the control during the four additional weeks on the intermediate diet it was not significant ( $p > 0.05$ ).

Mean cholesterol values showed no significant change from those of the initial two week control period when patients were given the intermediate diet for four extra weeks. In contrast, HDL-cholesterol rose significantly ( $p < 0.05$ ) with continuation of the control or intermediate diet. A mean rise of 5 mg/dl was observed at week four of the study portion of the diet following a steady rise from week 1. Again, these changes are comparable to those observed in patients who were



**Figure 22:** The effects of a prolonged intermediate (120g) sucrose control diet on a patient population subgroup. The composite graph represents some of the blood chemistry changes observed in 6 patients who continued the control diet after the initial 2 week period. All values, with the exception of urine glucose, were determined from fasting samples and were expressed as changes from the control period. Each point represents the mean and S.E.M. of all patients in this subgroup.

consuming the low sucrose diet during the study period.

As demonstrated in Figure 22, urine glucose decreased slightly. The decrease was non-significant when compared to control period values ( $p < 0.2$ ), but does reflect the general trend observed when the other two study diets were consumed. Fasting blood glucose in these control patients showed a drop after week two of the control period similar to high and low sucrose study groups. A mean drop of 15 mg/dl was observed during weeks 3 and 4 which was not significant ( $p > 0.1$ ).

Other values of interest were the insulin and glucagon values (data not shown) which did not change appreciably during the 4 weeks after the control period. These observations suggest that intermediate sucrose consumption seems to have little effect on lipid values in non-insulin dependent diabetic hypertriglyceridemic patients. Results with this group seem to reflect similar trends observed in individuals consuming sucrose restricted diets.

#### E. Serum Glucoregulatory Hormone Levels

The effect of high and low sucrose consumption on the glucoregulatory hormones is expressed in Figures 23-26 and Table 6. Insulin results are expressed as a weekly mean of all patients in each group during the control and study diet periods. Glucagon values and insulin/glucagon ratios were determined per group from one dietary period to another.

##### a. Fasting Serum and Postprandial Insulin Levels

Figure 23 illustrates the effect of sucrose consumption on serum



Fasting Insulin Concentrations  
High & Low Sucrose Diets

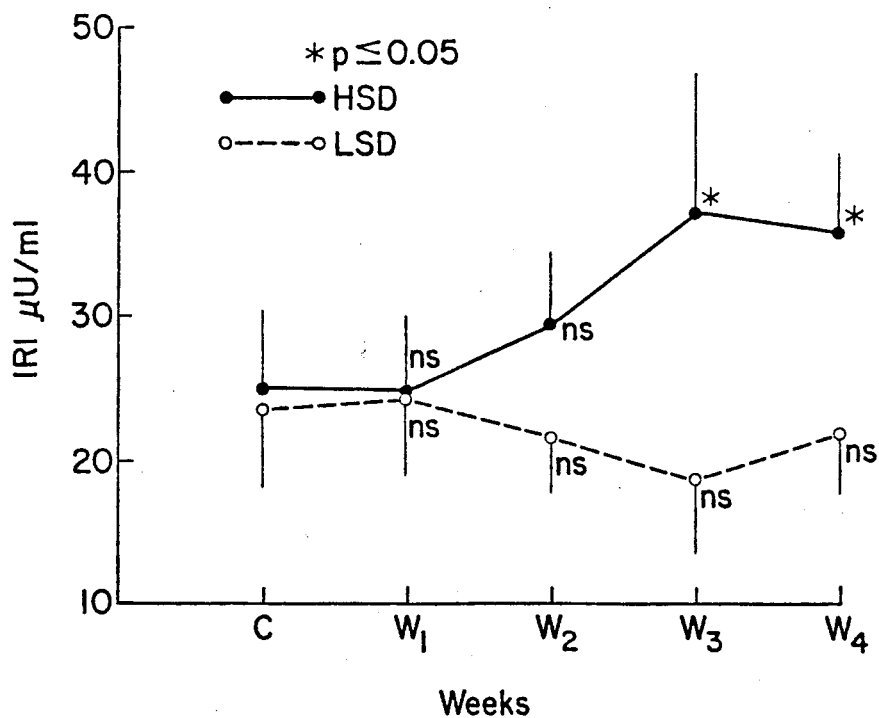


Figure 23: Fasting insulin concentrations observed during high and low sucrose consumption. Each point represents the mean and S.E.M. for all patients per group per time sequence studied. Significance is measured in difference from the control period value. Each patient's weekly sample is the average of 3 samplings throughout the week.

insulin following a twelve hour fast. The control values indicate that many non-insulin dependent hyperlipemic diabetics are also hyperinsulinemic (445). This patient population had a mean fasting insulin concentration of 23-25 uU/ml, higher than mean normal fasting insulin levels of 10-15 uU/ml. Patients consuming the low sucrose diet demonstrated a consistent decrease in I.R.I. (immunoreactive insulin) levels of 5 uU/ml below the control value of 23 uU/ml by the third week of the study. However, these values were insignificant compared to the effects of high sucrose consumption on fasting serum insulin. Patients on high sucrose diets after the control period showed increases in fasting serum insulin as high as 11 uU/ml over control levels by the third week of the study ( $p < 0.05$ ).

The effects of sucrose consumption on postprandial insulin levels are illustrated in Figure 24. Only weeks 2 and 4 of the study diet are illustrated along with the control period. Control period values in both groups were similar with mean insulin levels of approximately 50 uU/ml. A large highly significant increase was observed after two weeks of high sucrose consumption with mean values of 75 uU/ml after 2 weeks and 80 uU/ml after 4 weeks ( $p < 0.05$ ). Patients ingesting the low sucrose diet showed little change from control period insulin values with slight decreases of approximately 5 uU/ml observed during weeks 2 and 4 of the study diet period.

Figure 25 combines the results of both fasting and postprandial insulin values from the control period with those from weeks 2 and 4 of the study diets to demonstrate the concept of insulin increments. Increments in insulin concentration demonstrate the effect of diet on

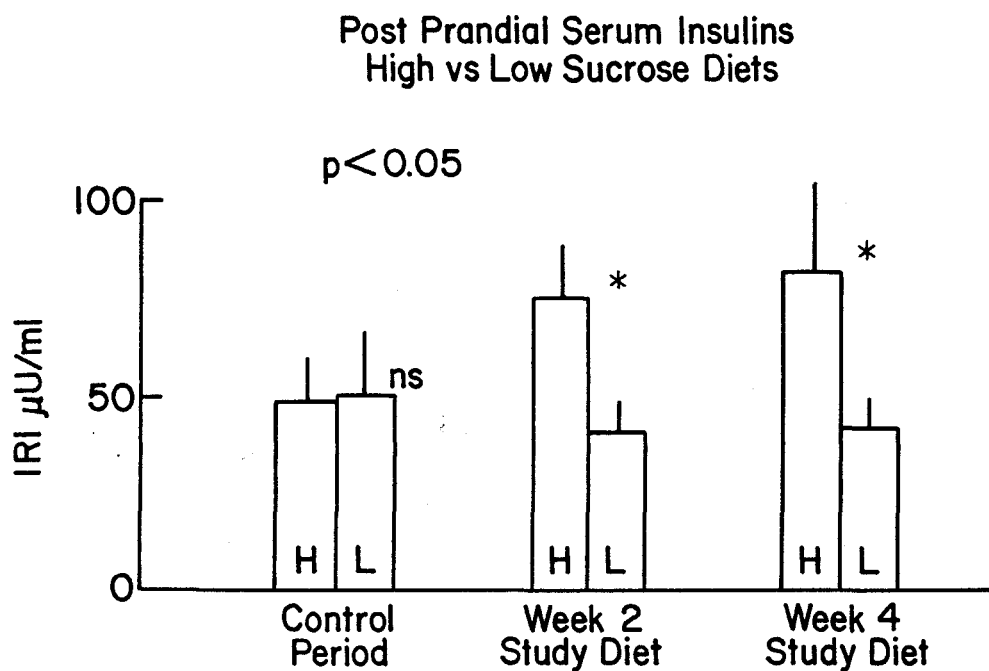


Figure 24: Postprandial serum insulin during the control period and 2 and 4 weeks of the study period. H represents the high sucrose diet, L represents the low sucrose diet. Significance is expressed as differences from the baseline on control period. All figures represent means and S.E.M. for all the patients measured per group.

### Fasting and Post Prandial Serum Insulins

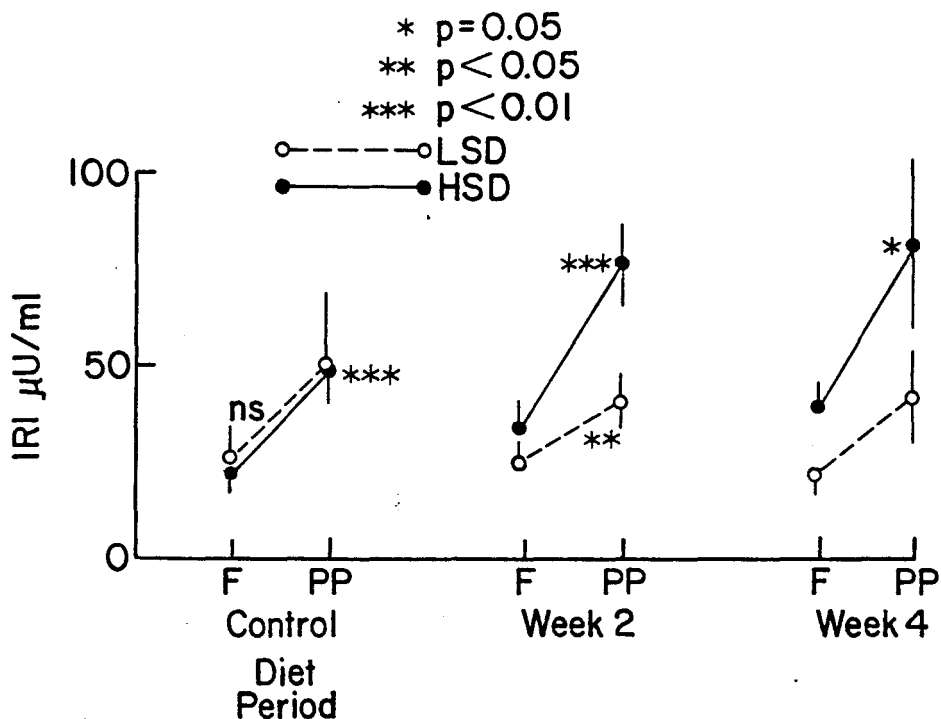


Figure 25: Fasting and postprandial serum insulin relationships during control period and week 2 and week 4 of the study diet period. F represents fasting and PP represents postprandial. Significance in this figure is determined by the difference between the fasting and postprandial insulins within each group. Points are determined by mean and S.E.M. of all patients per group per time period measured. All patients values are expressed as the mean of 3 values taken during the week of measurement.

insulin release. The significance obtained is the difference between fasting and postprandial levels of insulin in each group of patients during each study period measured. During the control period, both high and low sucrose study diet groups showed approximately a 24 uU/ml increase in insulin levels after a meal. During week 2 of the study diet, the patients consuming high sucrose showed a significant postprandial increase of almost 50 uU/ml ( $p < 0.01$ ) as compared to approximately 15 uU/ml for patients consuming low sucrose diets ( $p < 0.05$ ). This difference in incremental insulin response between high sucrose and low sucrose consumption was maintained through the fourth week of the study diets ( $p = 0.05$ ).

#### b. Fasting Glucagon Levels

Serum glucagon levels are shown in Figure 26. The results are expressed in mean concentration changes from control period values. The third week data is not included in this set because several of the third week samples were accidentally thawed and an incomplete number of results could only be obtained for the high sucrose group. This figure shows that fasting glucagon levels fluctuated from control values while consuming the low sucrose diets. But at 4 weeks, glucagon values were similar to control period levels. No substantial pattern of decrease or increase could be ascertained. Serum glucagon values from individuals on the high sucrose diet showed a significant decrease from control levels. Values dropped by 40 pg/ml during the first two weeks ( $p < 0.05$ ). These depressed levels were maintained with 4 week values approximately 25 pg/ml less than control diet values.

### c. Insulin/Glucagon Ratio, High and Low Sucrose Diets

Insulin and glucagon ratios were calculated for all patients during the control and fourth week of the study diet periods. The two study groups were then compared to their control diet values with the data summarized in Table 6.

I/G ratios increased by 30.2% from control values when patients consumed a diet rich in sucrose. This increase reflects the large rise in fasting insulin concentration and drop in glucagon concentration observed in this group. A decrease in I/G ratio was observed with the low sucrose diet group. This result reflects the smaller decrease in insulin concentration and little change in glucagon concentration observed in patients on sucrose restricted diets.

### F. Lipoprotein Levels During Study Diets

Blood was drawn twice weekly for lipoprotein electrophoresis during the control and study diet periods. Each group of patient results were averaged with mean values per week of study represented in Figure 27. All results were compared to control values and significance was determined as changes from control levels.

#### a. Effect of High and Low Sucrose Dietary Consumption on Lipoprotein Levels

Figure 27 shows circulating lipoprotein changes from control values while consuming sucrose enriched or sucrose depleted diets. All lipoprotein concentrations were expressed as percentage of total lipoprotein present. Patients who consumed low sucrose diets demonstrated

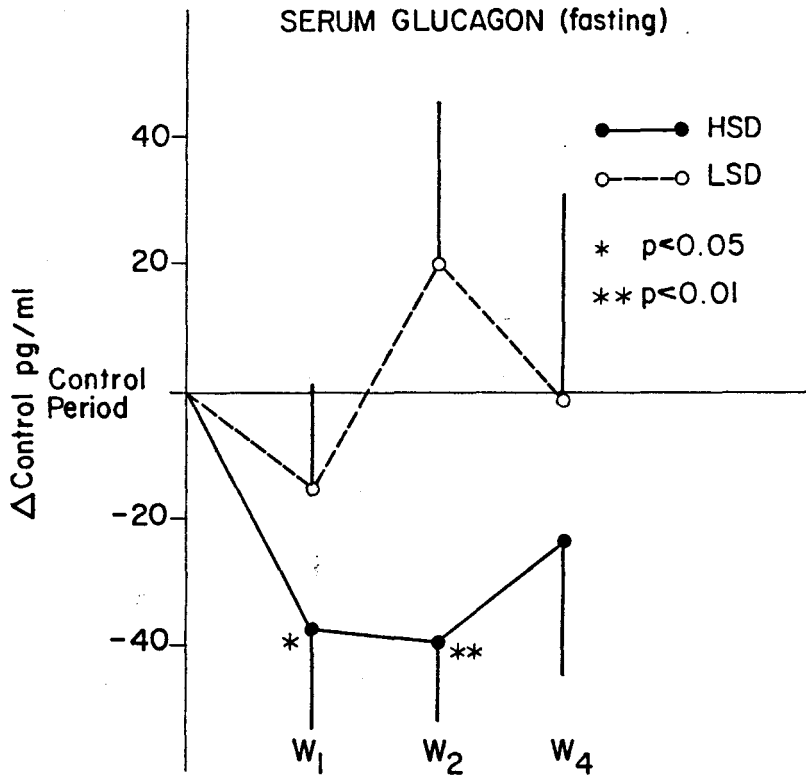


Figure 26: Fasting serum glucagon values expressed as changes from control period value. Each point represents the mean and S.E.M. of all patients per group per time period studied. Significance is expressed as the difference from the control period value for each group. The control period value is the average of the last 3 measurements during that period before patients were switched to a high or low sucrose diet.

TABLE 6

TABLE OF THE RATIO OF INSULIN OVER GLUCAGON

Experimental Situation	Change in I/G ratio due to diet		% Change in I/G
	From	To	
Control Period to High Sucrose Diet Period	5.1 ± 1.2	7.1 ± 1.0	↑ 39.2%
Control Period to Low Sucrose Diet Period	6.4 ± 1.3	5.5 ± 0.4	↓ 14.1%

Insulin to glucagon ratios were calculated from control diet period and week 4 of the study diet period values. The percent change expressed is that from the control value. The values are the means and S.E.M. of all patients from each group for the time period designated.



## Lipoprotein Distribution - High &amp; Low Sucrose

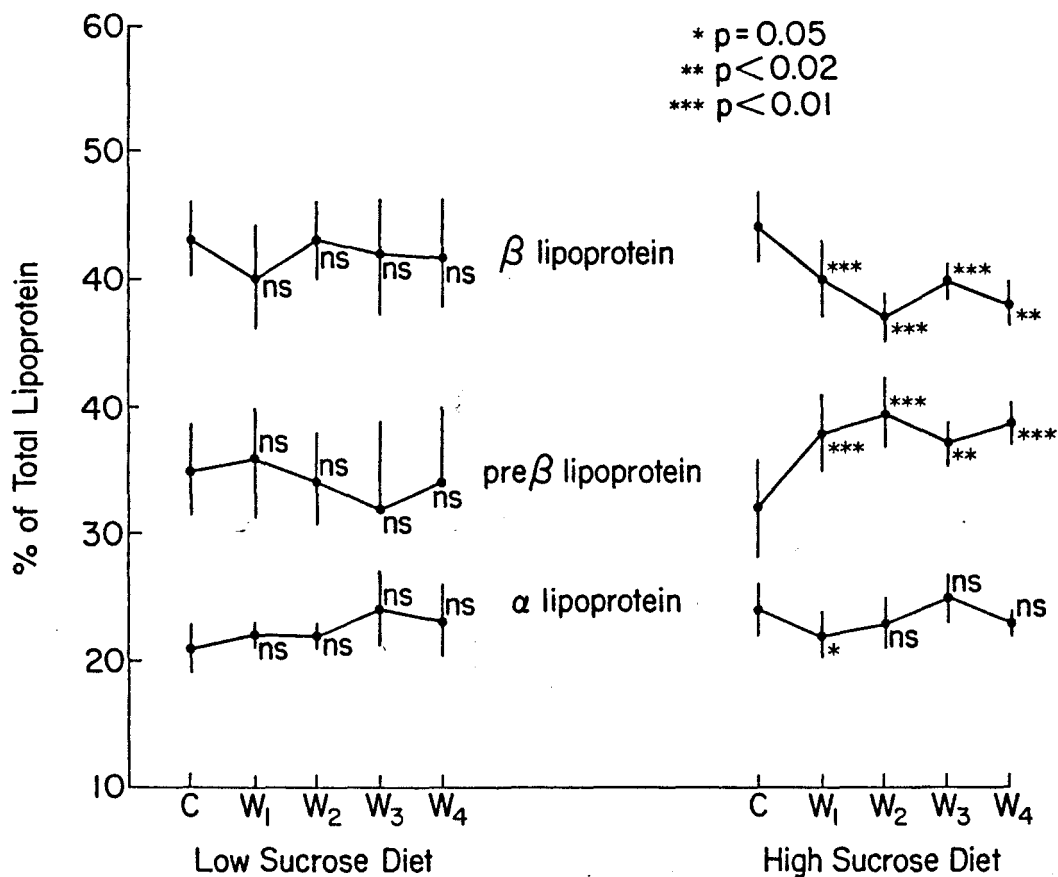


Figure 27: The percent of total lipoprotein present is expressed for  $\beta$ , pre  $\beta$  and  $\beta$  lipoprotein species. Each point represents the mean and S.E.M. of all patients per group per time period measured. All values are compared to the control period values (C) for each group. Significance is measured for each point with non-significant changes designated by (ns).

nonsignificant changes in  $\beta$ , pre  $\beta$  or  $\alpha$  lipoprotein percentages from control levels. The only lipoprotein fractions showing any change were pre  $\beta$  lipoproteins which dropped slightly, possibly reflecting the decreased fasting triglyceride concentration observed in low sucrose study group patients (Figure 17). An insignificant increase was also noted for  $\alpha$  lipoprotein which probably reflects the increase in HDL-cholesterol observed with consumption of low sucrose diets (Figure 19).

High sucrose study group patients showed highly significant changes from control values in all three lipoprotein fractions. The  $\beta$  lipoprotein fraction showed a significant decrease of approximately 10% from control values ( $p < 0.01$ ). This percentage was maintained from the second week of the study diet to the end. This drop in fasting  $\beta$  lipoprotein levels most probably represents the decrease in fasting cholesterol observed while consuming high sucrose diets (Figure 21). Pre  $\beta$  lipoprotein levels increased significantly, 10% over control levels while consuming high sucrose diets ( $p < 0.01$ ). This increase is probably due to the fasting triglyceride increase observed in high sucrose patients (Figure 17). Finally, a decrease in  $\alpha$  lipoprotein percentage was observed after the first week ( $p = 0.05$ ) on high sucrose diets with a rise back to control values by week three. This slight decrease could be a reflection of the decrease in HDL-cholesterol observed with increased sucrose consumption (Figure 19). Not surprisingly, when diets with different sucrose content are consumed lipoprotein pattern changes seem to reflect, in general, blood lipid alterations.

## G. Triglyceride Turnover

A commonly used method to estimate triglyceride production is based on the two assumptions that the decline in radioactivity of plasma VLDL triglycerides after pulse injection of a precursor into this pool is essentially monoexponential and the calculated rate constant obtained provides a valid measurement of the turnover rate of plasma triglycerides (419). Radioactive labeling of the triglyceride precursor glycerol is generally preferred to free fatty acid labeling because less recycling of glycerol occurs after pulse injection and a greater monoexponentiality in the decay curve is attained. This period is presumed to reflect less glycerol returning to the liver from hepatic triglyceride catabolism (recycling). Figures 28 and 29 represent single patient decay curves where linear regression analysis was used to derive patient's turnover results. Figures 30 and 31 are graphs depicting the fractional turnover rates (K values) of the patient population plotted against triglyceride concentration. Table 7 demonstrates changes in total turnover rate relative to the respective study diet. Figures 32 and 33 illustrate total turnover rates plotted against triglyceride concentration.

### a. Typical Patient Curve

Figure 28 illustrates the plasma radioactivity plotted semilogarithmically against time for one study patient. Two turnovers were performed, the first at the end of the control diet period and the second after two weeks on either the high or low sucrose study diet. A decay curve of the methanol fraction containing polar constituents is

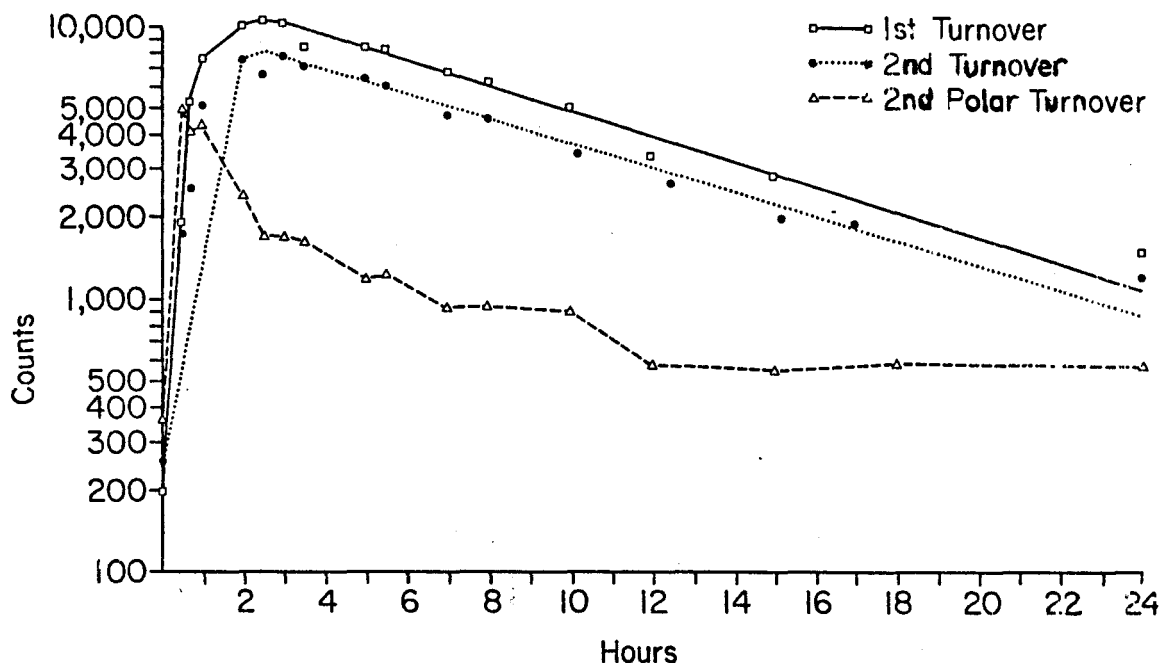


Figure 28: Triglyceride radioactivity curves after intravenous injection of tritiated glycerol in one of the patients who participated in triglyceride turnover studies. The open squares represent the turnover data collected immediately after the control diet period was completed. The closed circles represent the curve generated after two weeks on the study diet. The open triangles represent the counts found in the polar methanol wash after Folsch extraction procedures for nonpolar lipids were performed from the sample taken during the second turnover procedure. This fraction represents free glycerol, labeled free fatty acids and all other labeled material other than nonpolar lipid.

shown, representing free glycerol and glycerol that has been used to manufacture free fatty acids. Removal of the labeled triglyceride from this patient's plasma pool was monoexponential for approximately 17 hours. Figure 28 was not uniformly indicative of all the patients studied. In some instances, the monoexponential component only occurred between 1-12 hours. With these patients, a slower decay with a decreased slope was found to occur 12 hours after the test was initiated. This slow decay section of the curve could occur from recycling of the glycerol, from the plasma compartment, into new triglyceride or it could represent another slow synthetic triglyceride pool. In these cases, the portion of the curve with the greatest slope was used to calculate fractional turnover rates. A linear regression analysis was performed on this section of the curve and this line was extrapolated back to zero since this Y intercept value is considered the maximum radioactive incorporation of label into the plasma triglyceride pool. The patient represented in Figure 29 is given two pulse injections of glycerol, one at the end of the control diet and one two weeks after the initiation of the study diet. The Y intercept of 12, 115 disintegration in 20 minutes is obtained after the first pulse injection and the intercept at 95% disintegrations in 20 minutes is obtained after the second injection two weeks later.

#### b. Calculation of K Value Turnover

K values for this patient were determined by estimating the decay half life ( $T_{1/2}$ ) from the most linear part of the decay curve. This patient had  $T_{1/2}$  values of 8.5 hours and 7 hours for the first turnover

## RADIOACTIVITY vs. TIME

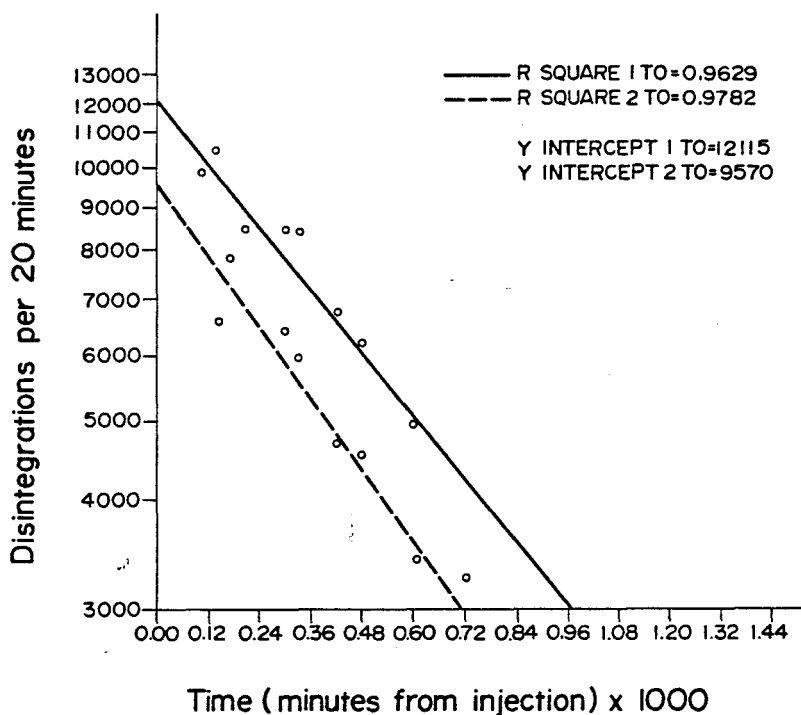


Figure 29: Representative plots of first and second turnover determinations in a patient demonstrating a biphasic decay. The slow decay component was dropped from the curve and linear regression analysis was performed on the rapid decay portion. The best fit lines are extrapolated back to the Y intercept, the value considered to be the maximum incorporation of labeled glycerol into the plasma triglyceride pool.  $T_{1/2}$  values are calculated directly from these figures by observing the time in which half of the total counts are lost.

and second turnover procedure respectively. The K values are calculated from the relationship

$$K = \frac{0.693}{T_{1/2}}$$

and for this particular patient were  $0.081 \text{ hr}^{-1}$  and  $0.099 \text{ hr}^{-1}$  for the first and second turnover procedures respectively.

c. Fractional Turnovers as Plotted Against Triglyceride Concentration

The fractional turnover rates (K value) obtained after consumption of the control diet from all patients studied were plotted against serum triglyceride values obtained during this same time period (Figure 30). A significant relationship between fractional turnover and triglyceride concentration was found with a coefficient of correlation of 0.812 and a significance of ( $p < 0.001$ ). It should be emphasized that as fractional turnover rate (K) decreases, triglyceride concentration increases. This inverse relationship was seen in all patients studied.

d. Plasma Total Turnover for Triglyceride Removal

Total plasma VLDL triglyceride turnover rates were calculated from the fractional turnover rate and the plasma VLDL triglyceride concentration using the following equation:

$$\text{triglyceride turnover} = \text{plasma triglycerides (mg/dl)} \times \text{plasma volume (dl/kg)} \times \text{fractional turnover rate (hr}^{-1}\text{)}$$

The relative plasma volume for this population was considered to be  $0.45 \text{ dl/kg}$  (57). This assumption has been validated by Adams and

## FRACTIONAL TURNOVER vs. TRIGLYCERIDE CONCENTRATION

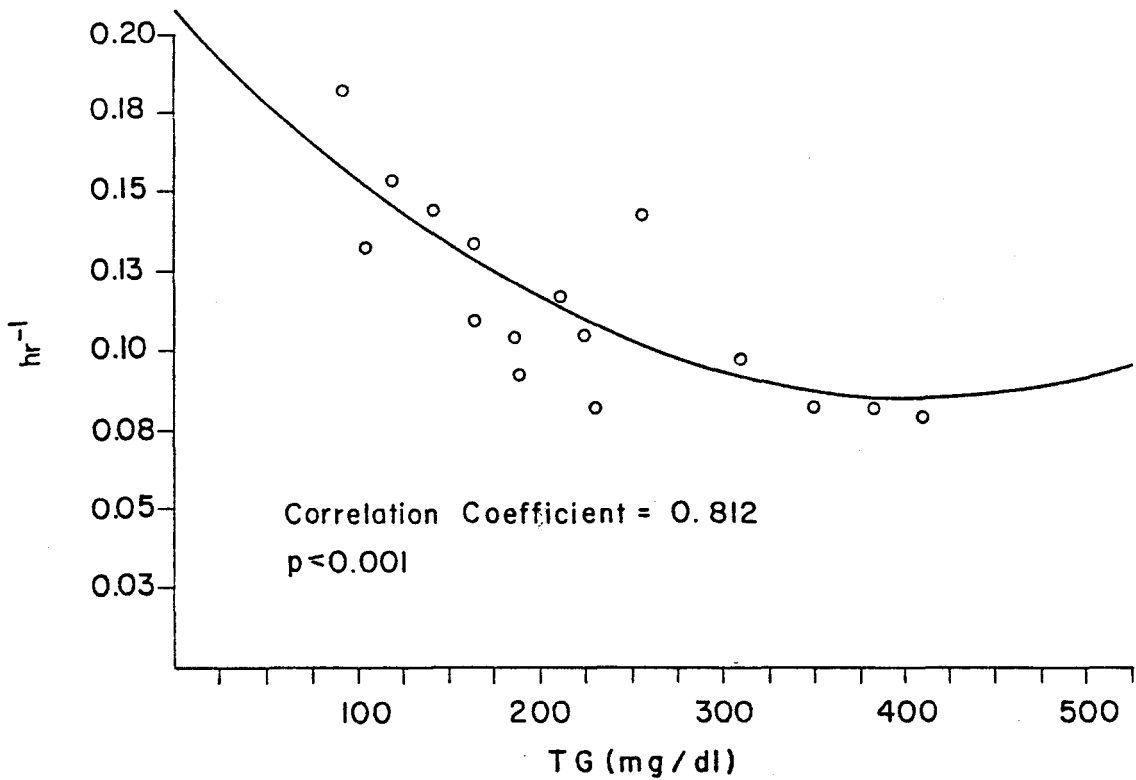


Figure 30: Fractional turnover rates, obtained from decay curves generated after the control diet period was completed, were plotted against triglyceride values in 19 patients. These control fractional turnovers were used to construct a line which was considered to be representative of the patient population studied. This control line was used to assess changes in the relationship between fractional turnover and triglyceride concentration due to diet.



kissebah (59). Changes in total triglyceride turnover when patients are switched from the control diet to the study diet (high and low sucrose) are illustrated in Table 7. The values listed in the "from" column are control diet total turnovers calculated from the patients who were randomized to either high or low sucrose diets. The 8.0 mg/kg/hr represents the mean of the control total turnover values from 10 patients before they were switched to a high sucrose diet. The total turnover value of 9.8 mg/kg/hr represents the mean value of total triglyceride turnover before nine other patients were switched from the control diet to the low sucrose study diet. When patients consumed high sucrose diets after control diets, their mean total turnover values increased significantly from a mean value of 8.0 mg/kg/hr to 10.8 mg/kg/hr ( $p < 0.01$ ). Low sucrose study patients showed the opposite effect with a decrease in total turnover values from 9.8 mg/kg/hr to 9.2 mg/kg/hr (not significant  $p = 0.2$ ).

e. Plasma Total Turnover Rates Versus Triglyceride Concentration

Figure 31 and Figure 32 demonstrate the relationship between total triglyceride turnover, which represents total body pool triglyceride turnover and triglyceride concentration. It has been suggested that the hyperbolic curve relating triglyceride concentration to triglyceride turnover is analogous to the relationship between substrate concentration and reaction velocity described by the Michaelis Menton equation (418). Figure 31 illustrates the patients who were

TABLE 7

TURNOVER RATES FROM  
CONTROL PERIOD TO STUDY PERIOD

GROUP	TOTAL TURNOVER VALUES mg/kg-hr		SIGNIFICANCE
	From	To	
High Sucrose Diet	8.0 ± 0.8	10.8 ± 1.0	p < 0.01
Low Sucrose Diet	9.8 ± 1.2	9.2 ± 0.9	NS

Changes in total turnover values from control dietary period to study diet. The significance was determined by paired t analysis which compared control values with study diet turnover values for each individual patient.

studied while consuming the control diet. Their control total turnover values were plotted against their triglyceride concentration during the same period. The results indicate that higher plasma triglyceride concentrations are generally associated with increases in triglyceride turnover ( $r = 0.851$ ,  $p < 0.001$ ). It can also be seen that triglyceride concentration and turnover rates are not linearly related throughout the entire range of triglyceride concentration. As total turnover rates exceed 12 mg/kg/hr the triglyceride concentrations increase much more rapidly.

The shape of the curve suggests that the removal rate for plasma triglycerides follow the kinetics of a saturable enzyme system. The  $V_{max}$  calculated from this curve is approximately 12.5 mg/kg/hr for this patient population. In normal adult populations the  $V_{max}$  for the enzyme system was found to be 30 mg/kg/hr (402). Therefore, this patient population demonstrates depressed total turnover rates relative to normal population studies. The comparison of data from HSD and LSD to the normal line represents the metabolic changes in the triglyceride removal system that occur when patients are switched from the control diet to either study diet. The patients who consumed the high sucrose diet demonstrated no change or a slight increase from control values. Patients consuming the low sucrose diet showed little change from control values when their points were plotted against the line generated during the control diet (Figure 32).

Finally, Figures 33 and 34 demonstrate the change from control values that occur in total turnovers when the study diets were consumed. These results were obtained after body weight correction fac-

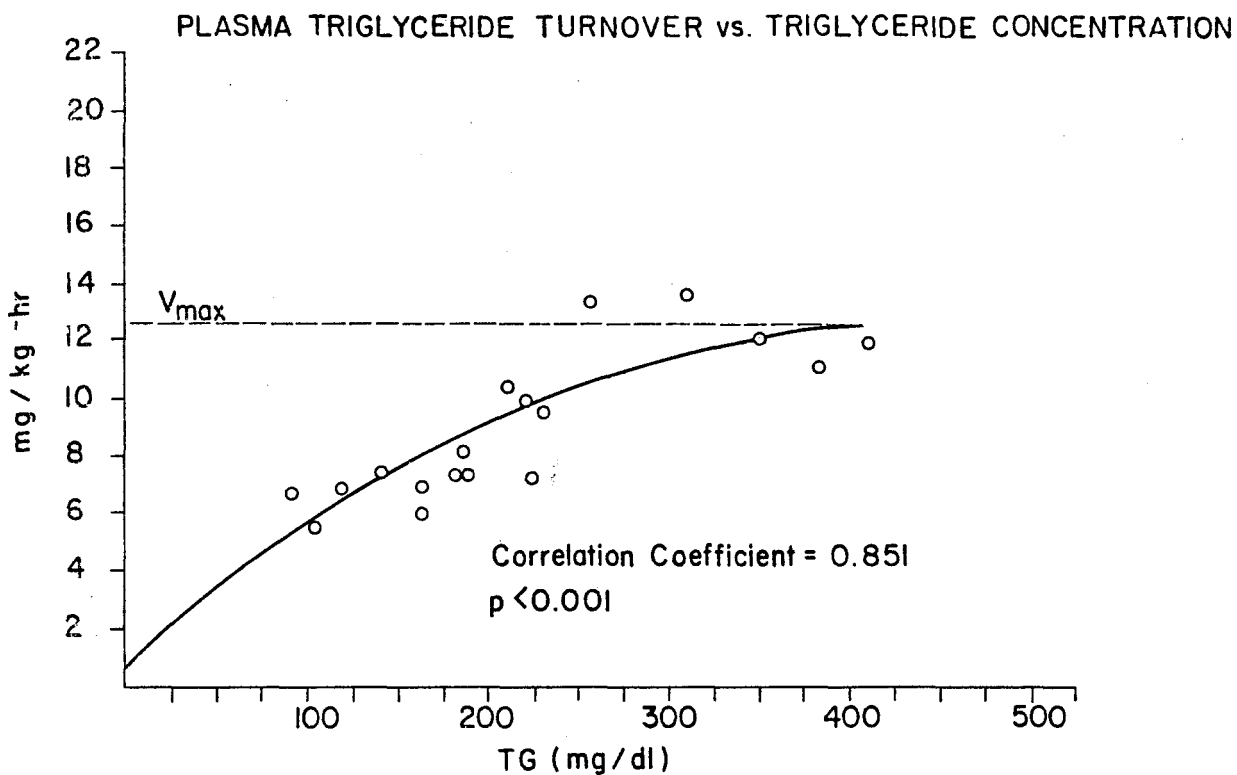


Figure 31: The relationship between plasma triglyceride total turnover and plasma triglyceride concentration in 19 patients after the two week control dietary period was completed. The line generated represents this patient population consuming a diet of average sucrose content.  $V_{max}$  was determined by the point at which the system demonstrated zero order kinetics and was found to be 12.5 mg/kg/hr.

tors were determined. Total turnover rates (T<sub>0</sub>) are increased when body weight factors are included. One observation predominates, patients who consumed the high sucrose diet demonstrated a slight increase in turnover rate with large TG increases (Figure 33). The small T<sub>0</sub>/TG ratio may suggest a removal defect predominates in the grossly lipemic individual (TG >200). The low sucrose study group predominantly showed the opposite effect whenever the changes in turnover rate exceeded 3 mg/kg/hr possibly suggesting synthesis stimulates removal (Figure 34).

#### H. Triglyceride Turnover Kinetic Evaluation of System

##### a. Linear Transform of Michaelis Menton System

Even though an approximation of V<sub>max</sub> is obtained by plotting total turnover versus triglyceride concentration (Figure 31). V<sub>max</sub> and K<sub>m</sub> values can be calculated more accurately from a linear transformation of the Michaelis-Menton equation. By this process a plot of [S /V] versus [S] is produced (Figure 35). Data reduction in the format of triglyceride concentration/turnover ( S/V) versus triglyceride concentration ([S]) was performed separately for patients consuming high sucrose, low sucrose, and control diets. The control group data is generated from all patients who consumed the control diet (n=19) before being switched to either the high sucrose (n=10) or the low sucrose diet (n=9). The three lines generated intercept the Y axis at approximately the same point. The X intercept of the line generated from patients consuming the control diet was found not to be statistically different from the X intercept of the lines obtained after con-

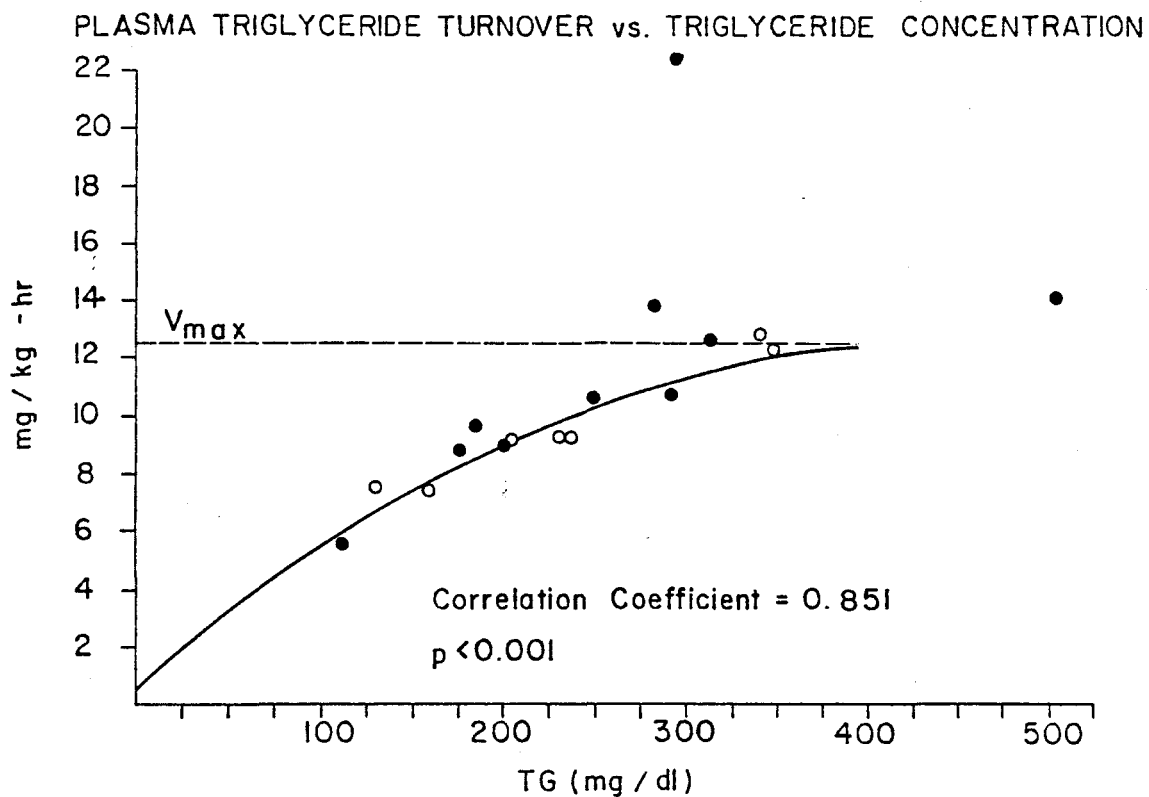


Figure 32: The relationship between plasma triglyceride total turnover and plasma triglyceride concentration in 10 patients consuming high sucrose diets (black circles) and 9 patients consuming low sucrose diets (open circles). The line and  $V_{max}$  shown are from Figure 31. The points demonstrate the changes that occurred in this relationship due to dietary manipulation.

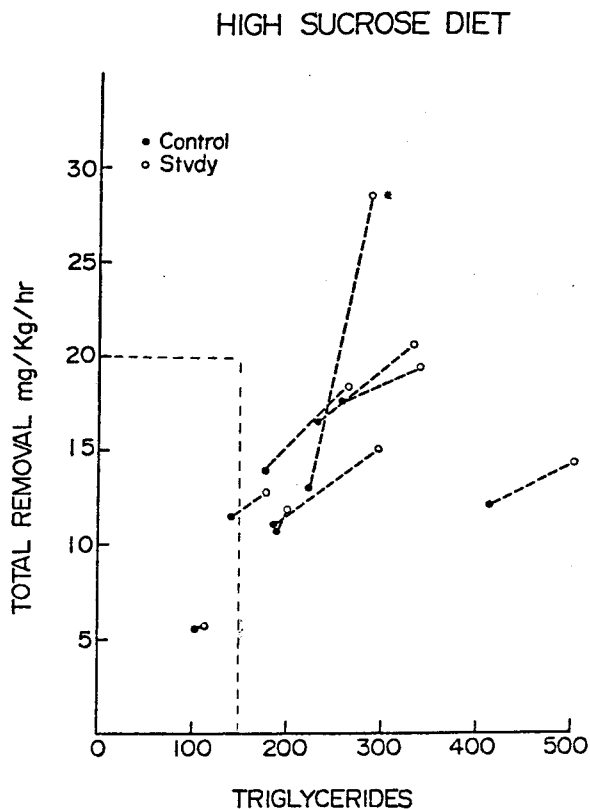


Figure 33: Plot of total turnover vs. triglyceride concentration in patients who consumed the high sucrose diet. Each line represents one patient whose control and study diet values are compared. Most lipemic patients demonstrated large increases in triglyceride concentration with small increases in total turnover with the exception of one patient who demonstrated the opposite relationship in this overall effect (\*). These results suggest a possible removal defect. The area designated by the box represents the normal patient response area (54).

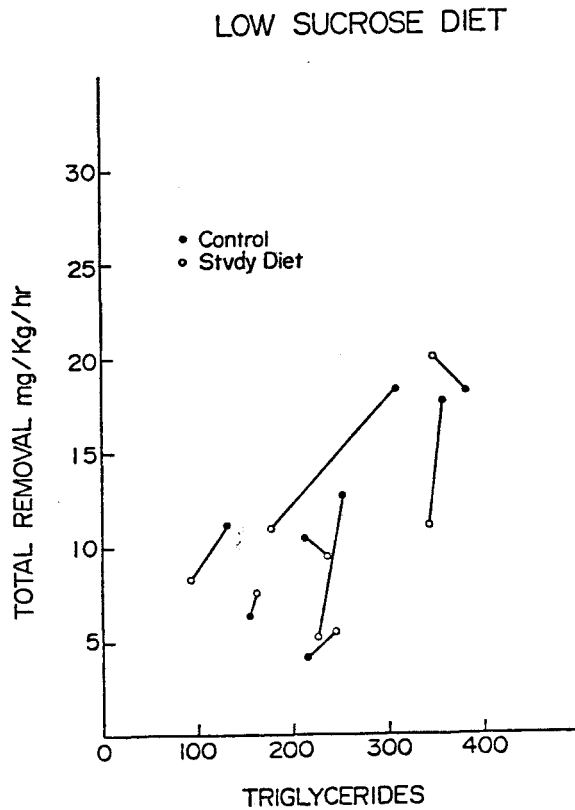


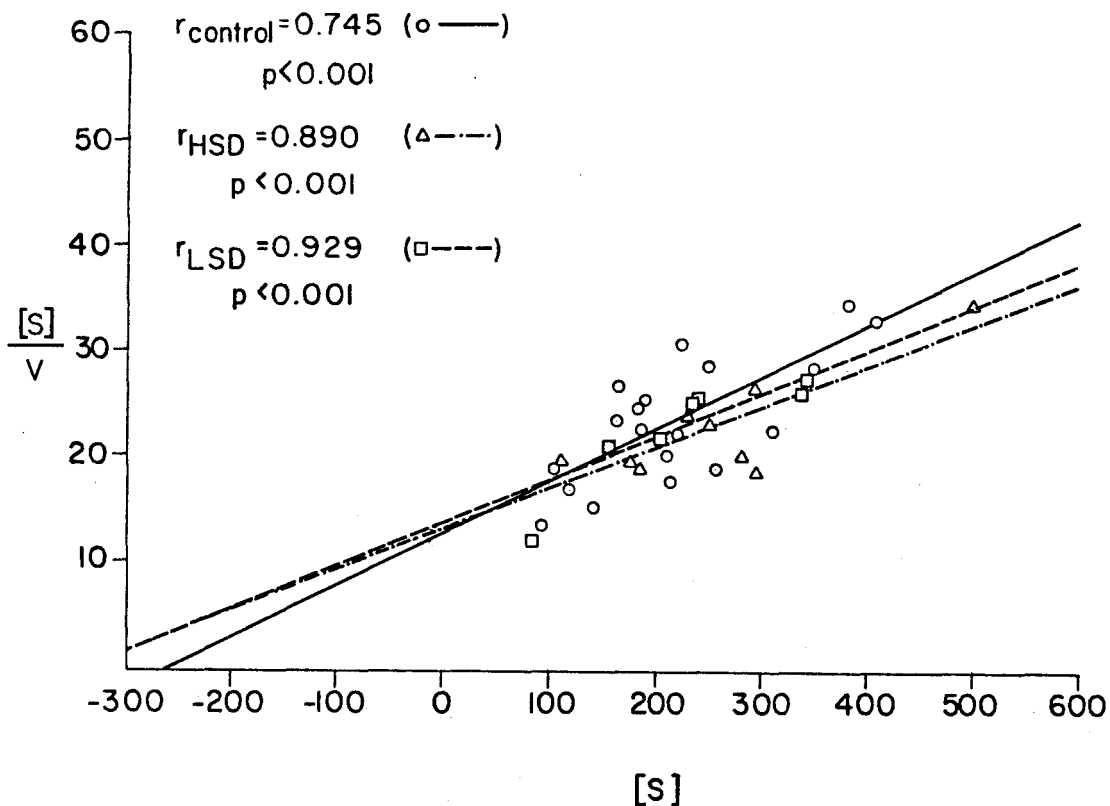
Figure 34: Plot of total turnover vs. triglyceride concentration in selected patients who consumed the low sucrose diet. Each line represents one patient whose control and study diet values are compared. Note the opposite trend in results as compared to Figure 33.



sumption of the study diets (high and low sucrose). Since the X intercept represents the triglyceride concentration at  $1/2 V_{max}$  ( $K_m$ ), this suggests the  $K_m$  for the patients consuming high or low sucrose diets remained unchanged from control  $K_m$  values. Linear regression analysis for all groups demonstrated a highly significant correlation of  $r=0.745$ ,  $p < 0.001$  for the control group,  $r=0.890$ ,  $p < 0.001$  for the high sucrose group and  $r=0.929$  with a  $p < 0.001$  for the low sucrose study group.

Figures 36 and 37 represent the results obtained from each group after consumption of the control diet compared with the results of the same study group after consumption of the study diet (high or low sucrose). In these plots,  $V_{max}$  equals  $1/\text{slope}$  and  $K_m$  equals the X intercept or the product of the vertical y intercept and  $V_{max}$ . The slope of the control line in Figure 36 is  $0.052 \pm 1.5 \times 10^{-2}$ . Therefore, the  $V_{max}$  for the high sucrose group consuming the control diet is  $19.3 \pm 5.76$  mg/kg/hr. Though this  $V_{max}$  value is higher than control group  $V_{max}$  from Figure 31 it still reflects a lower than normal  $V_{max}$  for the enzyme system. The higher  $V_{max}$  calculated above reflects a more accurate measurement of  $V_{max}$  done by this linear method of analysis. The  $K_m$  obtained for the HSD obtained group consuming the control diet is approximately  $260.7 \pm 101.3$  mg which is the product of the vertical intercept of the line ( $13.48 \pm 3.36$ ) times the  $V_{max}$  ( $19.3 \pm 5.76$  mg/kg/hr).

Consumption of a sucrose enriched diet after the control diet altered  $[S]$  and  $S/V$  values so that linear regression analysis produced a new line with a slope of  $0.039 \pm 6.5 \times 10^{-3}$  and a vertical y intercept of  $13.17 \pm 2.13$ . The  $V_{max}$  calculated for the triglyceride trans-



**Figure 35:** The relationship between  $[S]$  and  $S/V$ , where  $[S]$  is the serum triglyceride concentration (mg/dl) and  $V$  is the triglyceride turnover rate in mg/kg/hr, produces a linear transform of the turnover system. The solid line with open circles represents all 19 patients whose turnovers were performed immediately after the control dietary period had ended. The line with the open triangles and line dot configuration was generated from the 10 patients who consumed high sucrose and the open square dashed line represents the 9 patients who consumed the low sucrose diet. Both high and low sucrose diet turnover studies were performed after a two week interval in which individuals consumed the appropriate study diet. The control turnovers were done in the same patients after a two week interval while patients consumed the control diet of 120 gms of sucrose per day.

port system was determined to be  $25.6 \pm 4.3$  mg/kg/hr and the  $K_m$  (calculated as above) was  $337.6 \pm 78.4$  mg. Though slightly higher than control values these differences were found to be not statistically significant using two tailed t analysis.

Figure 37 shows the comparison of the triglyceride transport data obtained from the low sucrose study group after control and sucrose restricted diets were consumed. The line generated from the turnover data after consumption of the control diet had a slope of  $5.5 \times 10^{-2} \pm 1.54 \times 10^{-2}$  with a y intercept of  $9.36 \pm 4.16$ . The  $V_{max}$  obtained from the reciprocal of the slope was found to be  $18.2 \pm 5.1$  and the  $K_m$  for this group was determined to be  $170.2 \pm 89$  mg.

Consumption of the sucrose depleted diet after the control diet produced a new line with a slope of  $0.041 \pm 7.3 \times 10^{-3}$  and a y intercept of  $13.71 \pm 1.8$ . The  $V_{max}$  was  $24.3 \pm 4.3$  and the  $K_m$  of this group was determined to be  $332.8 \pm 73.4$ . Again no significant difference in  $K_m$  and  $V_{max}$  values could be obtained when the kinetic values from the control and low sucrose period were compared.

The results above indicate that the  $V_{max}$  for both study groups remained relatively unchanged from the  $V_{max}$  obtained during the control period. Both groups demonstrated no change in  $K_m$  values from the control group when two tailed t analysis was used. The lack of change in  $K_m$  values when the high sucrose diet was consumed demonstrates an unresponsiveness of the removal system to increased triglyceride which is released into the plasma pool after synthesis in the liver.

## I. Polyacrylamide Gel Electrophoresis and Isoelectric Focusing of VLDL Apoproteins

### a. Pattern of apo VLDL on SDS Polyacrylamide Gels

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was performed on samples which were obtained from each patient during both control and study diet periods. These assays were performed to assess the changes in apoprotein composition which occurred from dietary changes during the study. A typical pattern from several of the patients samples is shown in Figure 38. These patients have a large CIII<sub>1</sub> band which is always prevalent in hyperlipemic individuals. The CII band is observed as a thin, sharp band which migrates to a position above the CIII<sub>1</sub> band. The E band is seen as a large diffuse band which migrates a short distance into the gel. The B apoprotein, because of its large size and molecular weight, does not penetrate into the gel but remains as a large band between the interface of the stacking gel and separating gel. The CIII<sub>2</sub> band migrates furthest and is observed as a band occupying a position slightly below the large CIII<sub>1</sub> band. A typical densitometric pattern usually observed from these VLDL samples is diagrammed in Figure 39. Reading this pattern from left to right corresponds to the bottom and the top of the gel respectively. Again the typical apoprotein pattern of a hyperlipemic individual is observed. All apoprotein fractions from the VLDL sample are expressed as the percent of total soluble apoprotein present.

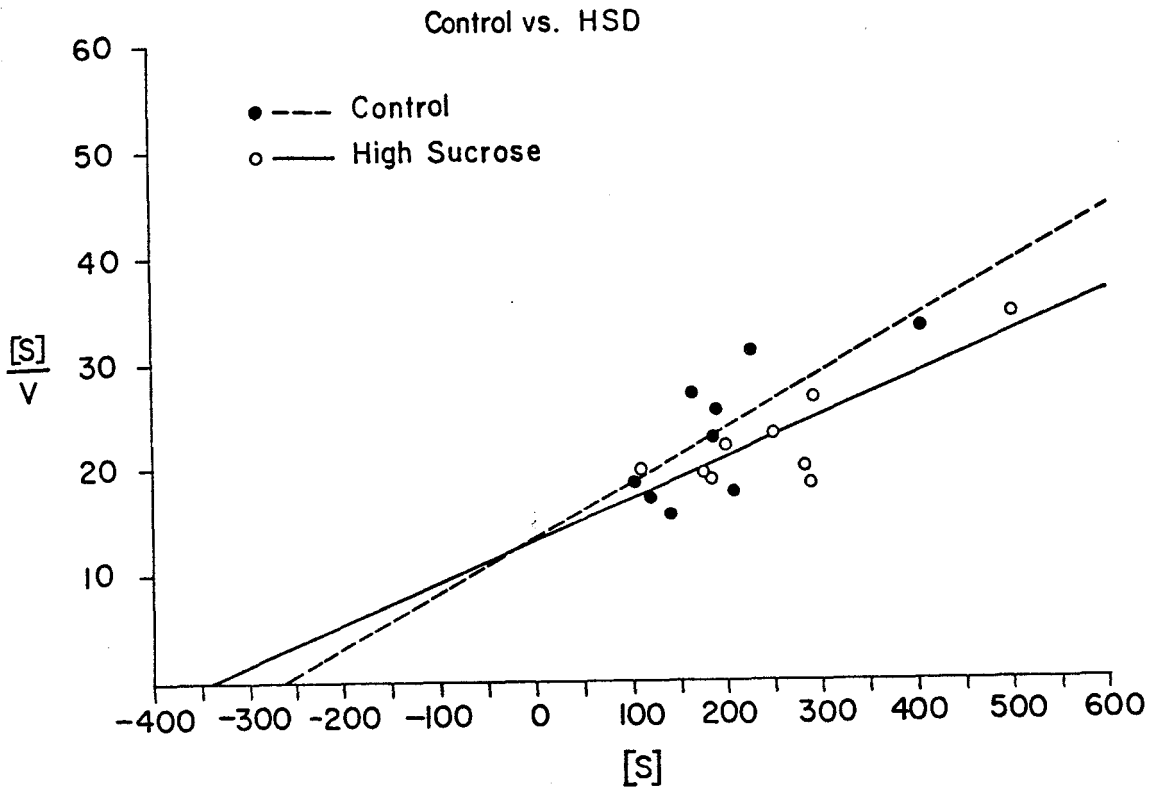


Figure 36: Plot of triglyceride concentration/turnover ( $[S]/V$ ) versus triglyceride concentration ( $[S]$ ) done separately for the group of patients who were randomized to high sucrose diets after control sucrose diets were consumed. Ten patients were studied in this group.  $r$  value for control equals 0.685 with a  $p < 0.01$ . The  $r$  value for the line generated after high sucrose consumption equals 0.890 with a  $p < 0.001$ .

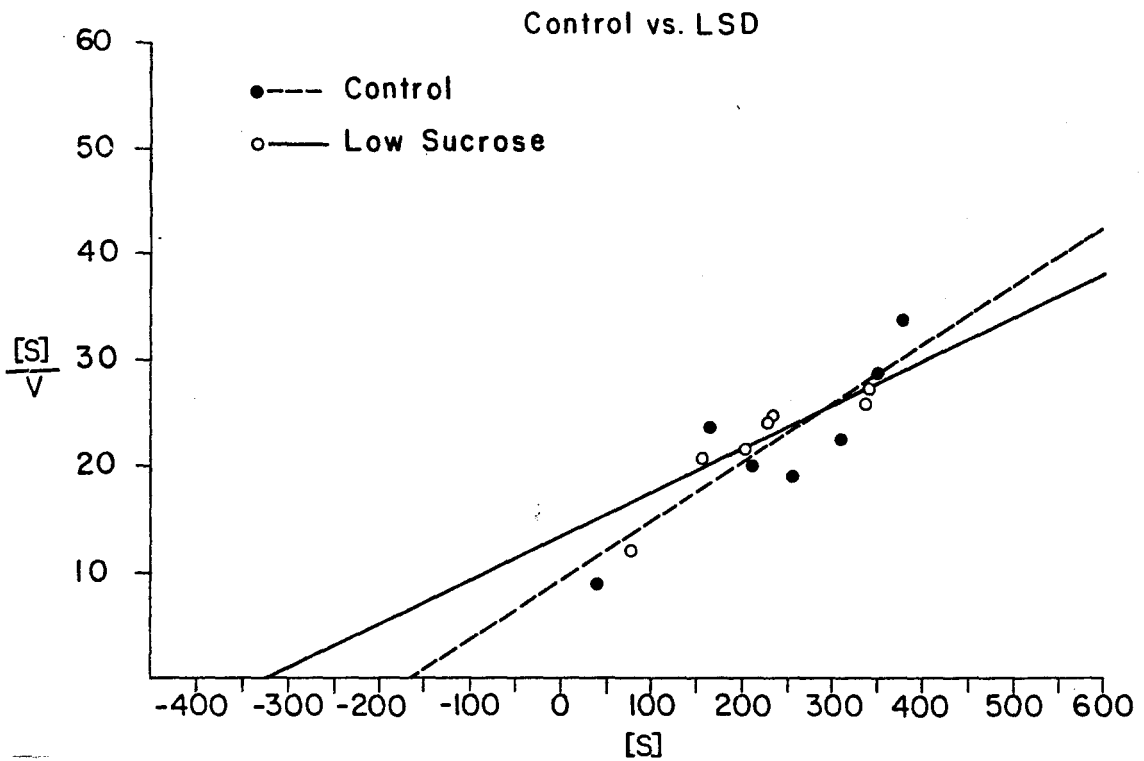
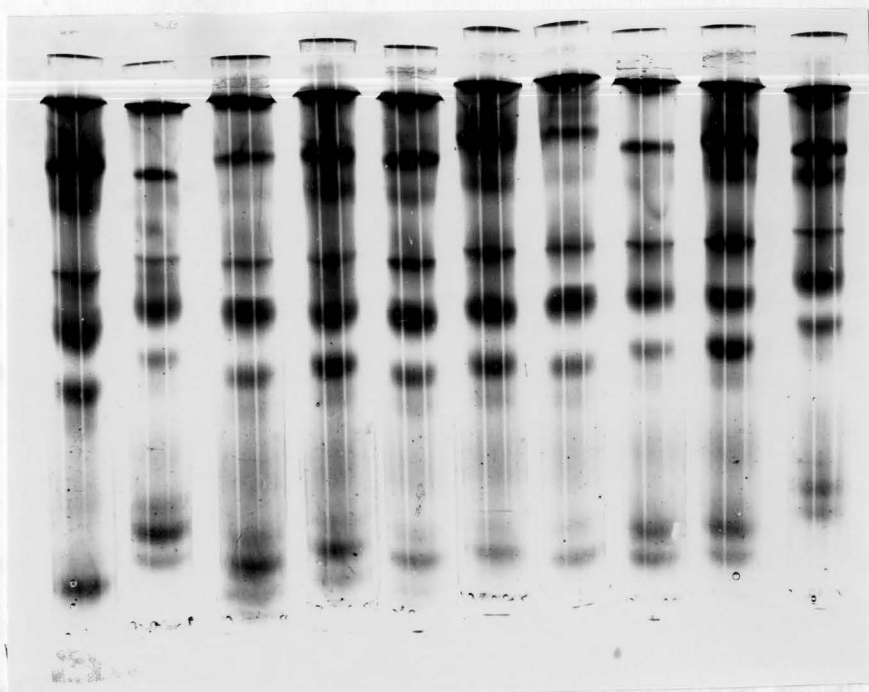


Figure 37: Plot of triglyceride concentration/turnover ( $[S]/V$ ) versus triglyceride concentration ( $[S]$ ) done separately for the group of patients who were randomized to low sucrose diets after control sucrose diets were consumed. Nine patients were studied in the group. The  $r$  value for the control equals 0.651 with a  $p < 0.01$  and the  $r$  value for the line generated after the low sucrose diet was consumed equals 0.929 with a  $p < 0.001$ .

### 5. Soluble Apoprotein Percentages Observed in This Patient

#### Population

Data from 20 study patients listing the composition of the apo-



---E

---CII

---CIII<sub>1</sub>

---CIII<sub>2</sub>

**Figure 38:** SDS polyacrylamide gel electrophoresis of VLDL apoprotein derived from ten of the patients who participated in the study. Ten percent polyacrylamide separating gel with 2.5% stacking gels were used. Two hundred ug of protein was added to each gel and electrophoresed for approximately 8 hours at 2.5 mA per tube. Gels were stained with a solution containing 1% Amido Schwarz in acetic acid.

b. Soluble Apoprotein Percentages Observed in This Patient Population

Data from 20 study patients listing the composition of the apoprotein fractions present at the time of entry into the study is seen in Table 8. The mean values of these fractions can be compared with a table of apoprotein fractions from normal individuals in Table 9. It should be observed that the greatest differences between the two groups of patients is that hyperlipemic individuals demonstrate a large increase in the apo CIII<sub>1</sub> fraction and a noticeable decrease in the apo E component relative to the normal patient population.

c. Isoelectric Focusing Patterns Observed for Apo VLDL

Isoelectric focusing in 7.5% polyacrylamide gels through a pH gradient of 4-8 was performed on all samples obtained for VLDL apoprotein determination. The major advantage of isoelectric focusing on apoprotein samples is the separation of isoprotein subunits of the apo E fractions. Figure 40 is a print of isoelectric patterns from several of the patients who were incorporated into the study. The apo E fraction can be divided into 3 isoprotein subunits. The E<sub>1</sub> isoprotein band migrates roughly to the midpoint of the gel and has an isoelectric point of approximately 5.31. The band is diffuse and is composed of several minor subfractions. Apo EII migrates to approximately the same position in the gel, but is found slightly above apo E<sub>1</sub>. Its isoelectric pH is 5.39. Apo EIII, the heaviest staining band, migrates the shortest distance into the gel with an isoelectric point of 5.44. The densitometric pattern of one of these gels is shown in Figure 41. Apo C proteins are the most acidic and migrate to an isoelectric point



## PAGE PATTERN VLDL

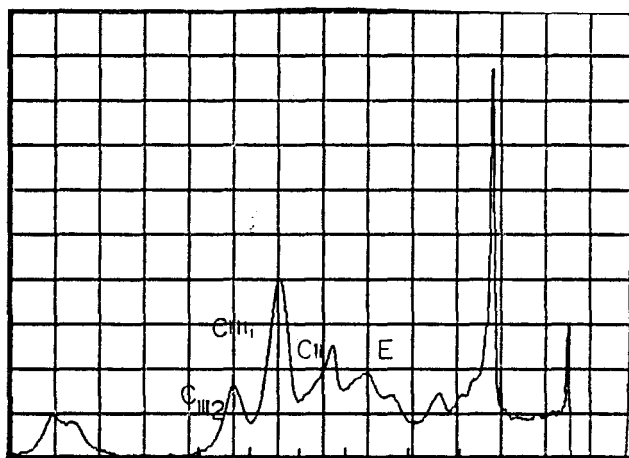


Figure 39: Typical densitometric scan of SDS polyacrylamide gel electrophoresis of VLDL apoprotein derived from hypertriglyceridemic patient. A Gelman, Model #ACD-18 was used to produce the scan represented.

TABLE 8

SOLUBLE APOPROTEINS AS PERCENT OF  
TOTAL SOLUBLE APOPROTEIN

PATIENT	APO E	APO C <sup>II</sup>	APO C <sup>III</sup>	APO C <sup>III</sup> <sub>2</sub>
1	21.5	16.3	51.0	11.3
2	19.4	20.6	37.2	22.4
3	29.8	20.7	31.9	17.5
4	10.1	13.4	55.3	21.2
5	13.6	27.7	27.6	28.2
6	25.7	18.2	44.7	11.4
7	22.2	28.1	25.3	20.3
8	30.7	22.0	29.4	17.9
9	20.3	12.0	41.6	26.0
10	23.3	17.3	37.5	22.0
11	25.3	14.4	47.5	12.8
12	20.9	15.8	45.0	17.9
13	24.4	21.4	23.3	28.9
14	22.6	27.3	21.6	28.5
15	23.0	18.8	32.7	25.4
16	36.3	19.8	31.3	12.6
17	23.4	8.5	45.3	9.5
18	16.8	8.7	48.2	26.3
19	28.6	15.3	25.9	30.2
20	37.8	17.9	18.5	25.5
$\bar{X}$	23.8	18.2	36.0	20.8
SEM	1.5	1.2	2.4	1.5

Table of soluble apoproteins represented as percent of total soluble apoprotein present in 20 of the patients who participated in the study. Values were obtained at time of patient incorporation into the study.

TABLE 9

Table 2. Distribution of Cholesterol in Lipoproteins

Group	No. in group	$\delta/\sigma$	Relative distribution, % of total (and SEM)				Absolute distribution, <sup>a</sup> mg/L serum (and SEM)			
			VLDL	IDL	LDL	HDL	VLDL	IDL	LDL	HDL
Control	17	12/5	6 (1)	1 (0.3)	65 (2)	27 (2)	130 (20)	30 (10)	1350 (90)	570 (50)
Diabetic	25	10/15	10 (3)	4 (1)	64 (2)	22 (2) <sup>b</sup>	250 (120)	90 (30)	1550 (100)	530 (50)
Normolipidemic	15	7/8	7 (1)	4 (7)	63 (2)	26 (1)	140 (20)	80 (10)	1310 (70)	580 (40)
↑ VLDL	6 <sup>c</sup>	2/4	16 (4) <sup>b</sup>	4 (1)	60 (3)	20 (8)	480 (120) <sup>b</sup>	110 (40) <sup>b</sup>	1760 (260)	590 (200)
↑ LDL	7 <sup>c</sup>	2/5	13 (3)	3 (1)	71 (4) <sup>b</sup>	13 (2) <sup>b</sup>	410 (100)	100 (20) <sup>b</sup>	2210 (110) <sup>d</sup>	400 (60) <sup>b</sup>

<sup>a</sup> Recovery of cholesterol in the ultracentrifugal fractions was 78–91% of the serum value; the absolute distribution is based on the relative distribution in the ultracentrifugal fractions, corrected to 100% recovery (disregarding chylomicrons).  
<sup>b</sup> Differs from control value,  $p < 0.02$ .  
<sup>c</sup> See Table 1.  
<sup>d</sup> Differs from control value,  $p < 0.001$ .

Table 3. Apoprotein Composition of Soluble VLDL Protein, in Percent ( $\pm$ SEM)

Group (no.)	(1) <sup>a</sup>	CI	E	CII	CIII <sub>1</sub>	CIII <sub>2</sub>	Minor components <sup>b</sup>	CII/CIII <sub>1</sub>
Control (12)	5.2 (0.8)	10.6 (1.7)	32.4 (3.1)	11.4 (1.1)	18.2 (1.9)	15.8 (1.5)	6.1 (1.8)	0.65 (0.04)
Diabetic (27)	3.7 (0.7)	11.0 (1.4)	28.1 (2.1)	14.3 (0.8) <sup>c</sup>	24.4 (1.9) <sup>c</sup>	17.9 (0.9)	<1 <sup>c</sup>	0.66 (0.2)
Normolipidemic (14)	3.8 (0.9)	13.1 (1.8)	28.6 (2.9)	13.4 (1.1)	21.9 (5.8)	17.5 (1.5)		0.68 (0.06)
↑ VLDL (6) <sup>d</sup>	3.6 (1)	8.7 (2.2)	24.7 (3.8)	15.2 (1.9)	29.2 (4.5) <sup>e</sup>	18.6 (0.9)		0.56 (0.06) <sup>e</sup>
↑ LDL (7) <sup>f</sup>	3.6 (0.7)	6.5 (1.2)	31.5 (3.8)	16.5 (1.2) <sup>e</sup>	23.4 (2.1)	18.4 (1.2)		0.71 (0.04)

<sup>a</sup> Band 1 on the electrophoretic gel, near the origin; identity not determined.

<sup>b</sup> Includes AI, AII, and one unidentified band moving between AII and CII.

<sup>c</sup> Analysis of variance: difference between diabetics and controls significant at  $p < 0.025$ .

<sup>d</sup> Includes three subjects with increase in VLDL (phenotype IV) and three subjects with increase in both VLDL and LDL (phenotype IIB). There was no difference observed between these two subgroups.

<sup>e</sup> Within analysis of variance, differs from control with  $p < 0.05$ .

<sup>f</sup> Phenotype IIA.

Distribution of cholesterol in lipoproteins and apoprotein composition in percent of soluble VLDL protein in percent from a normal control, diabetic, and normolipemic population as reported from reference #63. The apoprotein percent composition from these three groups should be used for comparison with the VLDL apoprotein composition of the hyperlipemic patients participating in the sucrose study.

of approximately 4.3. The most basic protein observed is apo C<sub>1</sub> which has an isoelectric point between pH 6.0 and 6.5. The isoelectric gradient which was generated during each electrophoresis is seen in Figure 41. The pH range was between 3.5 and 8.0.

d. Changes Observed in Apo VLDL as a Result of Different Sucrose Diets

The composition of apoprotein subunits in VLDL lipoproteins were compared between control diet and study diet periods in all patients who participated in the study. Samples were obtained three times weekly during both the control and study diet periods. The different VLDL apoprotein concentrations found in patients consuming the high or low sucrose diets as compared to the control diet are illustrated in Figure 42. All apoprotein concentrations are represented as the percent (%) of total peptide present. This includes total apo E and all apo C proteins, but the apo B protein, because of its size, does not penetrate the gel and is considered an insoluble apoprotein. Total apo E does not change significantly in these hyperlipemic patients from the control dietary period to either the high or low sucrose dietary period (Figure 42). Apo CII also remained stable throughout the study with no significant changes observed between control and study diet periods.

Significant changes were observed in the apo CIII<sub>1</sub> and apo CIII<sub>2</sub> protein fractions of the VLDL lipoprotein during the study. The apo CIII<sub>1</sub> fraction increased significantly in patients consuming the high sucrose diet, from a control value of 34% to almost 47% of total soluble peptide present. This increase was statistically significant

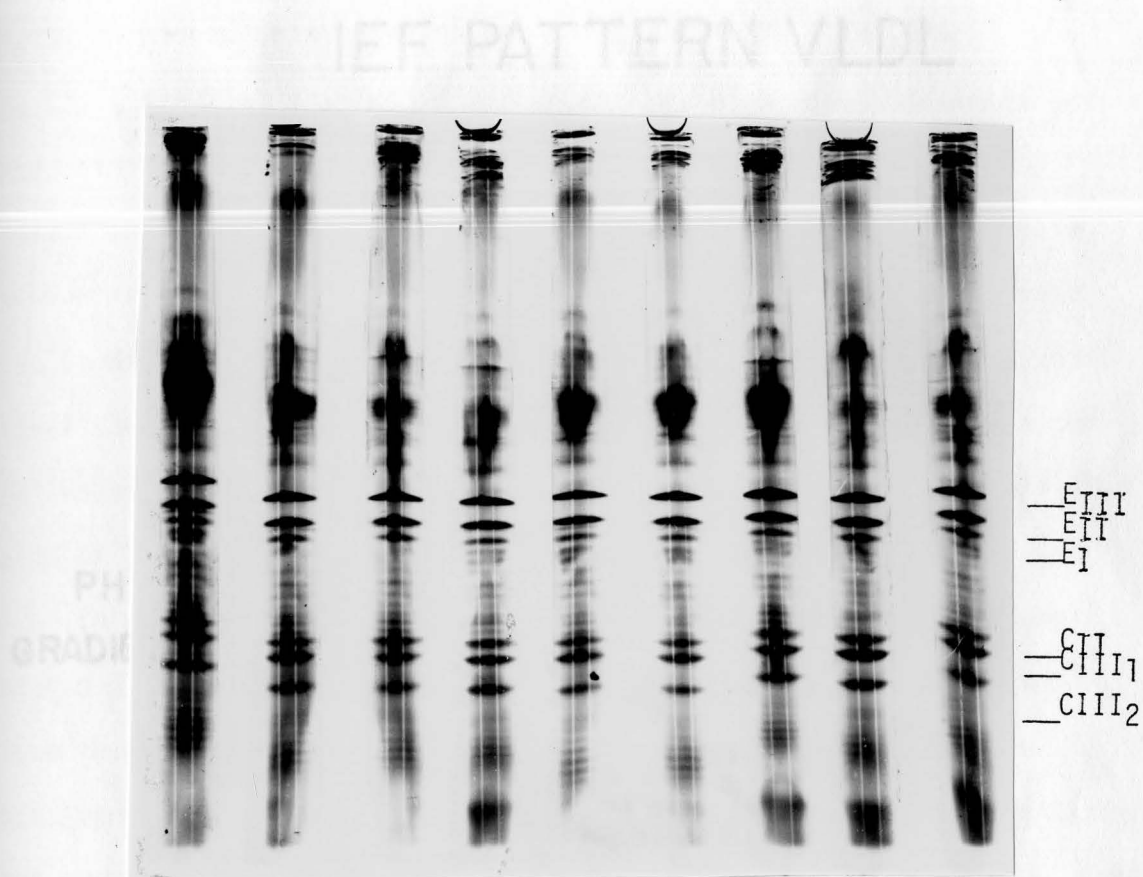


Figure 40: Isoelectrically focused gels of VLDL apoproteins derived from 9 of the patients who participated in the study. 7.5% polyacrylamide separating gels were used and samples were electrophoresed and stained as described in Materials and Methods.

## IEF PATTERN VLDL

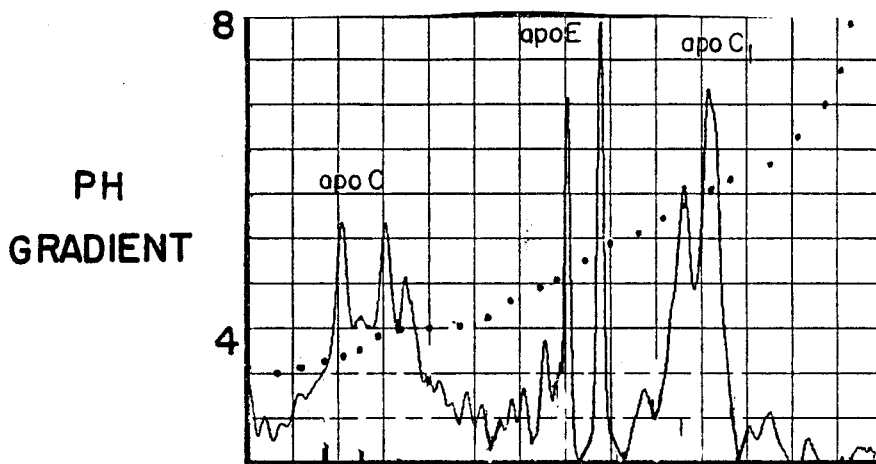
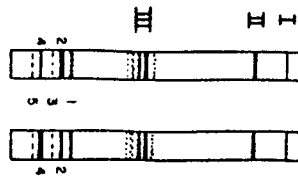


Figure 41: Typical densitometric scan of an isoelectrically focused gel containing apo VLDL derived from a hypertriglyceridemic patient. Gelman Model #ACD-18 was used for scanning and peak integration of samples.

( $p < 0.05$  using paired t analysis). The apo CIII<sub>1</sub> fraction decreased from the control period values in patients consuming the low sucrose diets. However, this decrease was not significant. In general, the apo CIII<sub>2</sub> fraction changed in an opposite fashion relative to changes observed for the apo CIII<sub>1</sub> fraction with consumption of both study diets. Specifically, the apo CIII<sub>2</sub> fraction decreased slightly from the control diet concentrations with consumption of the high sucrose diet (nonsignificant,  $p < 0.02$ ). However, apo CIII<sub>2</sub> fraction increased significantly when patients consumed the low sucrose diet, from a control value of approximately 20% of the total apoprotein to a study diet value of 28% of total apoprotein ( $p = 0.05$ ).

In summary, nonsignificant changes were observed in total apo E and apo CII concentrations from control values when patients consumed either high or low sucrose diets. Apo CIII<sub>1</sub> and CIII<sub>2</sub> showed the most significant changes observed. Apo CIII<sub>1</sub> increased significantly from control values when patients consumed a diet rich in sucrose. In contrast, apo CIII<sub>2</sub> increased significantly from control values when low sucrose diets were consumed.

Data generated from the isoelectric focusing measurements indicated relevant changes that occurred in the concentration of the apo E isoprotein subfractions with consumption of different sucrose study diets. Figure 43 graphically demonstrates changes in these subfractions from control diet values when diets rich in sucrose or low in sucrose were consumed. Minor changes from control values were realized in apo E<sub>1</sub> and apo E<sub>2</sub> isoprotein concentrations when either high sucrose or low sucrose diets were consumed (nonsignificant  $p > 0.30$ ). The apo EIII

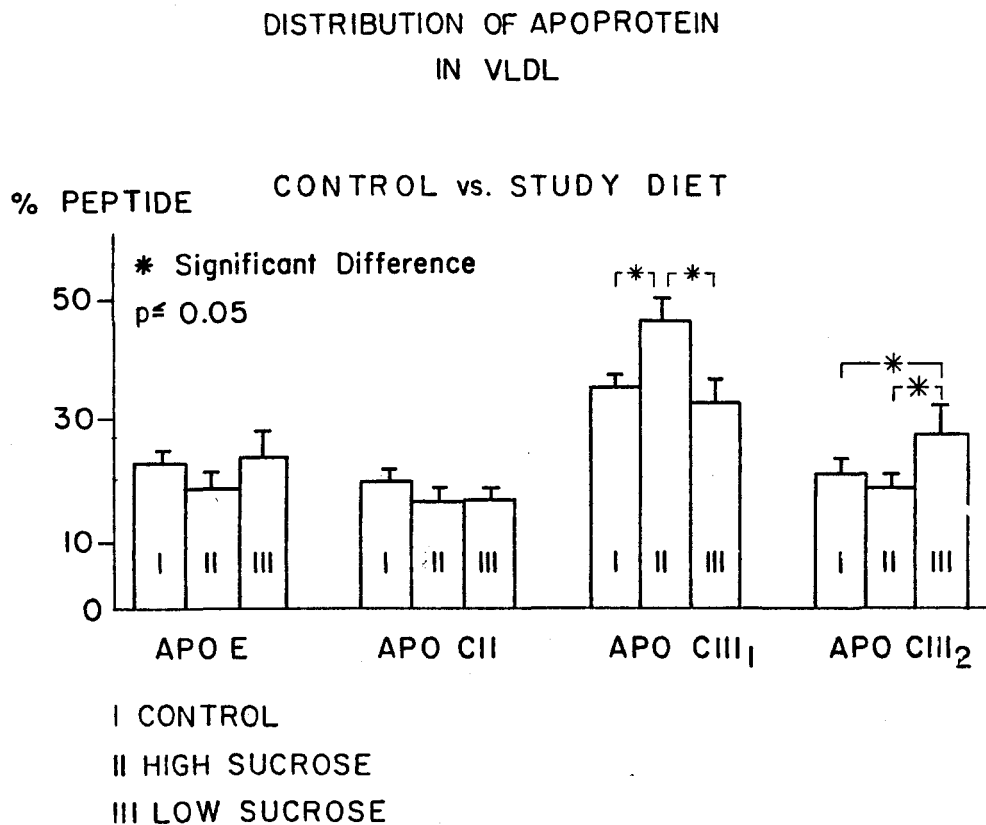


Figure 42: Effect of different sucrose content diets on apoprotein composition of VLDL. Group II high sucrose  $n = 17$ . Group III low sucrose  $n = 13$ . Group I control  $n = 30$ . Results represent soluble peptide in the apoprotein as percent of total soluble peptide present. Values represent mean  $\pm$  S.E.M. of all patients per group.



isoprotein subspecies showed a significant increase from control values when patients consumed high sucrose diets ( $p < 0.05$ ). Patients consuming sucrose restricted diets had relatively unaltered apo EIII levels from control period values.

e. Correlations Between Apoprotein Subfractions and Triglyceride Concentrations

The importance of alterations in apoprotein content of the VLDL is demonstrated in Figures 44 through 48. These illustrate the relationship between apoprotein content and triglyceride concentration observed in study patients. All of the correlation studies performed were done during the last week of the study diet for both high and low sucrose groups. Figure 44 illustrates the relationship between the percentage of apo CIII<sub>1</sub> observed and the triglyceride concentration measured in all patients consuming high sucrose diets. The correlation was significant ( $r=0.723$  and  $p < 0.001$ ). Figure 45 also demonstrates this same relationship in all patients who consumed low sucrose diets. The correlation was again highly significant ( $r=0.823$ ,  $p < 0.001$ ). From Figures 44 and 45, it is observed that as apo CIII<sub>1</sub> protein percentages increased, triglyceride concentration also increased. Patients who had apo CIII<sub>1</sub> concentration composing 50% or more of their soluble apoproteins present had triglyceride concentrations sometimes as high as 400 to 600 mg/dl. Thus, in these patients, apo CIII<sub>1</sub> levels correlate directly to triglyceride concentration.

The relationship between apo CII/CIII<sub>1</sub> ratios and triglyceride concentration is illustrated in Figures 46 and 47. In both groups of

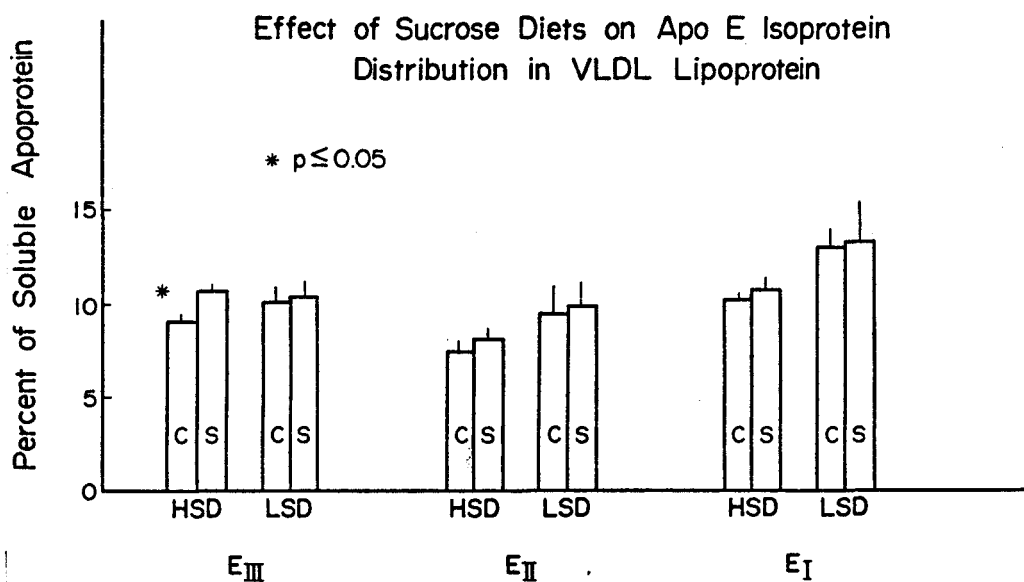


Figure 43: Changes in each apo E isoprotein subunit as a result of dietary manipulation. Values from all patients in a particular study group while consuming the control diet are compared to values from these same patients while consuming either high or low sucrose content diets. C represents the control diet and S represents the period of either high or low sucrose intake.

patients the ratio of apo CII/CIII<sub>1</sub> had an effect on triglyceride concentrations. As the apo CII/CIII<sub>1</sub> ratio increased, triglyceride concentrations decreased. A significant correlation was observed in apo CII/CIII<sub>1</sub> ratios and triglyceride concentrations in patients who consumed high sucrose diets ( $r=0.682$ , a  $p < 0.001$ ). Patients who consumed the sucrose restricted diet also demonstrated a strongly significant correlation between apo CII/CIII<sub>1</sub> ratios and triglyceride concentrations ( $r=-0.871$  and a  $p < 0.001$ ).

Finally, the relationship between apo CIII<sub>2</sub>/CIII<sub>1</sub> and triglyceride concentration in the high sucrose patient population is observed in Figure 48. The low sucrose population demonstrated a similar relationship. It should be observed that the negative correlation between apo CIII<sub>2</sub>/CIII<sub>1</sub> and triglyceride concentrations was significant ( $r = -0.60$  and a  $p = 0.05$ ). When triglyceride concentrations were elevated apo CIII<sub>2</sub>/CIII<sub>1</sub> ratios were depressed. Patients with apo CIII<sub>2</sub>/CIII<sub>1</sub> ratios of 0.25 or less had triglyceride concentrations of 350 mg/dl or more. Similarly, those patients with ratios of 1.25 or greater had triglyceride concentration of 150 mg/dl or less.

In summary, the above results show that apoprotein concentrations, especially apo CII, CIII<sub>1</sub> and CIII<sub>2</sub> had significant effects on triglyceride levels in this population of patients. As apo CIII<sub>1</sub> increased, triglyceride concentration also increased. Apo CII/CIII<sub>1</sub> ratios and triglyceride concentrations were inversely related. With more apo CII present relative to apo CIII<sub>1</sub>, lower plasma triglyceride concentrations were observed in the study group. In addition, a similar

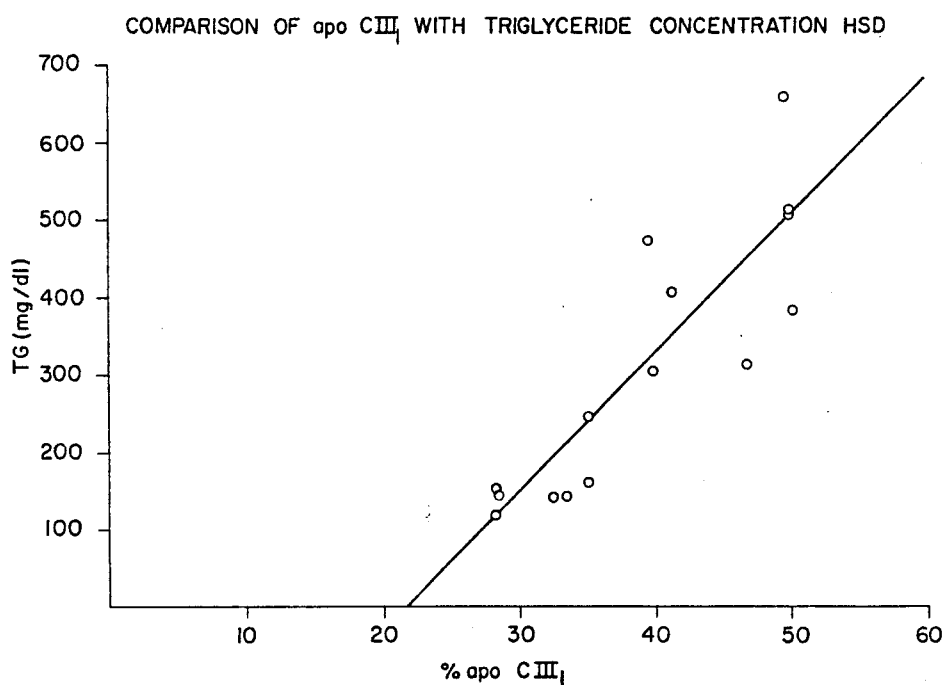


Figure 44: The effect of apo CIII<sub>1</sub> in VLDL on plasma triglyceride concentration in 17 patients on the high sucrose diet. A significant positive correlation was found ( $r=0.723$ ,  $p < 0.001$ ). These values were generated from patients during the final week of the high sucrose diet.

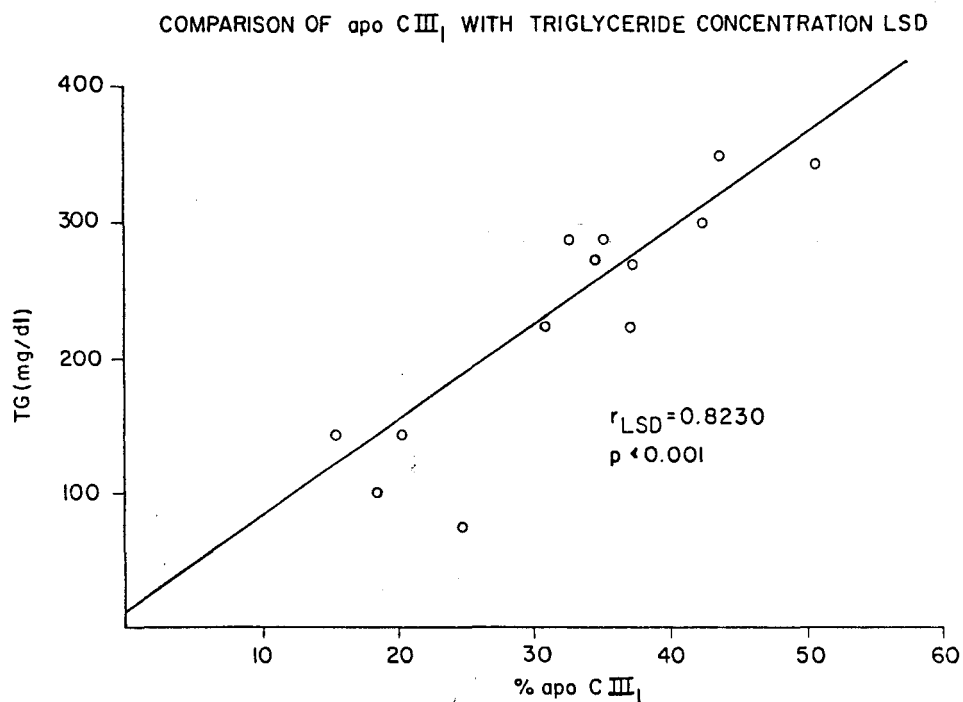


Figure 45: The effect of apo CIII<sub>1</sub> in VLDL on plasma triglyceride concentration in 13 patients on the low sucrose diet. A significant positive correlation is observed ( $r=0.823$ ,  $p < 0.001$ ). These values are taken from patients in the final week of the study after being randomized to the low sucrose diet.

relationship was observed between apo CIII<sub>2</sub>/CIII<sub>1</sub> ratios and triglyceride concentrations in this patient population. With more apo CIII<sub>2</sub> present relative to apo CIII<sub>1</sub>, triglyceride concentrations found in individual patients were decreased.

#### J. Abnormal VLDL Found in Several Patients Participating in the Sucrose Study

##### a. Observations of VLDL Lipoprotein Electrophoresis

A particularly interesting observation was found in 4 patients who consumed sucrose enriched diets. While the lipoprotein electrophoresis measurements were done during both control and study diet periods, it was noticed that these patients demonstrated a variable hyperlipemic electrophoretic pattern. This pattern could be classified as type IV while they were consuming the control diet, but changed to a type III hyperlipemic pattern when they consumed the high content sucrose diets. A sequential electrophoretic pattern from one of these patients is illustrated in Figure 49. The relatively large pre  $\beta$  band associated with type IV hyperlipemic individuals gradually disappears during the study diet and is incorporated into the  $\beta$  band. This pattern is characteristic of an individual with type III hyperlipemia having the broad band pattern.

##### b. Isoelectric Focusing on Abnormal VLDL Lipoproteins

Since the possibility existed that these patients could have a VLDL different in composition from the rest of the high sucrose patient population, their apoprotein electrophoresis results were compared to

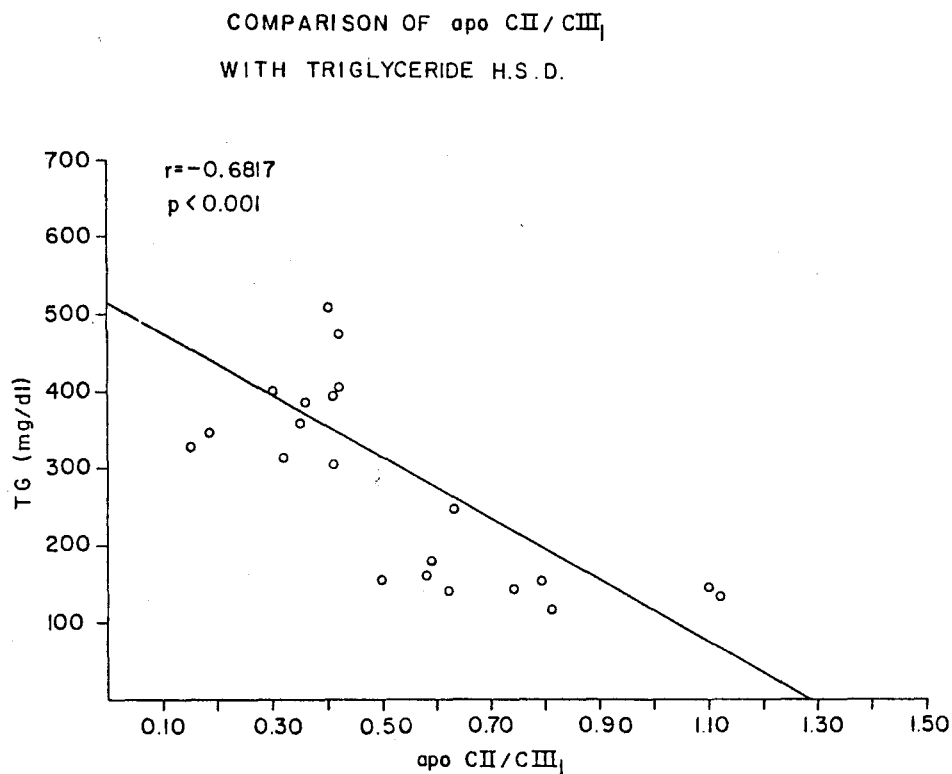


Figure 46: The apo CII/CIII<sub>1</sub> ratio and its effect on plasma triglyceride concentration in 17 patients who consumed high sucrose diets. A significant negative correlation was obtained ( $r = -0.6817$ ,  $p < 0.001$ ). These values were obtained after patients had consumed the high sucrose study diet for a 4 week period.

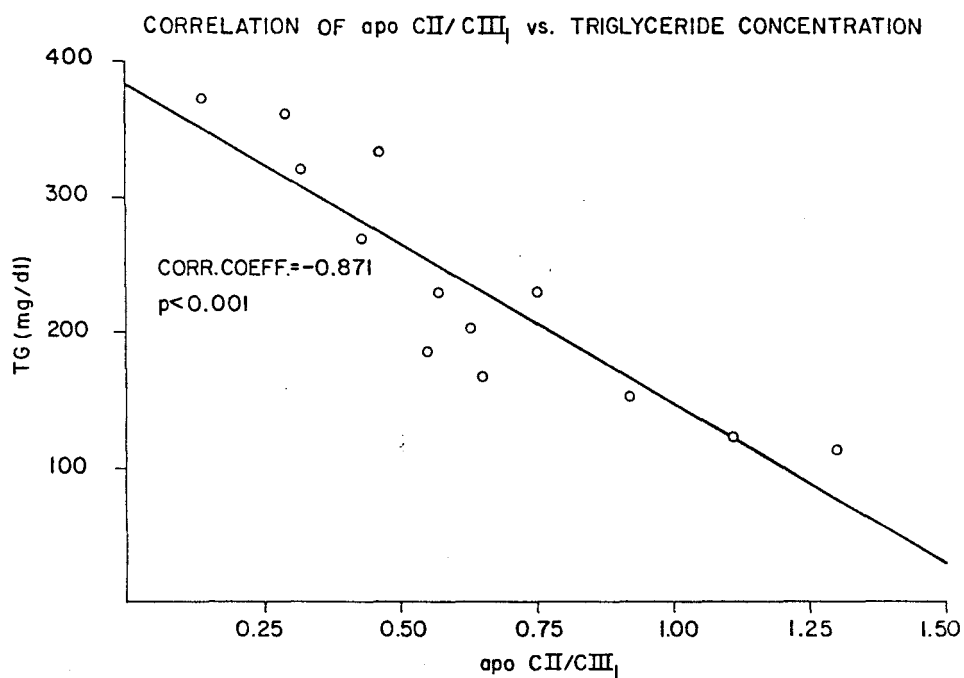


Figure 47: The apo CII/CIII<sub>1</sub> ratio and its effect on plasma triglyceride concentration in 13 patients who consumed the low sucrose diet. A significant negative correlation was obtained ( $r = -0.871$  and a  $p < 0.001$ ). These values were obtained after patients had consumed the low sucrose study diet for a period of 4 weeks.



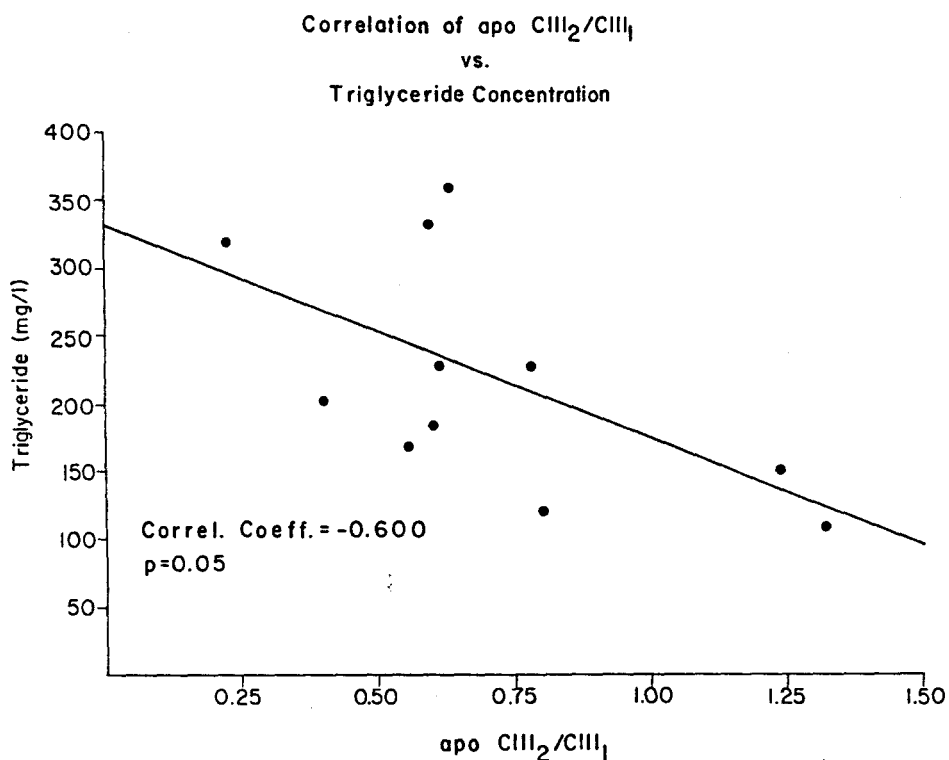


Figure 48: The apo CIII<sub>2</sub>/CIII<sub>1</sub> ratio and its effect on plasma triglyceride concentration in patients who consumed the high sucrose diet. A significant negative correlation was obtained ( $r = -0.600$  with a  $p = 0.05$ ). These values were obtained after patients had consumed the high sucrose study diet for a period of 4 weeks. Eleven patients were used to obtain the results shown. Similar results were obtained from patients who consumed the low sucrose study diet for the 4 week period.

that from the remaining high sucrose patient population. No appreciable differences could be found in the SDS PAGE patterns from the designated abnormal VLDL subgroup and the rest of the patient population. Comparison of the patterns obtained from isoelectric focusing procedures on abnormal VLDL and the VLDL from other patients consuming high sucrose diets illustrated an interesting difference. A typical isoelectrically focused gel containing the VLDL from a hyperlipemic individual consuming the high sucrose diet is shown in Figure 50. Particular attention must be paid to the apo E isoprotein pattern of this patient. The typical apo E pattern with the most acidic apo E<sub>1</sub> being a diffuse band, and two thick apo EII and apo EIII bands is observed. Figure 50 also represents the isoelectrically focused VLDL from the 4 patients in question. Again observe the apo E isoprotein pattern. The diffuse apo E<sub>1</sub> band is present and the apo EII band is directly above. However, no apo EIII band can be observed in these patients. Several repeat electrophoreses were performed on these patients with the same apo E isoprotein pattern observed. It is possible that the loss of this particular apo E isoprotein band could be responsible for the differing pattern observed in these 4 patients during lipoprotein electrophoresis.

#### K. The Effect of Variable Sucrose Content Diets on Platelet Aggregation

##### a. Effects of Sucrose Content on Total Platelet Aggregation

The possibility that variable sucrose content diets could also affect platelet aggregation was studied in all the patients who parti-

## High Sucrose Diet

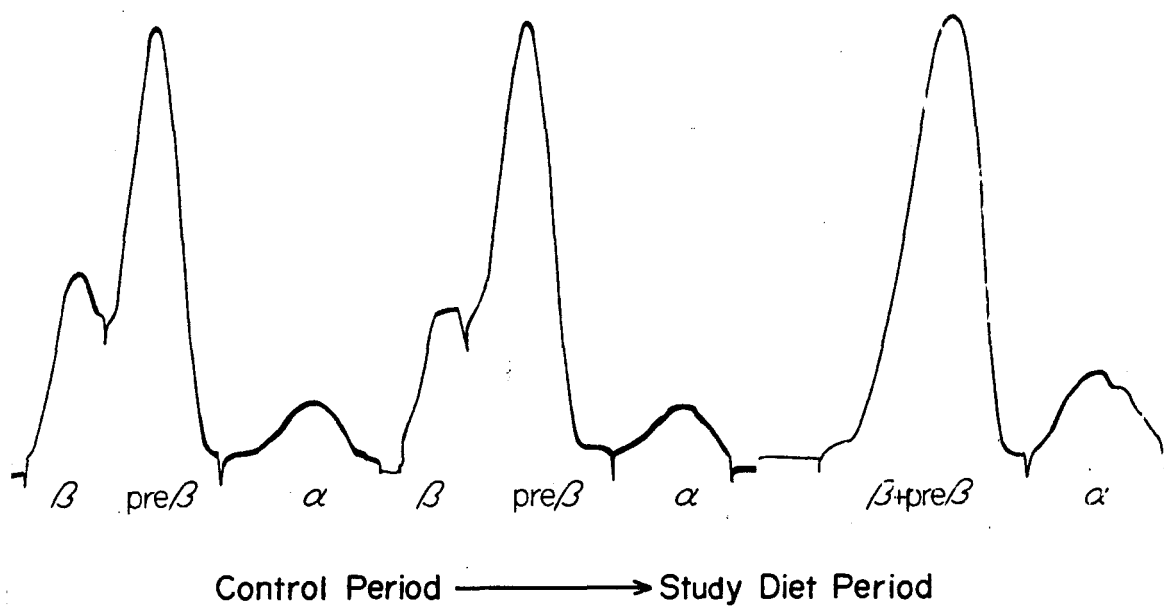


Figure 49: Typical lipoprotein electrophoretic pattern obtained from VLDL abnormal individual during the control and study diet period. Observe how the pre- $\beta$  band seems to migrate along with the  $\beta$  band after high sucrose dietary consumption. This pattern was found in 4 individuals who consumed the high sucrose diet.

cipated in the project. Platelet aggregation measurements were done once a week during both control and study diet periods. The aggregations done during the control period were compared to those done during either high or low sucrose consumption. In this way, changes in platelet aggregation due specifically to diet could be ascertained. Figure 51 shows the effect of high sucrose dietary consumption on platelet aggregation. Data from 4 representative patients from the high sucrose study group are shown. All results are represented as percent light transmittance through the mixing cuvette, where more light is transmitted when more platelets have aggregated. In all patients consuming the high sucrose diet illustrated in Figure 51, an increase in aggregation occurred over the control aggregation values. In some cases the increase was as high as 55 units on the percent transmission scale. In other cases, the increase observed was slight, consisting of only 10% units. However, in all cases, an increase in platelet aggregation was realized. Figure 52 illustrates the effect of low sucrose diets on platelet aggregation using data from 3 representative patients. All the patients shown in Figure 52 demonstrated a slight decrease in aggregation while consuming the low sucrose study diet as compared to the control diet. However, relatively little change from control diet values was observed in many of the patients who consumed the low sucrose diet and these patterns far outnumbered patient data demonstrating a slight decrease.

The overall effect of sucrose content in diets on platelet aggregation is shown in Figure 53. The figure represents measurements done on 30 patients, 17 consuming high sucrose diets and 13 consuming low

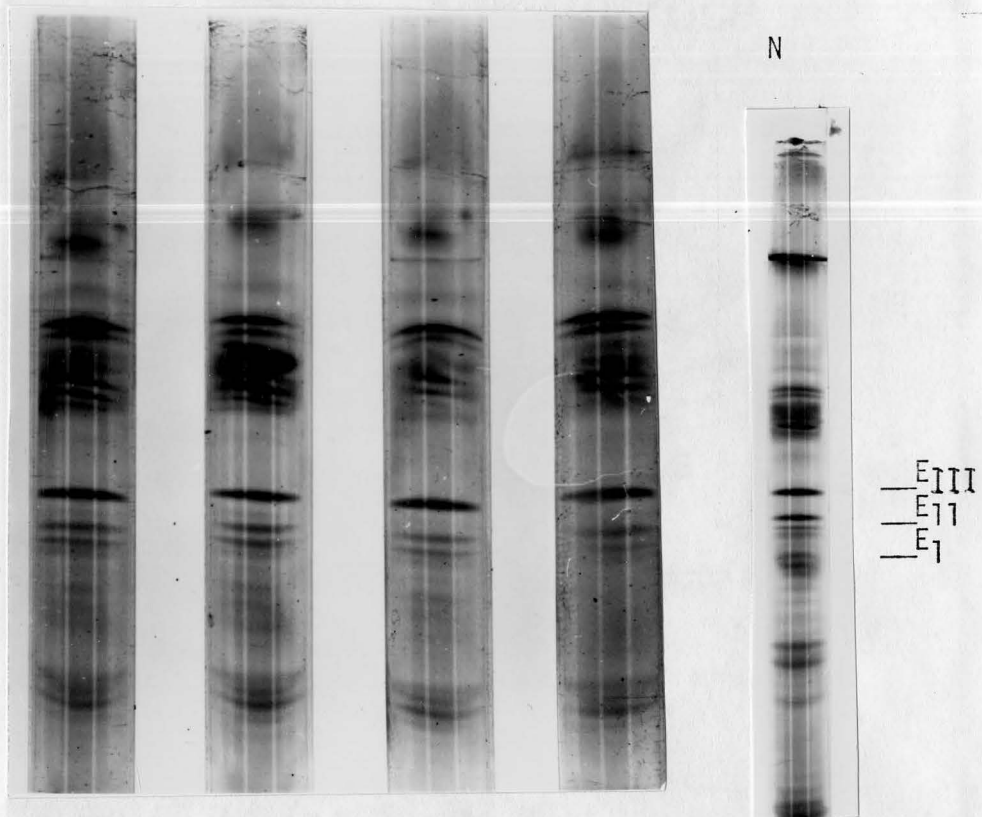


Figure 50: Isoelectrically focused samples of VLDL from patients who consumed the high sucrose diet and demonstrated a shift in their lipoprotein pattern from type IV to type III. This VLDL designated VLDL abnormal (VLDL abn) was found in only 4 of the patients studied. Observe the apo E isoprotein pattern as compared to the normal. No apo EIII band exists in the VLDL of these 4 patients. These samples were obtained at the end of the high sucrose dietary period. Gel preparation and focusing procedure done as described in Materials and Methods.

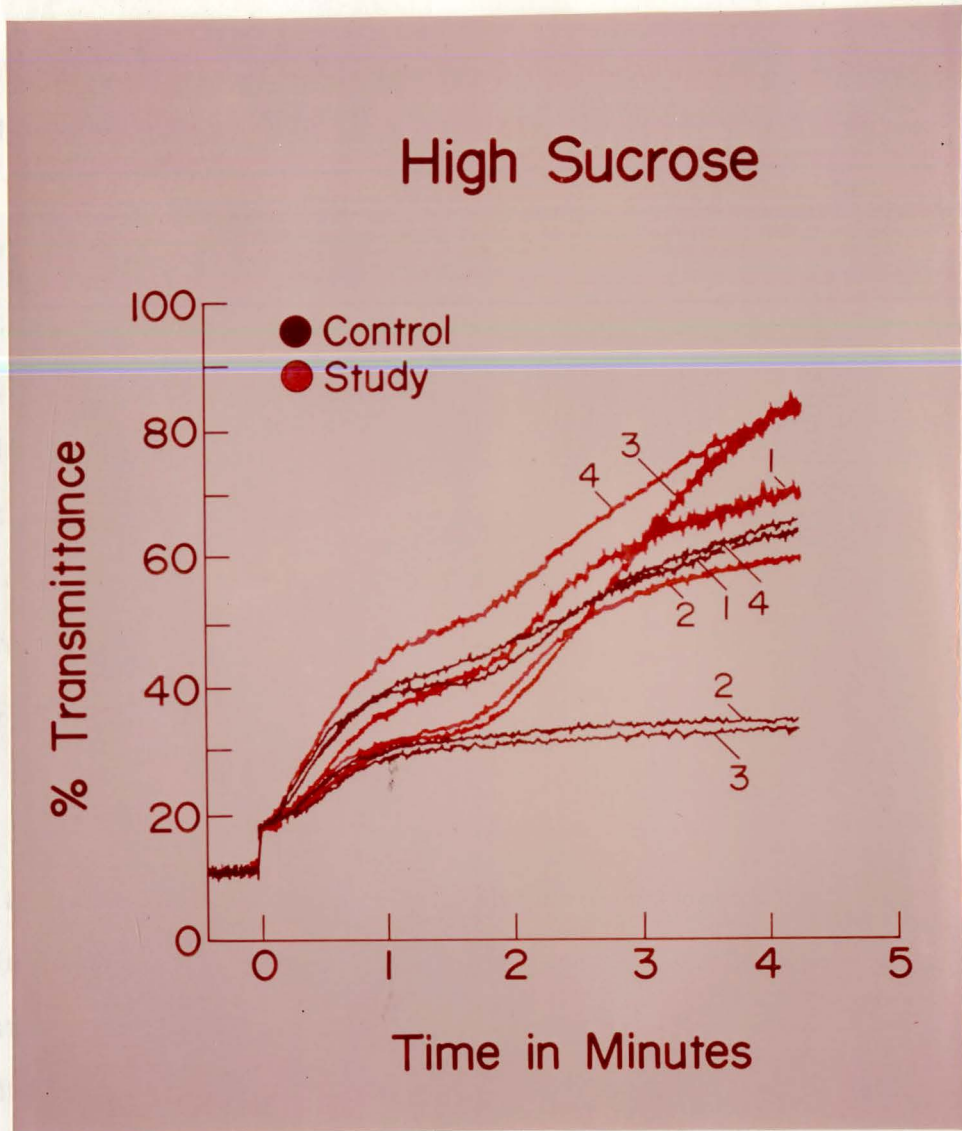


Figure 51: Platelet aggregation curves from 4 patients who consumed the high sucrose diet after consumption of the control diet. Each patient is designated by number. The dark line represents that patient's total aggregation at the end of the control period. The light line represents that patient's aggregation at the end of the high sucrose study diet. Aggregation results are represented as % light transmitted through the measuring cuvette.

sucrose diets. Each group is an average of the platelet aggregations obtained while consuming the study diet as compared to that groups' average aggregation while consuming the control diet. A slight, but insignificant decrease in total aggregation was observed from control aggregation values in the patient group consuming the low sucrose diet. However, in patients consuming the high sucrose diet, a significant increase in average aggregation was observed over the control platelet aggregations. The patients who consumed high sucrose diets had mean control aggregation values of approximately 50% which increased to 65% light transmitted after high sucrose consumption. This 15% increase in light transmission was significant ( $p < 0.05$ ).

#### b. Platelet Sensitivity Studies

Platelet sensitivity studies were also done in both study groups by varying the concentration of the aggregating agent, epinephrine. Seven patients from each group had 30 cc of blood drawn. Appropriate plasma dilutions with platelet rich and platelet poor plasma were made to obtain enough samples to perform 20 aggregations per study. The platelet sensitivity assays were performed during both control and study diet periods. Each patient's platelet sensitivity results during the study diet were compared to the results obtained when the same patient consumed the control diet. The concentration of aggregating agent at which the second phase platelet response disappeared was considered to be the lowest concentration of aggregating agents at which the platelets could adequately respond.

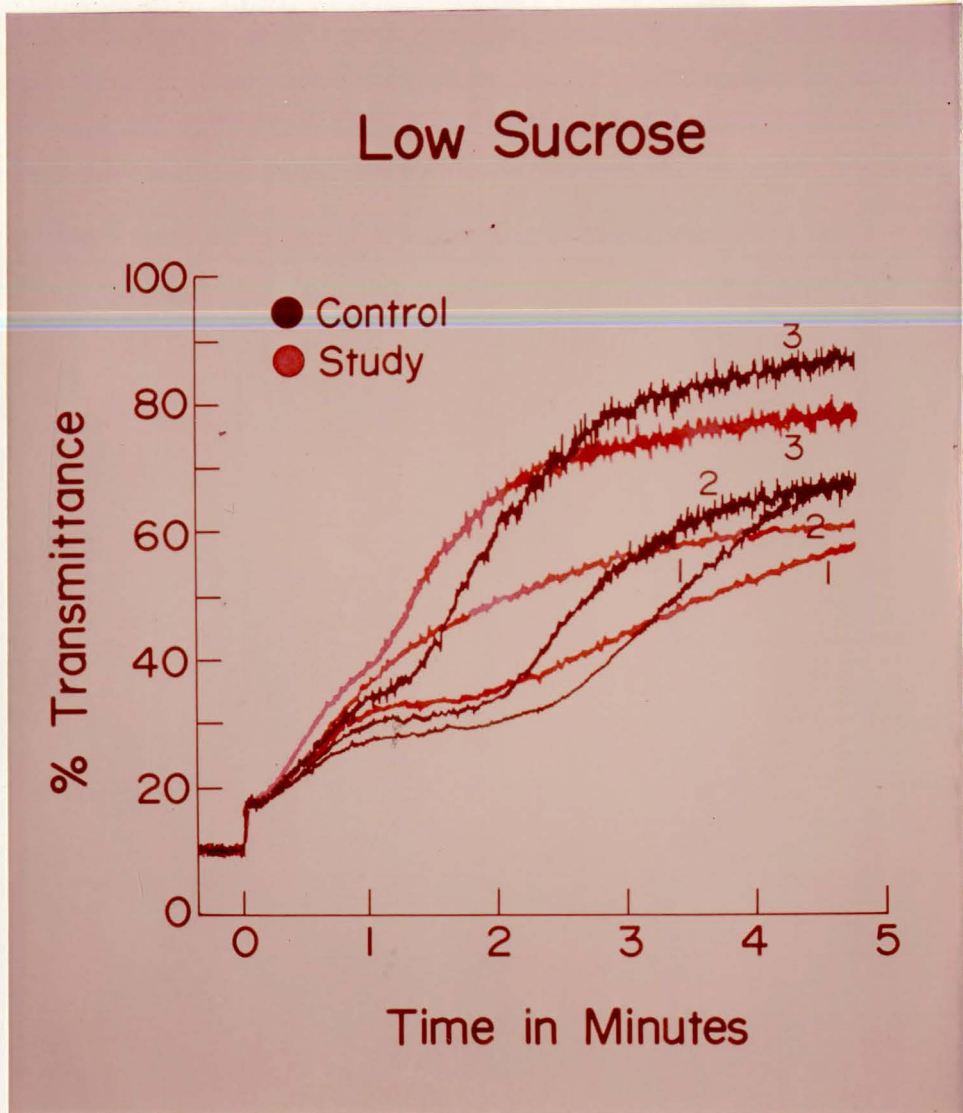


Figure 52

Figure 52: Platelet aggregation curves from 3 patients who consumed low sucrose diets after consumption of the control diet. Each patient is designated by number. The dark line represents that patient's total aggregation at the end of the control period. The light line represents that patient's aggregation at the end of the sucrose dietary period. Aggregation is measured in % light transmittance through the measuring cuvette. (\*) is at the  $p < 0.05$  level.



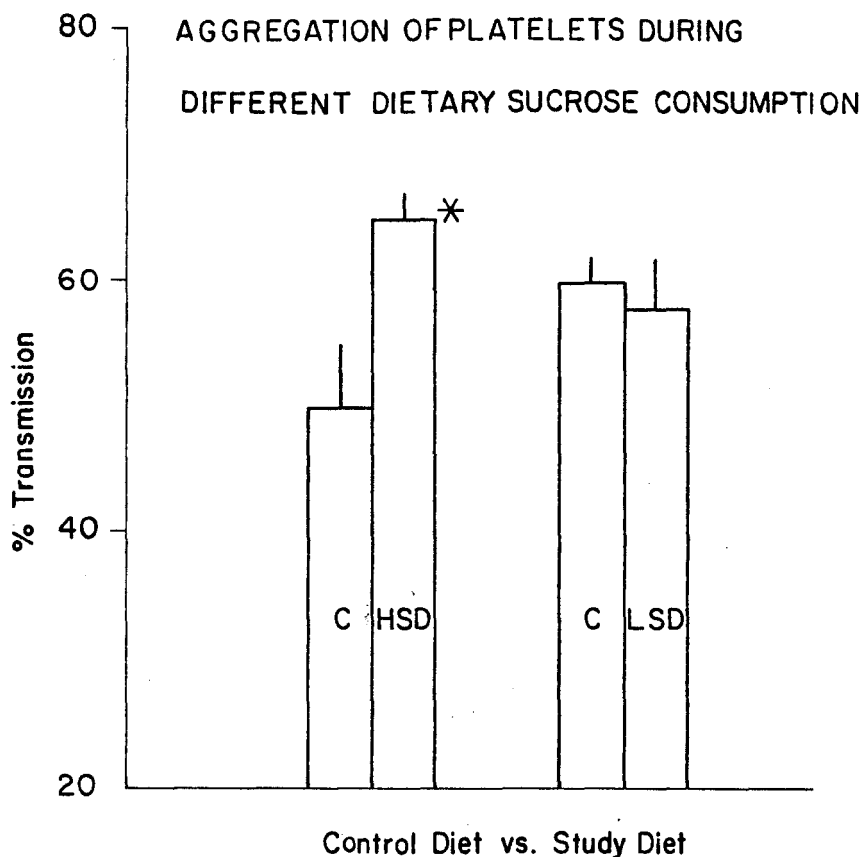


Figure 53: The effects of dietary sucrose on platelet aggregation in 30 patients: 17 consuming high sucrose and 13 consuming low sucrose diets. Each group consuming either study diet is compared with itself after consumption of the control sucrose diet. C represents the mean SEM aggregations of all patients from the study group after consumption of the control diet. HSD and LSD represent the mean SEM aggregations after consumption of the high and low sucrose diet respectively. Significance (\*) is at the  $p < 0.05$  level.

The platelets of patients consuming the low sucrose diet showed little change in sensitivity when compared to the control diet values. However, the patients who were placed in the high sucrose regimen after the control period demonstrated an increased sensitivity to the aggregating agent as compared to the control diet. Table 9 illustrates the effect of sucrose enriched diets on platelet sensitivity. Platelets of all patients consuming sucrose-enriched diets were more sensitive to the aggregating agent. In some cases, concentrations of epinephrine as low as 0.25 and 0.1  $\mu\text{M}$  elicited the second phase response. The concentration of aggregating agent that elicits the second phase response in a normal population is between 2.5 and 5  $\mu\text{M}$ .

TABLE 10

## PLATELET SENSITIVITY WITH VARYING CONCENTRATIONS OF EPINEPHRINE C vs.S

	<u>50</u>	<u>10</u>	<u>5</u>	<u>2.5</u>	<u>1.0</u>	<u>0.5</u>	<u>0.25</u>	<u>0.1</u>	<u><math>\mu</math>M</u>
1. C	51	31	51	22	NS				
1. S	75	70	69	62	60	16	NS		
2. C	66	68	63	54	20	NS			
2. S	57	53	58	51	49	28	15	NS	
3. C	70	68	69	63	66	23	NS		
3. S	71	74	67	75	64	63	14	NS	
4. C	22	23	NS						
4. S	58	54	54	40	24	NS			
5. C	55	56	49	42	NS				
5. S	66	56	40	41	32	NS			
6. C	71	69	70	67	28	NS			
6. S	63	66	66	61	72	63	65	24	
7. C	56	56	53	52	42	25	NS		
7. S	70	72	69	61	62	61	19	NS	

Platelet sensitivity in 7 patients who consumed the high sucrose study diet after consumption of the control sucrose diet. C represents the sensitivity results at the end of the control diet period. S represents the sensitivity results at the end of the study diet period. Results are expressed as the % light transmittance through the cuvette after 5 minutes of incubation with aggregating agent. The effect of the aggregating agent is considered non-significant (NS) if no second phase response is initiated after addition of aggregating agent.

## 6. DISCUSSION AND SIGNIFICANCE

This study sought to ascertain the metabolic consequences of high carbohydrate diets in diabetics with hypertriglyceridemia. This was initially prompted by the observations of Anderson et al. (10) as to the effect of carbohydrate restriction and high carbohydrate diets in men with chemical diabetes. The clinical relevance of this question was underscored in 1979 when the American Diabetes Association (ADA) released its new dietary guidelines for diabetics. The ADA recommended allowances for carbohydrate intake be increased to 50 or 60% of the total calories consumed in contrast to 40% as recommended only a few years earlier (6). Reduction of dietary fat was the main objective of these proposed changes since excess fat was presumed to be the pathogenic factor in accelerated arteriosclerosis observed in diabetics.

In a recent editorial, Reaven states that "although it is commonly considered to be a temporary phenomenon, the transient nature of carbohydrate induced increases in triglyceride levels is not supported by a great deal of experimental data" (102). Reaven succinctly reviewed the incidence of favorable and unfavorable effects of high carbohydrate diets and concluded that most of the reported studies dealt with normal well controlled diabetics and treated type I or type II diabetics on high fiber diets (102). He felt the need of a study with "diets . . . similar in all but proportion of carbohydrate and consisting of food stuffs that patients will be able to tolerate chronically". He particularly expressed concern for the effect of high carbohydrate diets on hypertriglyceridemic, non-insulin dependent diabetics.

The need to know the advantages, if any, of a truly sucrose

restricted diet in the treatment of the 85% of diabetics having type II (non-insulin dependent) diabetes, a form of the disease in which a third of the patient population is hypertriglyceridemic, is of great importance. It is also important to ascertain critical levels of dietary sucrose at which glycemic control and triglyceride metabolism may become altered in these patients.

Our experimental protocol specifically focused on sucrose ingestion in the above group. Sucrose is the least studied component in diabetes, yet is the largest chemically homogenous component in the regular diet (11). Sucrose intake amounts to 40 to 60% of dietary carbohydrate in the non-diabetic population of industrialized countries (11). Since a great proportion of processed foods have variable but large proportions of added sucrose not reported or quantitated (137), the increased consumption of processed carbohydrate containing foodstuffs by the diabetic population predisposes these patients to increased sucrose ingestion.

As discussed in the literature review, most reports dealing with the effects of sucrose on a given population used artificial diets, either composed of liquid nutrients or extremely high in carbohydrate proportion. Albrink and Newman (38) found that high and low fiber diets also affected insulin and glucose responses to a meal rich in carbohydrate. In this study, the diets were composed of natural nutrients which closely mimic the intake pattern and fiber content of a normal outpatient diet.

Initial experiments were concerned with the specific effects of sucrose enriched or sucrose restricted diets on relevant blood chemistry

parameters of the diabetic patient. The effects of different sucrose content diets on levels of serum glucose, urinary glucose, triglyceride, cholesterol, HDL-cholesterol, and patterns of lipoprotein electrophoresis are summarized in Table 11. Specific effects of dietary sucrose content are represented since each patient's results, while consuming either low or high sucrose diets, are compared to the results obtained when the same patient ingested the control sucrose diet. Basically (and intentionally), sucrose content is the only parameter of the diet that changes significantly.

Surprisingly, alteration of dietary sucrose levels had essentially no effect on serum or urine glucose levels (Figures 13 and 15). These results are in contrast to the findings of Reiser (40), Cohen (26), and Akgün (31) who state that increased sucrose ingestion causes a rise in blood serum glucose levels in normals and diabetics. Reiser (40) found that high sucrose diets caused an increase in mean glucose levels compared to low sucrose intake in non-diabetic subjects. Cohen (26) administered high and low sucrose diets with subsequent testing of glucose tolerance. He found impaired glucose tolerance after high sucrose consumption and suggested that because sucrose consisted of one half glucose, its ability to stimulate insulin release was also halved. Akgun (31) also found plasma glucose levels were lower when sorbitol or fructose was ingested, but when sucrose content in the diet was increased, plasma glucose levels rose. He could not find any link between plasma glucose levels and insulin response.

The results of the other studies discussed above are questionable, since in many cases other dietary factors which could effect

TABLE 11

	HIGH SUCROSE DIET		LOW SUCROSE DIET	
Triglyceride	AM	+ p < 0.001	No Change	
	PM	+ NS	+ p < 0.001	
HDL-Cholesterol	AM	- NS	+ P < 0.005	
	PM	No Change	No Change	
Cholesterol	AM	- p=0.05	No Change	
	PM	- p=0.05	- NS	
Serum Glucose	AM	- p < 0.02	- NS	
	PM	- NS	- NS	
Urine Glucose	No Change Low Spiller	- p < 0.01 High Spiller	No Change Low Spiller	-p=0.05 High Spiller
$\alpha$ Lipo		- NS	+ NS	
$\beta$ Lipo		- p < 0.01	No Change	
Pre $\beta$ lipo		+ p < 0.01	- NS	

Summary of the effects of different sucrose diets on blood and urine chemistries in the patient population, (+) designates an increase in values, (-) designates a decrease. NS signifies no significant change observed. Significant changes demonstrated by p value. AM values signify the fasting state, PM values represent the postprandial state.

plasma glucose concentrations were ignored. The studies of both Akgün (31) and Reiser (40) utilized sucrose diets which were artificial and not consistent with normal dietary intake patterns. In both studies, sucrose solutions were given and no dietary fiber content considerations were made. Dietary fiber has been clearly documented as having favorable effects on postprandial glucose concentrations in normal and non-insulin dependent diabetic subjects (460, 461). When sugar and pectin were incorporated into a large breakfast, postprandial glucose levels were significantly reduced as compared to the same individual eating fiber free meals containing similar nutrients. When patients with mild diabetes were given a water soluble fiber along with an oral glucose tolerance test solution, postprandial glucose concentrations were significantly lower compared to similar test solutions without fiber. Other investigators (15) have confirmed the short term beneficial effects of fiber supplements on glucose and meal tolerance tests. Others have demonstrated long term improvement in glucose tolerance or metabolism with fiber supplemented diets (462, 463). The extent to which plant fiber induces carbohydrate malabsorption has not been quantitated. The total absorption of simple sugars ingested with soluble fibers does not appear to be diminished, but the hydrolysis and absorption of complex carbohydrates may be decreased. With high fiber diets, fasting and average blood glucose concentrations were significantly lower and urine glucose excretion was also significantly impaired (464). Glucose metabolism was also improved with the high fiber diet, despite lower endogenous insulin concentrations. When patients were subsequently placed on a low fiber



diet, the improvement observed with the high fiber diet was reversed and average glucose concentration was similar to values observed on the initial low fiber diet.

Plant fibers influence glucose metabolism and insulin requirements by altering intestinal absorption of carbohydrate and by indirectly affecting hormone secretion probably by influencing insulin sensitivity and glucose metabolism in various tissues (464). The effect of plant fiber on the rate of carbohydrate absorption has been examined in greater extent than other possible mechanisms that would affect glucose metabolism. However it seems unlikely that it would account solely for the observed effects. Many plant fibers and fiber rich foods appear to delay gastric emptying thus providing carbohydrate to the intestines for digestion and absorption more slowly than after a low fiber meal. The gelatinous globules presumably formed in the small intestine by water soluble fibers may retard starch and sugar hydrolysis by separating carbohydrate to the distal jejunum where carbohydrate is less efficiently absorbed due to lower concentrations of disaccharidases. In addition, fiber may inhibit digestive enzyme activities and slow the digestion and absorption of carbohydrate, proteins and fats. Since our study diets contained high concentrations of sucrose and relatively standard amounts of natural fiber, the effect of sucrose content in a diet of normal composition was adequately measured. Thus, the differences in our results, as compared to other investigators most probably reflect the structural components of the diet and are much more representative of the specific effect of sucrose intake on a diabetic population consuming disaccharide in a non-hospital, home situation.

Restricting sucrose intake in diabetic patients is based on the premise that sucrose might cause rapid oscillations of blood glucose, result in glucosuria and cause interference with metabolic control (2). Our studies demonstrate no such oscillation. The fact that urine 24 hour glucose concentrations decrease when isocaloric carbohydrate diets, which have high sucrose content, are given is extremely interesting. Diets which are isocalorically similar should affect urinary glucose output in a similar fashion. It can only be concluded from our study that sucrose enriched diets have no effect on causing an increase in urinary glucose output. The decreased excretion of glucose must mean that the carbohydrate moiety is metabolized and is not excreted by the patient. The decrease in glucosuria on the low sucrose diet is expected since the total carbohydrate intake of these patients was slightly less and the refined sucrose intake was minimal (the majority of carbohydrate being starch). The observation that the control group also demonstrated a slight decrease in 24 hour glucosuria suggests that hospitalization could also lower glucose excretion. Though this could be a factor, we feel that it could not alone lead to the significant reduction in 24 hour urine glucose excretion observed in patients consuming the study diets. The lack of any significant increase in serum glucose concentration or urinary glucose output while consuming the high sucrose diet points to the possibility that the increased proportion of calories as sucrose, may be utilized metabolically to produce triglyceride. Examination of the data obtained on fasting triglyceride concentrations indicates large increases in circulating triglyceride levels after high sucrose intake and relatively little change in these

levels following low sucrose intake (Figure 17). These results are substantiated by other reports which stress the effect of sucrose content in the diet on circulating blood lipid. Nikkila (465), MacDonald (30, 34) and Kuo (23) studied normal and non-diabetic subjects finding that increased dietary sucrose intake significantly alters the metabolism of triglyceride in human subjects. Studies by Sunt (466), Cohen (28), and Chlouverakis (142) have demonstrated that sucrose appeared to be responsible for the increased levels of circulating lipids observed in some diabetic patients. These studies suggest that the absence of fat in the diet was being met by the synthesis of lipid due to carbohydrate induction. Further evidence to support this possibility was seen in the altered fatty acid pattern observed in patients on a high sucrose regimen in which the observed increase in oleic acid is consistent with the conversion of carbohydrate to fat (23).

The effect of sucrose induced carbohydrate hyperlipemia was challenged by Albrink and Newman (38) who ascertained the effects of high and low fiber diets on plasma lipid levels. They gave two high carbohydrate diets, one with high fiber, the other with low fiber and suggested that carbohydrate induced hyperlipemia does not occur if the carbohydrate diet is rich in dietary fiber. Our results refute these findings. We demonstrated a significant sucrose induced hypertriglyceridemic effect even though our diets had standard fiber contents. Mann et al. (33) were able to reduce serum triglyceride levels an average of 22% in non-diabetics through voluntary sucrose restriction. Though we were able to reduce sucrose intake to 3 g/day, much lower than possible in the self-selected diets of Mann, we observed no reduction in

triglyceride levels with restricted sucrose intake. Fasting triglyceride levels remained virtually the same as those observed when the 120 g/day sucrose control diet was given. Postprandial triglyceride concentrations significantly increased from control diet values when patients consumed the low sucrose diet, probably reflecting the slightly higher lipid content of the low sucrose diet (Figure 18). Again, no effect on fasting triglyceride levels were observed, either as a result of sucrose restriction or altered postprandial lipid values. With this in mind, the introduction of sucrose into the diabetic diet at levels of less than 120 gm/day might be safe. This might allow easier compliance with the moderate fat restriction now recommended for the population at large which is even more compelling for the diabetic patient.

The changes in triglyceride concentration were not paralleled by a rise in serum cholesterol levels in patients on either study diet (Figure 21). Both fasting and non-fasting cholesterol levels decreased relative to control diet values with consumption of the high sucrose diet. This indicates that high carbohydrate ingestion does not increase cholesterol levels, a principal advantage in diabetic patients when carbohydrate intake is increased (6). The observation that high carbohydrate, high sucrose diets calorically similar to the control, resulted in a lowering of cholesterol levels indicates that the sucrose content of the diet has no effect on cholesterol levels in the blood. This is further illustrated by the fact that although sucrose content was reduced to an extremely low level, no improvement in serum cholesterol concentration was realized. Again, a dietary regime in which small amounts of sucrose are included could be beneficial because the

increased palatability would lead to greater dietary compliance.

Though HDL-cholesterol concentrations are often insensitive to diet (467), levels increased significantly while patients consumed the low sucrose diet. HDL-cholesterol levels showed no change, or in some cases, a slight decrease in patients on high sucrose diets (Figure 19). This inverse relationship between HDL-cholesterol and plasma triglyceride concentration has been extensively documented (453-455). Blum *et al.* (457) showed that dietary substitution of carbohydrate for fat is accompanied by an increase in VLDL, a decrease in HDL and a reduction in the ratio of HDL<sub>2</sub> to HDL<sub>3</sub>. The negative association with total triglyceride concentration is stronger for HDL<sub>2</sub> than HDL<sub>3</sub>. There also appears to be a sex related difference in the inverse relationships between HDL-cholesterol and VLDL triglyceride though this difference was not tested in this study (456). In both study groups, HDL-cholesterol levels may be lowered since these individuals were diabetic. The HDL-cholesterol results obtained after low sucrose intake represent a small metabolic benefit. It must be pointed out that high sucrose diets did not result in any significant deterioration of HDL-cholesterol plasma levels (Figure 19).

The recognition of HDL-cholesterol as a negative risk factor for the development of premature cardiovascular disease is well known (454, 455). Two general mechanisms have been proposed by which HDL is a primary deterrent for the development of cardiovascular disease. The first mechanism involves the proposed role of HDL in cholesterol transport in which HDL facilitates removal of cholesterol from peripheral cells to the liver for ultimate removal by the body. The second mecha-

nism involves the competitive inhibition of LDL binding to peripheral cells by HDL. Decreased plasma levels of HDL, in both cases, could be postulated to cause an increased cholesterol build up in the body. The ingestion of low sucrose diets did result in a significant increase in serum HDL cholesterol levels, the only benefit obtained by sucrose restriction. However, in contrast to Blum (457) who demonstrated a decrease in HDL-cholesterol following increased carbohydrate intake, this study shows that ingestion of high sucrose diets caused minimal reductions in HDL-cholesterol levels.

Lipoprotein concentrations changed predictably in conjunction with the changes in the lipid concentrations discussed above (Figure 27). The greatest change occurred in the  $\beta$  and pre- $\beta$  lipoprotein concentrations during consumption of the high sucrose diet. A large increase in pre- $\beta$  lipoprotein concentration with reciprocal decrease in  $\beta$  lipoprotein concentration was observed. Since little significant change was observed in these fasting parameters while low sucrose diets were consumed,  $\beta$  and pre- $\beta$  lipoprotein levels also changed little in this study group.

The dramatic rise in fasting serum triglyceride concentration and the decrease in urinary glucose output with consumption of the high sucrose diet indicates that retained carbohydrate might be used to produce triglyceride in the liver de novo. Increased triglyceride synthesis may be due to a metabolic condition peculiar to the diabetic. Normal patients, in many instances, did not demonstrate the extreme fluctuation in triglyceride concentration seen in the type II diabetics in this study (24, 29, 31, 23).

If indeed the metabolism of sucrose is different in diabetics, then differences might be reflected in the concentration of hormones required to metabolize the disaccharide. Insulin and glucagon results obtained from patients consuming the different study diets confirm this speculation. In general, the observed changes in hormone concentrations in these patients when high and low sucrose diets were administered were quite different. Relatively high fasting insulins were observed in all patients upon admission to the study. This was generally expected since many type II diabetics may demonstrate insulin resistance and thus elevated fasting insulin concentrations (446). When high sucrose diets were administered, fasting and non-fasting insulins increased dramatically. This effect of hyperinsulinemia with increased sucrose ingestion has also been observed in other reports (33, 34, 39, 40, 46). Low sucrose diets however, resulted in relatively little change in fasting and non-fasting levels.

Insulin increments, the difference between fasting and non-fasting insulins, clearly increased when high sucrose diets were consumed, as seen in Figure 25. This significant increase in non-fasting insulins over the control diet clearly demonstrates that dietary sucrose content affects insulin secretion in these hyperlipemic diabetics. As an additional consequence, Beck-Nielsen and Pederson (39) demonstrated that high sucrose feeding can result in a 36% decrease in specific cell binding fractions for insulin. A decrease in insulin binding is associated with reciprocal increases in plasma insulin concentration. This situation may contribute, along with increased insulin secretion, to the hyperinsulinemic status of these patients.

Another mechanism by which this hyperinsulinemia could occur is explained by adaptive changes in the intestine in response to increased sucrose intake. Sucrase activity was greater in humans who consumed isocaloric diets containing sucrose as compared to glucose (41). The concept of specific dietary regulation of jejunal sucrase activity has been investigated by Rosenweig and Herman (136). Reiser demonstrated that a high sucrose diet caused a large secretion of GIP as compared to isocaloric diets which were composed of starch (44). He also found the disaccharidase enzyme and GIP secreting cells were in close approximation on the brush border of the intestinal wall. Since disaccharidase activity has been shown to occur in proximal areas of the small intestine, the sucrose absorbed in this part of the small intestine has been found to be an important secretagogue for GIP (138). GIP is known to be an important stimulator of insulin release (48, 139). The more rapid absorption of glucose after a sucrose load in these adapted individuals may lead to an enhanced secretion of GIP. Overall, the effect of disaccharide in producing hyperinsulinism is probably the result of increased insulin resistance due to loss of peripheral binding sites and of increased insulin secretion due to greater stimulation of GIP hormone.

In this study, hyperinsulinism develops at precisely the same time as the development of hypertriglyceridemia. These results directly contradict the findings of Hayford and Danney (36) who found negative correlations between triglyceride and insulin levels. However, our results are in agreement with the findings of Olefsky and Eaton (37, 46) who demonstrated a positive effect of insulin upon the production of



hypertriglyceridemia. Evidence that insulin promotes VLDL-triglyceride secretion was shown by Salans (447). Using perfused liver slices, Letarte and Fraser (448) demonstrated that insulin promotes the incorporation of <sup>14</sup>C glucose into fatty acid moieties of hepatic lipid and increases their subsequent release. Further support for the concept of increased dietary sucrose causing a hyperlipemic response, through increased triglyceride synthesis, was demonstrated by Michaelis (140) who found lipogenic enzymes in the rat liver were induced to a greater extent when animals were fed sucrose than when consuming equivalent amounts of glucose. Bruchdorfer (141) found increases in hepatic fatty acid synthetase enzymes after high sucrose diets. Olefsky (37) believed, as the results of our own study indicated, that hyperinsulinemia acts upon the liver to accelerate hepatic triglyceride synthesis and secretion resulting in elevated triglyceride levels.

The changes observed in circulating glucagon levels in response to different sucrose content diets probably occur due to the insulin response previously discussed. This assumption is reasonable since the cells of the pancreas belong to a family of cells which are sensitive to insulin concentration (451). Unger (42) has postulated that reduction in sensitivity of the cells to chronic elevations of insulin resulted in decreased suppressibility of glucagon release. Our studies do not confirm this postulate since the circulating levels of glucagon decreased with increasing insulin concentration.

Other reports have supported the concept of increased triglyceride synthesis causing hypertriglyceridemia in response to dietary carbohydrate type and load (50-52). Because a removal defect of triglyceride

from the plasma could also be responsible for this patterned lipemic response, the possibility of the existence of a defect in removal was determined by in vivo techniques utilizing radiolabeled glycerol. Two possibilities could exist to increase plasma triglyceride levels, synthesis could saturate the removal mechanism, or alterations could occur in the removal of triglyceride from the plasma caused by a defect in the lipase enzymes or the substrates on which the enzymes act.

Results of this study agree with those of Nikkila and Kekki (57) who found the enzyme system (lipoprotein lipase) followed Michaelis Menton kinetics and could be saturated. We found that patients who consumed the high sucrose diet showed no statistically significant change in  $K_m$  when compared to those values obtained while consuming the control diet. These results do not agree with those of Adam's and Kissebah (59) who demonstrated an increased  $K_m$  but a decreased  $V_{max}$  in their patients who consumed high carbohydrate diets. However, it must be emphasized that the  $V_{max}$  values obtained from our patient population on both high and low sucrose diets appeared to be still lower than those obtained from normal patient populations (59). Patients who consumed the low sucrose diet showed no change in  $K_m$  or  $V_{max}$  from the control values and it must be assumed that sucrose restriction offered no improvement in peripheral triglyceride removal efficiency.

The apparent inability of these patients to activate their triglyceride removal mechanisms, by lowering  $K_m$ , after sucrose induced triglyceride production is intriguing. This possible faulty removal mechanism, not observed in normal patients (402) could be an additional causative factor in the hyperlipemic response observed after high

sucrose intake. The inability to activate the triglyceride removal system, when high sucrose diets are consumed, could be related to a change in VLDL apoprotein cofactors first identified by Bar-On (64). Endogenous lipoproteins are presumed to be the natural substrates for the lipoprotein lipase enzymes. The results obtained in this study could be a reflection of two possible mechanisms: the VLDL isolated from the different groups may not be identical or the lipoprotein lipase system may contain two different enzymes which have different characteristics.

Experiments performed to substantiate sucrose induced changes in the apoprotein constituents of the VLDL demonstrated that high sucrose diets resulted in an increase in apo CIII<sub>1</sub> protein, an inhibitor of lipoprotein lipase (218) (Figure 42). Furthermore, apo CIII<sub>1</sub> levels were significantly correlated with triglyceride concentration in this study (Figure 44). Apo CIII<sub>2</sub> levels were found to decrease in these same patients. Apo CII was slightly decreased or remained the same.

Overall, the ratio of apo CII/apo CIII<sub>1</sub> decreased and significant correlations between this ratio and triglyceride concentrations were observed (Figure 46). These results corroborate the findings of Gabor and Spain (63), and Lambert (68) and Catapano (69) who believed the relative amounts of apo CII as compared to apo CIII<sub>1</sub> enhanced the interaction between lipoprotein lipase and triglyceride within the cell surface monolayer. These results also substantiate the postulate put forth by Alaupovic (154) who proposed that the type of apoprotein found in VLDL reflected the nutritional and metabolic status of the indivi-

dual.

We did not observe an increase in apo CII levels during the high carbohydrate sucrose intake as suggested by Falko (67). The initially low apo CII concentration observed in these patients may be best explained by the depressed HDL-cholesterol levels found in all patients who entered the study. Havel (271) demonstrated that chylomicron and VLDL metabolism involve net transfers of apo CII protein from HDL to VLDL and back to HDL. The nascent VLDL that is released from the liver is stabilized by apo CII from circulating HDL. While the transfer of apo CII back to HDL is proportional to triglyceride hydrolysis (275), insufficient levels of HDL, and a decreased capacity to supply VLDL with apo CII, will prevent normal triglyceride hydrolysis resulting in increased triglyceride concentration.

Speculation on the effect of apo CIII<sub>1</sub> and CIII<sub>2</sub> on VLDL metabolism was suggested by Shelburne and Windler (281, 282). These investigators observed that apo CIII<sub>1</sub> not only inhibited lipoprotein lipase but also demonstrated a pronounced inhibitory effect on hepatic uptake of VLDL. Bar-On and Roheim (64) noted that apo CIII<sub>1</sub> and apo CIII<sub>2</sub> were different only in sialic acid content. It is possible that this glycoprotein is similar to other sialated glycoproteins which demonstrate correlation between sialic acid levels and hepatic receptor affinity (283). Apo CIII<sub>1</sub> may provide a mechanism for denying lipoproteins hepatic access until appropriate peripheral metabolism has occurred. If apo CIII<sub>1</sub> were not present, ineffectual recycling of the lipoprotein by the liver would occur. The uptake of remnant VLDL will be decreased if apo CIII<sub>1</sub> is not removed in the periphery and could

result in hyperlipemia. In contrast, apo CIII<sub>2</sub> does not inhibit lipoprotein lipase but can diminish the effect of apo CIII<sub>1</sub> by binding to the same active site but still allowing the lipase to be activated by apo CII (269, 270). It has been observed that the sialic acid content of these two proteins is determined by their residency time in the Golgi apparatus of hepatic cells (217). The fact that apo CIII<sub>1</sub> has less sialic acid residues indicates the amount of time spent in the Golgi is less than that for apo CIII<sub>2</sub>.

Increased insulin found in patients who consumed the HSD stimulates triglyceride production. Since hepatic triglyceride synthesis is increased, VLDL production is increased as well. The residency time of total apo CIII in the Golgi apparatus is decreased resulting in an increased proportion of apo CIII<sub>1</sub> in these patients. Since apo CII is also depressed, because of lower circulating HDL levels, the decreased ratio of CII/CIII<sub>1</sub> apoprotein does not stimulate increased lipoprotein lipase activity even in the presence of increased circulating triglyceride levels. Finally, since the triglyceride and apo CIII<sub>1</sub> are not removed as rapidly as normal, the VLDL circulates for an extended period of time. The observations above could account for the decreased uptake of a portion of partially hydrolyzed lipoproteins which contain relatively high levels of apo CIII<sub>1</sub>. Patients who consumed the low sucrose diet exhibited little change in the triglyceride removal Km values from control to study diet. In these patients apo CIII<sub>2</sub> levels increased while apo CIII<sub>1</sub> levels decreased or remained the same. Thus, inhibition of lipoprotein lipase can not be postulated as above. In fact, these results would point to an increased removal of VLDL by

plasma lipase. However, studies from several laboratories suggest that human plasma lipoprotein lipase activity is a combination of several lipases which have different properties (266, 458). It has been found that insulin levels could play a role in specific lipase activity (266). Certain lipoprotein lipase enzymes appear to be more susceptible to insulin concentrations than others. Since the low sucrose study group had slightly lower or unchanged insulin levels, it could be possible that one set of enzymes remains inactive because of lower insulin concentrations resulting in no change in  $K_m$  observed in this patient population. Also, Persson (61) demonstrated that patients who had normal or increased cholesterol levels with steady triglyceride levels would have normal lipolytic activity. Cholesterol levels remained stable or increased slightly in the LSD study patients. A possibility exists that the kinetic data generated by low sucrose diet individuals reflects a two enzyme system. One enzyme is inhibited, resulting in an increased  $K_m$ , while the other is stimulated, to clear triglyceride more rapidly, resulting in a decreased  $K_m$ . This compensatory mechanism allows for little observable change to occur in both plasma triglyceride levels and  $K_m$  values in these patients.

The data on the effect of sucrose enriched diets on apo E levels were partially corroborated by Mahley (226) who demonstrated that dietary changes could elicit an increase in apo E concentration. His diets contained high amounts of cholesterol however, and to date, the specific effect of sucrose on apo E is unreported. This study demonstrated no significant change in total apo E, but a significant increase in one of the isoprotein subunits, apo EIII, was observed in

high sucrose diet patients (Figure 43). Low sucrose diets demonstrated no effect on isoprotein composition of apo E. Increase in the apo EIII subunit would seem plausible since Schneider (321) has demonstrated that apo EIII is one of the main protein cofactors needed for proper hepatic uptake of remnant VLDL. Studies by Havel and others have demonstrated that patients with type III hyperlipoproteinemias, characterized by a dysfunctioning apo EIII protein, generally were observed to have decreased hepatic uptake of intermediate density lipoprotein (223, 227-228). The increase in apo EIII observed in our patients may be a mechanism used by the individual to produce accelerated clearance of these remnant VLDL lipoproteins and aid in triglyceride clearance from the plasma.

Other experimental data corroborating the above mechanism was obtained from four patients whose response to high sucrose consumption differed from the others. Their hyperlipoproteinemic pattern shifted from type IV to type III (Figure 49). These patients demonstrated a complete absence of the apo EIII isoprotein subunits (Figure 50). The remnant VLDL have prolonged circulating half-lives due to decreased apo EIII and are devoid of most apo C proteins due to the action of lipoprotein lipase. Therefore, the remnant VLDL of these patients have more  $\beta$  character since there is more apo B as total apoprotein. Since the charge of this lipoprotein now resembles that of the lipoprotein, electrophoretic migration is similar or identical to the broad  $\beta$  pattern. Loss of apo EIII inhibits the uptake of these remnants by the liver and increases their circulating half-lives. Since these remnants still contain nominal amounts of triglyceride, this

mechanism increases the hyperlipemic response observed in these patients.

The above assumptions cannot be made to explain the change in the lipoprotein pattern when high sucrose diets are consumed by this patient subgroup. However, it could be possible that a small amount of apo EIII is produced by these patients. In these study patients, increased synthesis of triglyceride by the liver stimulates VLDL production to such an extent that apo EIII synthesis lags behind and the VLDL produced either have extremely low concentrations of the apo EIII protein or are totally lacking in the structural component.

Therefore, in general, the results of this series of experiments demonstrate that sucrose causes increased synthesis of triglyceride, but also alters the apoprotein content of nascent VLDL released by the liver, and possibly functions to decrease removal of peripheral VLDL by lipoprotein lipase.

Another line of approach to study the effect of sucrose consumption on type II diabetics is to investigate platelet adhesiveness and platelet aggregation. Increased platelet aggregation has been observed in diabetics (89-92). Since hyperlipemia has also been demonstrated to increase platelet function (97), a natural relationship exists between the effects of sucrose on circulating lipids and platelet aggregation. Renaud (387) was the first to demonstrate the effect of dietary-induced hyperlipemia on platelet aggregation. His results, and the results of this study, demonstrate that increased hyperlipemia causes increased platelet aggregation (Figure 53). Szanto et al. (101) found that increased insulin levels correlate well with increased



platelet adhesiveness. Our studies confirm these findings as well. The low sucrose study patients, who demonstrated insignificant alterations in insulin concentration, also had unaltered platelet responses to diet. But patients consuming high sucrose diets had increased insulin levels, platelet sensitivity and platelet aggregation. Farbiszewski et al. (389) also observed a distinct effect of lipoproteins on platelet aggregation. In our study, patients who consumed high sucrose diets had increased circulating VLDL and increased platelet responses to aggregating agents. It is possible that lipoproteins interact with platelets in the circulation altering platelet membrane components. Bolton (98) found the  $\beta$  lipoprotein interacts with certain receptor sites on the platelet surface resulting in altered platelet mobility. Since VLDL contain similar apoprotein constituents, extended circulating half lives of these lipoproteins, due to decreased clearance, might allow the platelets to incorporate these lipoproteins or their components into their membranes.

A mechanism for the increased platelet sensitivity observed in these patients, other than changes in membrane fluidity, was reported by Sinha (398). This investigation demonstrated that increased lipid incorporation into the platelet membrane is associated with decreases in the activity of membrane bound adenylyl cyclase and depressed cAMP levels. Insulin, a known inhibitor of cAMP production, is elevated in the patient who consumed high sucrose diets. Since cAMP is a potent inhibitor of platelet aggregation, a decrease in cAMP levels induced by both increased lipid incorporation and increased insulin concentrations could cause increased platelet aggregation in these hyperlipemic

patients. Thus, the impact of the high sucrose diet through either mechanism results in the development of increased platelet aggregation and responsiveness of platelets to aggregating agents.

The deleterious effects of sucrose seem to occur only when extremely high levels of the disaccharide are ingested. Our results show that even when sucrose intake is reduced to a point which would be impossible to obtain in an outpatient dietary regime, lipid production, blood glucose, urinary glucose output, VLDL composition and platelet aggregation are altered minimally or unchanged when compared to a diet consisting of 120 g/day sucrose intake. Fasting triglyceride levels increased only with the highest sucrose levels, demonstrating that exquisite sensitivity of hypertriglyceridemics to another normally used nutrient, alcohol (52) does not seem to apply for the more ubiquitous disaccharide. But the general conclusion is one that we share with Frank Nutall that "Actually, there is little scientific basis for the complete exclusion of refined sugars from a diabetic persons diet" (51).

#### A. Conclusion and Future Directions

This study was designed to ascertain the effects of sucrose consumption in a diabetic population which is predisposed to hyperlipemia. This was a relevant problem since many studies, as alluded to previously, presented conflicting results concerning the effect of sucrose on blood lipid levels, glucoregulatory hormones and lipid transport mechanisms. Diabetic dietary therapy has shown a pronounced shift toward increased carbohydrate consumption. Therefore, the ingestion of refined sugars, especially sucrose, which remains unquantitated and

hidden in certain foodstuffs, has possibly increased. Also dietary acceptance is an important factor in any chronic therapy. The palatability that small amounts of sucrose adds to a diet lends an increased likelihood of compliance with the dietary regime and ultimately, improved blood glucose control. Thus, it is mandatory to know the effects of graded amounts of sucrose in natural mixed diets and the chemical response of these type II diabetics to such diets.

Ideally, an experimental search for a cause of hyperlipemia would be to investigate whether the suspected factor actually produces the disease. But, none of the ordinary laboratory animals can be made to develop the hyperlipemic state as seen in man, and of course, it is out of the question that one should attempt to do so in humans. The alternative is short term experiments in man in which attempts to reproduce some of the characteristics of hyperlipemia are made. The experiments designed here enabled us to answer a number of questions concerning the effects of sucrose as an etiological factor in diabetic control and the development of hypertriglyceridemia. The primary goal was to see whether sucrose produced, not simply an increase in triglyceride concentration, but as many other potential abnormalities associated with poor diabetic control.

This study demonstrated that under controlled conditions, no special sensitivity to variation in sucrose restriction can be observed. Essentially, no effect of widely variable sucrose levels on blood glucose and glucosuria was identified. In addition, no deleterious changes in the levels of serum HDL-cholesterol or cholesterol were seen when diets rich in sucrose were ingested. Furthermore,

no improvements to normal levels could be detected when sucrose restricted diets were given to this patient population. On the other hand, high sucrose consumption had a most significant effect resulting in a great increase in fasting triglyceride levels. The changes in the plasma lipoprotein constituents reflect, predictably, changes that occurred in the lipid constituent of the plasma.

Glucoregulatory hormones showed a pronounced increase in circulating levels of fasting insulin with significantly larger insulin increments and a reduction in fasting glucagon levels with consumption of high sucrose diets. This suggests that the hypertriglyceridemia associated with increased sucrose intake is due to increased triglyceride synthesis. Kinetic studies of the plasma triglyceride pool demonstrated that sucrose rich diets had no effect on the affinity of the removal system for plasma triglyceride. Thus the lack of any stimulated increase in peripheral triglyceride removal, while consuming (HSD) could be a contributing factor to the hyperlipemic response. Again, complete sucrose restriction and complex starch replacement offered little improvement in fasting insulin concentration and plasma triglyceride removal mechanisms over those obtained by intermediate (control) sucrose levels.

Studies of the very low-density lipoprotein (VLDL), the main carrier of triglyceride in the fasted state, and the apoproteins that comprise them were carried out in order to identify whether the proportions of dietary sucrose affect these transport proteins. It was found that high sucrose consumption alters the apoprotein content of the VLDL. The apo CII/CIII ratio decreased which could have resulted

in decreased stimulation of the lipoprotein lipase system and an altered removal of VLDL from the plasma pool. The increase in apo CIII may be a reflection of either a decreased residency time in the hepatocyte Golgi apparatus of VLDL apoprotein components or an increased plasma residency time of the VLDL in the peripheral pool. The former is a reflection of a possible increased liver VLDL secretion due to increased triglyceride synthesis. The latter is a result of an elongated plasma pool half life as demonstrated by the extremely low K values observed in these patients.

We have also demonstrated that elevated sucrose intake associated with hyperlipemia results in increased platelet aggregation and sensitivity of platelets to aggregating agents, never before reported in the literature.

The minimal effects of a daily consumption of sucrose in quantities of less than 100 gm per day suggests that its cautious introduction in mixed meals might allow easier compliance with the moderate fat restriction now recommended for the diabetic population at large. Furthermore, by contributing to palatability, dietary sugar will ease the life of many diabetics perhaps contributing to better adherence to diet as part of the whole health care program in diabetic treatment. In view of this, we strongly recommend that sucrose intake from all sources be monitored in a valid manner. The implementation of this study would entail a radical new outlook in dietary therapy for the diabetic. Perhaps, it is reasonable to suggest that sucrose content of packaged, frozen, and canned foods be determined and that a campaign be launched to inform the susceptible population.

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The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Ph.D.

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