EFFECT OF LOCUST BEAN GUM ON SERUM CHOLESTEROL, TRIGLYCERIDES AND HISTOPATHOLOGY OF LIVER, AORTA AND HEART TISSUES IN ALBINO RATS

By

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A C K N O W L E D G E M E N T

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I. INTRODUCTION

'Atherosclerosis resulting in Ischaemic heart diseases is the greatest epidemic that man-kind has ever faced' (World Health Organization, 1977). A review of the statistics of the Government General Hospital, Madras, shows that the myocardial infarction is the number one killer (about 40 per cent) among the medical emergencies (Senthilnathan, 1979). With better living conditions and urbanisation of society, atherosclerosis and coronary heart disease are assuming increasing importance in our country as it is in the more privileged countries (Gopalakrishna, 1979).

Specialists from the world over agree that the basic cause of coronary heart disease is atherosclerosis (Heart News, 1977; 1978). Atherosclerosis recognised as the leading cause of death today, and one of the greatest contributors of physical and mental incapacity remains an enigma in modern medicine (Levadas *et al.*, 1979; Senthilnathan, 1979; Reddy, 1979). It is a multifaceted disease of the large and small arteries characterised by plague like intimal deposits, composed of collections of lipids, complex carbohydrate, blood and blood products, fibrous tissue and calcium deposits and associated with medial changes (Reddy, 1979).

In the light of several experimental, epidemiological and clinical investigations, it is generally accepted that the risk of developing coronary heart disease in population groups is positively correlated with the level of serum cholesterol (Padmavati, 1977; Stamler, 1978; Gopalakrishnan, 1979 and Marmol, 1979). This risk is relatively small at levels less than 220 - 250 mg/100 ml but increases progressively with each increment in the plasma cholesterol above this level depending upon age (American Journal of Clinical Nutrition, 1973; and Heart News, 1977).

There is also a positive relationship between plasma cholesterol, low density lipoprotein, very low density lipoprotein, triglycerides and incidence of coronary heart disease (British Heart Journal, 1978; Gordon et al., 1977 and Kumari et al., 1976). It has been observed in several well-documented studies is that there is an inverse relationship between plasma level of high density liporprotein and high density pipoprotein cholesterol and incidence of coronary heart disease and other forms of occulusive vascular disease (Miller et al., 1977; Gordon et al., 1977; Bradly et al., 1978).

Serum cholesterol can be successfully reduced in a great majority of people by dietary modification alone (Heart News, 1976). The role of nutritional factors such

as the type and concentration of dietary fat, the type of carbohydrate and dietary fibre in altering plasma cholesterol have been widely publisized (Zeiser, 1973 and Kritchevsky et al., 1975; WHO Chronicle, 1977). Many of these dietary constitutents have been used to check hypercholesterolemia though not as liberally as drugs. It is now widely accepted that vegetable foods can lower serum cholesterol and are less atherogenic than animal foods (Carroll et al., 1975; Yadav et al., 1977).

Pulses and legumes which are the major sources of protein in India, have been of interest because of their availability to modify cholesterol concentration. Cereal-legume combinations are a hall mark of the traditional diets of most of the developing world. Soyabean, lima bean, chick pea (Cicer arietanum), cow pea (Vicia fabia, Mungo bean (Black gram), Guar (Cyamposis tetragonoloba), gum arabic and locust bean (Ceratonia siliqua) have been shown to possess hypocholesterolemic properties (Mathur et al. 1984 and 1971; Madhava, 1971; Vijayagopal, 1973; Soni et al. 1978; Nisha et al. 1980; Zavoral, 1981).

The use of Locust bean gum as a dietary constituent in India is not very popular though the bean is available. However, it is used as stablizer in ice creams and in similar food products. The possible use of this gum in higher quantities, was thought of interest from the point

of view of its effect on lowering the cholesterol and trigylceride levels. Hence the main objective of this investigation.

The investigation has been designed to study the effect of Locust Bean Gum (*Ceratonia Silicua*) incorporated at varying levels in the diet of albino rats on cholesterol levels, triglyceride levels and histopathological changes.

II. REVIEW OF LITERATURE

The literature pertaining to this study on the "Effect of Locust Bean Gum on Serum Cholesterol, Triglycerides and Histopathology of Liver, Aorta and Heart Tissues in Albino Rats", is reviewed in the following sequence:

A. Pathological changes in the Atherosclerotic tissues.

B. Risk factors predisposing to Coronary heart disease.

C. Role of legumes in preventing hypercholesterolemia.

A. Pathological Changes in the Atherosclerotic Tissues:

Atherosclerosis is a pathological entity and multi-faceted disease of the large and small arteries, characterised by plague like intimal deposits, composed of collections of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits, and associated with medial changes (Reddy, 1979). The disease may involve any vessel, but the commonest are the aorta, the coronaries, the cerebral vessels and the renal vessels (Ananthasubramaniam, 1979).

The lesions are first seen as fatty streaks or

spots on the inner linining of arteries (Anderson et al. 1968). They appear as short, thin, slightly raised yellow lines (Davidson et al. 1975), consisting of free cholesterol, cholesterol esters and triglycerides in proportions approximate those of circulating blood lipids (Anderson et al. 1969), and gradually harden into fibrous bulges called plaques as it ages. As the plaques become numerous, the arteries become roughened and narrowed, the elasticity is lost and the flow of blood through the vessels is curtailed (Mitchell, et al. 1968; and Robinson et al. 1974).

The largest class of lipid in atherosclerotic plaques is cholesterol esters, in which the fatty acid pattern resembles that of plasma cholesterol ester, with linolate the most abundant followed by oleate (Lawrie et al. 1964; Smith et al. 1968). Fibrinogen is the only other plasma protein present. Phospholipids are reduced in plaques, compared with plasma lipoproteins, and the predominant type is sphingomyelin.

The intracellular lipids of fatty streaks are mostly cholesterol esters, with a bizarre of fatty acid pattern. They contain a high proportion of oleic acid, low linoleic acid and raised $C_{20:3}$ (Smith et al. 1968; Lang and Insull, 1970). Fatty streaks contain less low density protein than the normal intima (Smith and Slater, 1972), and it would appear that their lipids have mostly been esterified in situ.

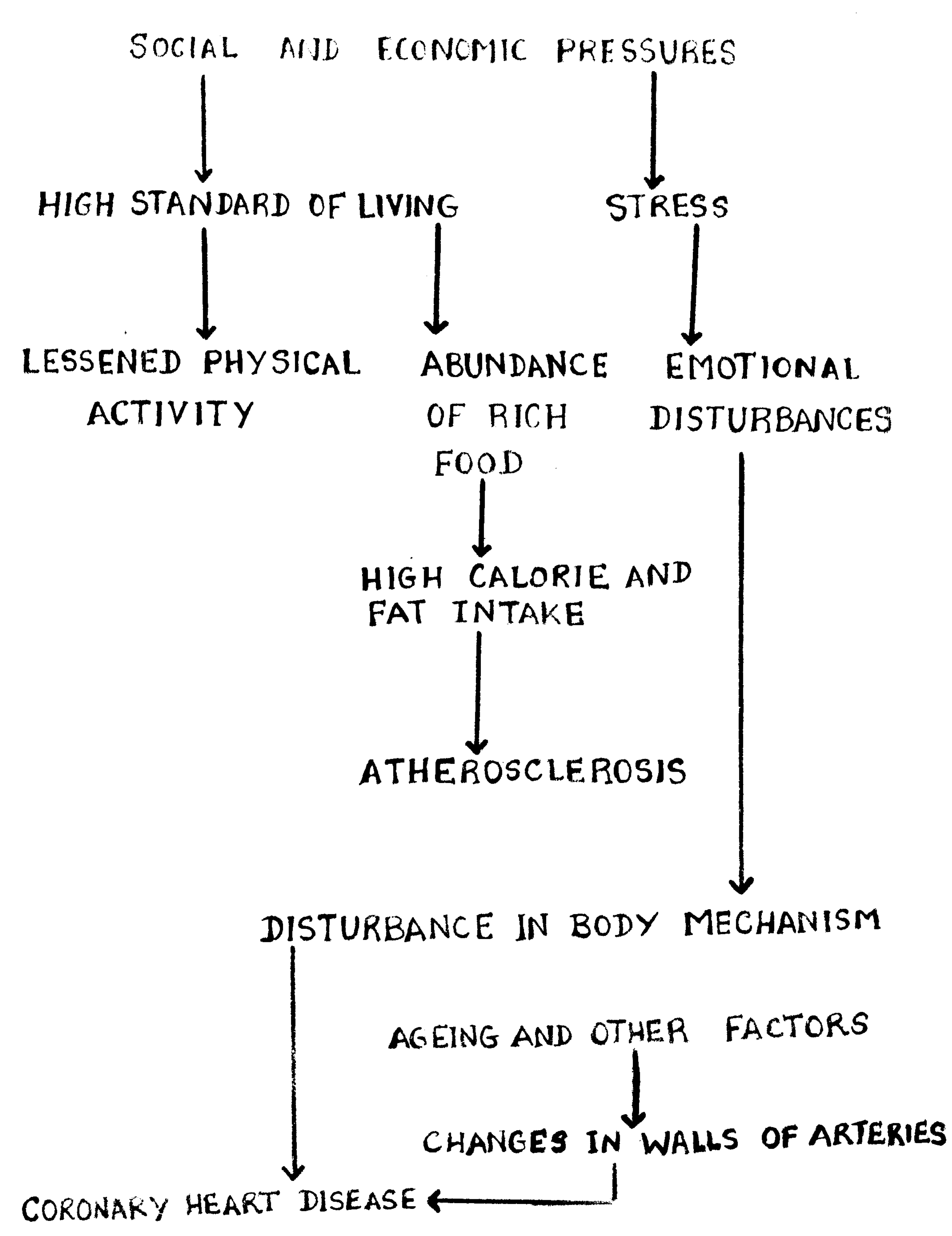


FIG. 1. RISK FACTORS IN HEART DISEASE

B. <u>Risk Factors Predisposing to Coronary Heart Disease:</u>

As could be seen from Figure 1, a chain of factors are in the formation of atherosclerosis which leads to coronary heart disease (Devadas *et al.* 1979).

1. <u>Non-Nutritional risk factors:</u>

a) <u>Hypertension:</u>

Hypertension causes an increased chance of coronary heart disease, or heart failure, stroke, blindness and kidney failure (WHO, 1976; Heart News, 1978 and Bhatia, 1978). According to Stanley (1977), this elevated blood pressure seems to increase the production of cholesterol in the body. Obese hypertensive persons experience a greater risk of coronary heart disease and mortality than persons with either hypertension or obesity alone (Lowenstein, 1974).

b) <u>Hypercholesterolemia:</u>

The elevated risk of coronary heart disease is linearly related to levels of plasma cholesterol(Barines and Kodicek, Nutrition Review, 1972; Deoda, 1973; Miller and Miller, 1975; Barr *et al.* (1978).

c) <u>Hyperlipidemia:</u>

Populations which consume a diet high in saturated

fats have a higher incidence of hyperlipidemia and coronary heart disease than those who do not (Deutsh, 1976 and Levy et al. 1978).

d) Diabetes mellitus:

Diabetics are particularly prone to hypertension and proliferative lesions of the small blood vessels (Davidson et al. 1975). Garcia et al (1974) have shown an undoubted relationship between various degrees of glucose intolerance and the development of vascular disease. Gordon et al. (1982) are of the view that diabetes, is associated with obesity, elevation of plasma lipids, hyperinsulinaemia and possible hypertension. Again it is associated with increased calcification in the coronary artery and a combination of hypertension, raised coronary lesions (WHO Chronicle, 1974).

e) Obesity and physical activity:

Obesity is a predominant factor predisposing to hypercholesterolemia, hypertension and diabtes melletus, and therefore emerges as a distinctly important cause of atherosclerosis (WHO Chronicle, 1974; and Devadas et al. 1978; Ganapathy, 1979; Thavari, 1978 and Scharffenberg, 1980).

Vigorous physical activity may help in maintaining

normal weight and avoiding obesity (Blackburn, 1974; and Stamler, et al. 1972). Greater percentage changes in triglyceride, but not in cholesterol were observed in those assigned to the formal exercise programme (Shorey et al. 1976; Masironi, 1978 and Kramsch et al. 1981).

f) Other non-nutritional risk factors:

Cigarette smoking has been unequivocally shown to increase the risk of myocardial infarctions and sudden death, and there is a prompt and dramatic decline in the mortality on cessation (Heart News, 1980; Health Information Exchange, 1982). The nicotine present in the cigarettes (Aronowm et al. 1971; Ball et al. 1974; Kershbaum et al. 1961; Oliver et al. 1971; Castelli et al. 1966) and the carbonomonodixe of the heavy smoke are laid to be the causative factors (Gopalakrishna, 1979; Horn, 1978; Viswanathan, 1979; Ball, 1980 and William et al. 1981).

Alcohol increases the caloric intake, stimulates the production of beta-lipoproteins (Antia, 1973; Jhavari and Edwards, 1978 and Ineswell, 1978 and Mishkel et al. 1977). Caffeine in coffee and tea may act as cardio-vascular stimulants through effect on the adenyl cyclose system (Stare and Gortin, 1976; Thrash, 1977; Truswell and Lavy, 1978; Bonnott et al. 1970; Antia, 1973; and Yudkin, 1979).

Epidemiological and pathologic studies have shown that the progression of this disease is age related (Strong and Mc Gill, 1969; Haust, 1971).

Women in child bearing age are seen to be protected from the effects of coronary heart disease, which is attributed to ovarian hormone (Grande, 1975; and Gorden et al, 1978; Grigorove, 1971; Hjortland, 1976; Mc Namara et al, 1976). Women using oral contraceptives have been found to have a greater risk of developing cardiovascular disorders. Oestrogen containing oral contraceptive tend to rise plasma lipids and lipoproteins while the combination type oral contraceptive rise plasma cholesterol and triglycerides (Yeung, 1976; Mann et al, 1975; Bradley et al, 1978).

Environmental stress and intense emotions, tension, worries, raise the blood pressure, blood volume increases and the pressure of the heart chamber is increased (Shebas, 1977).

2. Nutritional and Related Factors Affecting Cholesterol and Triglyceride Levels.

(a) Type and concentration of fat:

Both quantity and quality of dietary fat have an

important bearing on serum cholesterol levels (Srikanthia, 1971; Grande, 1975 and Moore *et al*, 1977) and they also have a role in blood coagulation and plasma fibrinolytic activity. Populations which consume a diet high in saturated fats have a high incidence of hyperlipidemia and coronary heart disease (Williams *et al*, 1977). A diet predominent in cholesterol rich foods such as organ meats, eggs, and saturated fats and oils can significantly rise serum cholesterol levels (Turner, 1978). Emphasized widely is the fact that poly unsaturated fats like vegetable oil have a marked cholesterol lowering effect (Vergroesen, 1977; Sreekumar *et al*, 1978; Senthilnathan, 1979 and Jacotot *et al*, 1979).

b) Type of carbohydrate:

Ingestion of a diet containing clories in excess of the daily requirements for maintenance and physical activity in the form of refined carbohydrates leads to an elevated cholesterol and blood triglyceride levels (Burton, 1976). Sucrose, starch, fructose, galactose, and lactose are atherogenic in declining order. (Yudkin *et al*, 1969; Keys, 1971; Ahrens, 1974 and Kritchevsky *et al*, 1973).

Carbohydrates present in the cereals, pulses and vegetables in the form of cellulose and hemicellulose

interferes with the absorption of dietary cholesterol and therefore have a serum cholesterol lowering effect (Devadas et al, 1979). It is suggested that pectins decrease endogenous absorption of cholesterol and increases cholesterol turnover (Mokady, 1974).

c) Type of protein:

Animal protein has been associated with high and vegetable protein with low plasma cholesterol levels (Connor and Connor, 1972) and this differential effect is thought to be related to amino acid composition (Carroll, 1978; and Carroll et al, 1978). Nair et al, (1971); Mann (1977) and Hermus (1978) reveal that yogurt and milk have cholesterol lowering property.

d) Vitamins:

Nicotinic acid (Key, 1970; Kodicek, 1972; Truswell and Levy, 1978). Vitamin B_6 (Mc Cully (1979), Gruberg et al (1979) Truswell, (1978)), Vitamin C (Barnes Kodicek, 1972 and Huges, 1976), Initial studies by Spittle (1971), Vitamin E (Wilson et al, 1978), and co-enzymes (Science Digest, 1979) all have a role to play in atherosclerosis.

e) Minerals:

Trace elements like chromium, manganese, vanaolium, zinc, having a beneficial effect, while others such as lead and particularly cadmium have a detrimental effect (Masironi, 1973; WHO Chronicle, 1973; and Truswell, 1978) in Coronary heart disease.

f) Dietary fibre:

Dietary fibre the non-digestible component of our diet, has been implicated in recent years as causing a reduction in serum cholesterol, and it has been referred to as a natural hypocholesterolemic agent (Trowell 1972; Balmar et al. 1974; Domingo et al. 1978; British Department of Health and Social Security, 1975; Trowell, 1975 and British Nutrition Foundation Annual Lecture, 1978; Schwarg, 1977; Vijayagopal et al, 1973; Nisha and Sharma, 1980). The lipid lowering effect of fibre is advocated to be due to the increase in the excretion of bile salts and faecal sterols (Trowell, 1972; Mokady, 1974; Stary et al. 1978; Bremner et al. 1975; Kritchevsky, 1977; Lin Chen, 1979 and Chen and Anderson, 1979).

3. Other dietary factors and related agents reducing cholesterol levels:

Onions as such and essential oil of onions reduce

cholesterol levels significantly (Jain *et al*, 1978; Sainani *et al*, 1979). Garlic and garlic oil possess marked cholesterol lowering properties due to an active principle sulphide of allyl (Jain *et al*, 1978). Gujral *et al*, 1978) points out that ginger significantly reduces both serum and hepatic cholesterol levels and simultaneously increases cholesterol excretion in the faces.

C. Role of Legumes in Preventing Hyper Cholesterolemia:

Pulses and legumes which are the major sources of protein in India, have been of interest because of their availability to modify cholesterol concentration. Cereal - legume combinations are a hall mark of the traditional diets of most of the developing world. Soyabeen, lima bean, chick pea, cowpea, vicia fabia, and pisum sativum are some of the commonly consumed legumes (Mathur *et al*, 1968; Madhava, 1971; Vijayagopal, 1973; Soni *et al*, 1978).

Mathur and Coworkers (1979) demonstrated the cholester(lowering effect of chick pea in rats, rabbits and man. Siddiqui and Siddiqui (1979) suggested that the hypocholesterolemic principles in chick pea may be due to two isoflavins, namely biochanin, A and formononetin. Siddiqui (1979) and Sharma (1979), isolated Biochanin A an formononetin from chick pea, fed the recrystallised

compounds to albino rats and demonstrated their ability to reduce serum cholesterol. It appears that isoflavone with a methoxy group possesses hypocholestrolemic activity. A high ratio of glutamic acid to essential amino acid in cereal protein leads to hypocholestorolemia in man (Nutritional Reviews, 1980).

The guar gum obtained from Guar (*Ayamopsis tetragonoloba*, cluster bean) has recently been reported to decrease the serum cholesterol level and serum lipid levels (Nisha and Sharma, 1980). In a study conducted by Chen and Anderson (1979) it was found that certain water soluble plant fibres such as guar gum or pectins have greater cholesterol lowering effects than do certain insoluble plant fibers such as wheat bran or cellulose. In human studies, feeding of guar gum lowered serum cholesterol levels in normal subjects and in hypercholesterolemic subjects. In animal studies, guar gum incorporated into the hypercholesterolemic diets lowered serum cholesterol, liver cholesterol and total liver lipids in rats (Nisha et al, 1980). The mechanism of hypolipemic action of guar, might be related to diminished caloric in take or less and delayed absorption of nutrients due to the presence of unabsorbable carbohydrate in the diet. Guar is composed of glactomannan which is not hydrolysed in the Gastro Intestinal tract and is classified as unavailable carbohydrate (Jenkins et al, 1975).

Locust bean gum (LBG) (Ceratonia Cilliqua) belongs to the family of leguminosa. The endosperm material contains insoluble materials such as cellulose and proteins. Structurally locust bean gum is a polymer of D-mannose and D-galactose. According to a study conducted by Zavoral (1981), locust bean gum was more effective than some pharmocologycal agents in lowering serum lipids. Zavoral and his colleagues (1981), conducted a study on familial hypercholesterolemia (FHC). FHC is associated with severe premature coronary artery disease, death and is resistant to dietary treatment. So a special diet was developed to evaluate the lipid-lowering effect of locust bean gum in food products fed to adults and children from families with familial hypercholesterolemia as a potential aid in preventing coronary artery disease. They found that total cholesterol was lowered 11 per cent at four weeks. High density lipoproteins did not change. The HDL/LDL ratios increased. There was no significant side effects of the LBG diet. LBG in food products appears to be a safe, effective means of lowering serum lipids in normal and hyperlipidemic members of familial hypercholesterolemic families.

III. EXPERIMENTAL PROCEDURE

The main objective of this study was to evaluate and analyse the cholesterol and triglyceride altering properties, and also the histopathological changes of the heart, aorta and liver tissues in albino rats fed on Locust Bean Gum. The procedure involved may be categorised as:

A. Selection and grouping of animals.

B. Formulation of the diets.

C. Feeding and evaluating the effect of Locust Bean Gum.

A. Selection and grouping of animals:

Twentyfour male albino rats were selected from the laboratory stock colony and grouped into four groups, and housed in individual cages. The rats selected for the experiment were 60 days old. The rats were grouped such that the difference in weight allowed within the groups was $\pm$ 5 gms, and the difference between the group was $\pm$ 1 gm. A number of scientists investigating on the lipid profile, seem to have selected animals of different ranges of weight for their studies. For example, Mathur *et al* (1979) used rats weighing between 150-200 gms, Kelley 100-120 gms, and Anusaya Devi *et al* (1979) used albino rats weighing 200-250 gms.

In this study rats weighing 130-150 gms were chosen. The study conducted in two phases. In the first phase of the experiment, all 24 animals were fed with an atherogenic diet for a period of 45 days. Food and water were provided libitum. At the end of this period, one rat from the group was sacrificed and the liver, heart and aorta were removed and presered for the histopathological study.

In the second phase of the study, groups II, III and IV were fed the experimental diets, namely Locust Bean Gum diet at 10 percent, 20 per cent, 30 per cent level respectively. Group I was the control group, fed on a diet containing skimmed milk powder, groundnut oil, vitamin and mineral mixtures and starch. The diet was fed at ad libitum for 31 days (ie) one month, and the animals sacrificed at the end of this phase, samples of the liver, heart and aorta were removed and preserved in 10 per cent formalin for the histopathogical study.

B. Formulation of the diets:

The sample of Locust Bean Gum was first analysed for nitrogen content by macrokjeldal method (NIN, 1971) in triplicate, and the amount of protein (N_2 x 6.25) determined.

Totally five diets were formulated for the whole experimental period. These were the artherogenic diet and the diet containing Locust Bean Gum at three levels, namely 10 per cent, 20 per cent and 30 per cent levels respectively. These three levels were arrived at after conducting a preliminary feasibility study in food products, incorporating Locust Bean Gum at different concertrations. Locust Bean gum acceptance was found to be good, up to 30 per cent level in all the preperation.

The autherogenic diet provided skim milk protein at 18 per cent level. The experimental diet was formulated in such a way, that the amount of protein supplied by 10 per cent, 20 per cent and 30 per cent Locust Bean Gum was found out. The rest of the 18 per cent protein was supplied by the skim milk powder. Thus the experimental diet also provided protein at 18 per cent level.

In the atherogenic diet, the source of fat was coconut oil at a level of 20 per cent (SreeKumar and Kurup 1978). In the experimental diets containing Locust Bean Gum, ground nut oil at 9 per cent level was used as a source of fat. The reason for using ground nut oil as a source of fat in the second phase of the study, was to stabilize cholesterol levels as far as possible (Landes et al, 1974).

The Vitamin mixture (Appendix I) was supplied at 2 per cent level, and mineral mixture (Appendix I) at 4 per cent level respectively, to supply adequate quantities of these nutrients. The rest of the weight was made up with corn-starch. All the diets were prepared at the start of the experiment and stored in the deep freezer. The detailed composition of these diets are presented in Table I

TABLE I

PERCENTAGE COMPOSITION OF THE ATHEROGENIC AND EXPERIMENTAL DIETS

		Experimental			
	Atherogenic	Control	I	II	III
Skim milk powder	47.37	47.37	46.05	44.47	43.16
Vitamin	2	2	2	2	2
Mineral	4	4	4	4	4
Fat	20 (Coconut oil)	9 (Ground nut oil)	9	9	9
Starch	26.63	37.63	28.95	20.53	11.84
Locust Bean Gum	..	..	10.00	20.00	30.00
Total	100	100	100	100	100

C. Feeding and evaluating the effect of Locust Bean Gum:

1. Food intake

The animals were given food and water adlibitum every day. Food for each individual animals were weighed out separately and transferred into separate containers. It was then mixed with sufficent water and steamed, to a semi-solid consistancy. The food intake of the animals were assessed by the amount of food consumed every day. The left overs and spilt food was cleaned of hair, excreta and placed in separate watch glasses and heated in the oven overnight, till completely dry. This dry mass was weighed and substracted from the amount of food originally given. Thus the actual intake of food was calculated.

2. Weight gain:

Weight gain records were maintained by weighing the animals every fort-night, to the nearest milligram.

3. Cholesterol levels:

The cholesterol levels were estimated before starting the experiment, and then once in 15 days using Zak's method (Zak, 1957). The details of the procedure is given in Appendix II.

4. Triglyceride levels:

As triglyceride is also a risk factor in atherosclerosis attempt was made to measure the serum triglyceride levels. To ensure the initial triglyceride levels, before the start of the experiment, one rat was selected at random, and blood was collected for normal triglyceride level and estimated subsequently at the end of the atherogenic period, at the beginning of the experimental period, and latter everyfortnight, till the termination of the experiment. Blood was collected from live animals by cutting the tip of the tail, with a sharp blade. The cut end was then lowered into a clean test tube. A piece of cotton dipped in xylene was rubbed over the whole length of the tail to facilitate the flow of blood. Xylene was used to dialate the major vein. About 2 to 3 ml of blood was collected for each animal by this method. After collecting the blood, the cut end of the tail was plugged with cotton dipped in detol.

From the blood collected, the serum was separated and the estimation of cholesterol was done in duplicate, using Zak's method (Zak 1957), as given in appendix II.

5. Histopathological studies:

Slides of heart, aorta and liver tissues were prepared by fixing, dehydrating, embedding and impregnating in wax,

cutting and staining the tissues with haemotoxylin - eosin for histopathological observation. Micro photography of the slides were also taken.

The details of the procedure is given in Appendix III.

IV RESULTS AND DISCUSSION

The results obtained in this study pertaining to the effect of Locust Bean Gum on some physiological parameters in albino rats is discussed under the following headings:

A. Food intake

B. Weight gain

C. Cholesterol levels

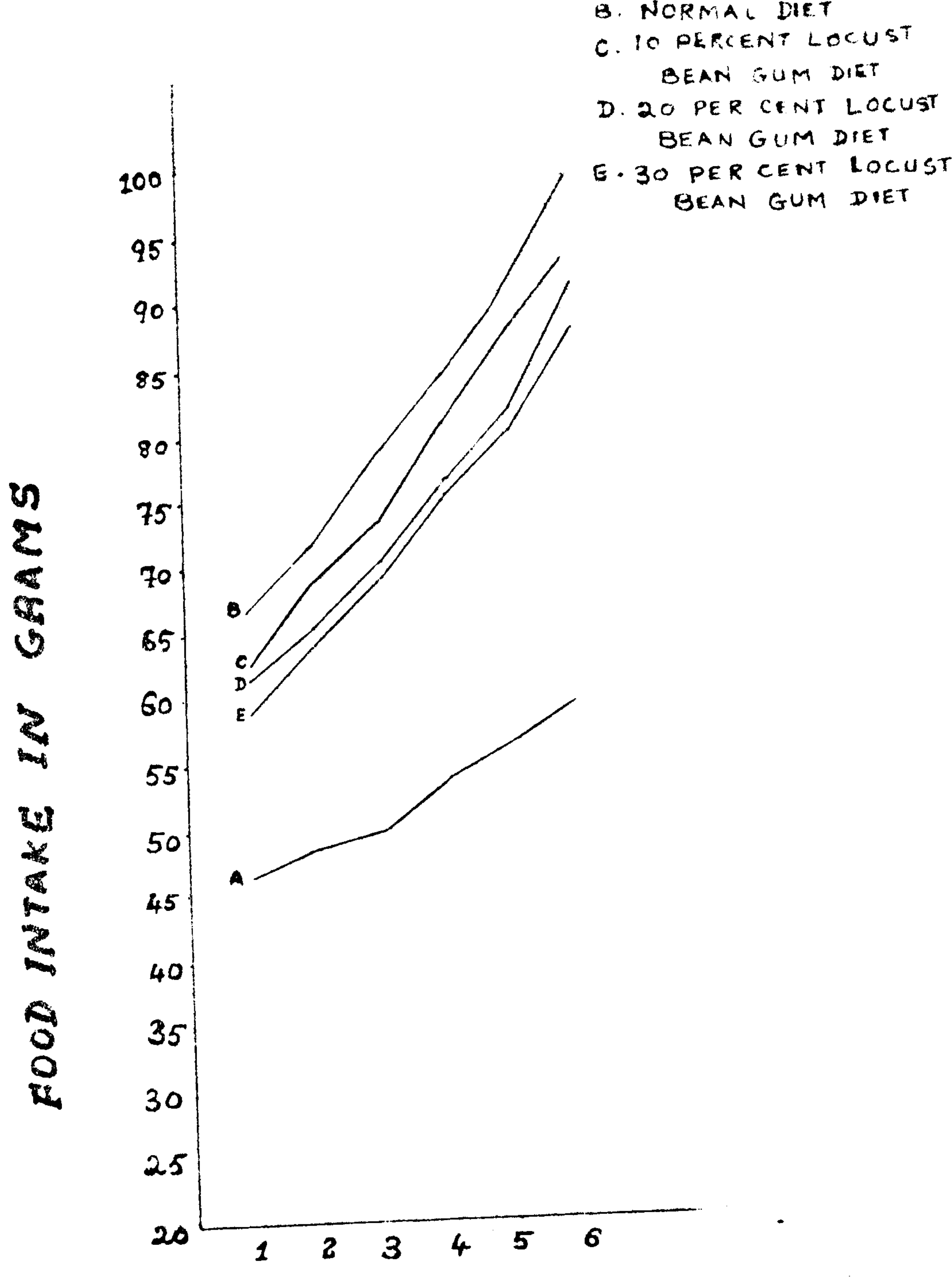
D. Triglyceride levels

E. Histopathological changes of the selected tissues

A. Food intake:

The twenty four male albino rats selected were divided into four groups according to their weights and designated as groups I, II, III and IV. In the first phase all the twentyfour rats were fed on an atherogenic dietary regimen and in the second phase, group I was fed on a normal diet, while groups II, III and IV were fed on a diet containing Locust Bean Gum at 10 per cent, 20 per cent and 30 per cent levels respectively. The food intake record was maintained throughout the two phases.

It was noted that the food consumption on an average during the first phase was around eight grams. The average intake in group I was 8.91 g., in group II was 8.61 g., in group III was 8.64 g., and in group IV was 8.56 g.



PATTERN OF FOOD INTAKE

FIGURE 11

Since the first phase namely the atherogenic diet phase ended after six weeks, when considerable increase in cholesterol and triglyceride levels were evidenced by all the rats, two rats were sacrificed one from group I and the other from group IV, in order to study the histopathological changes due to the atherogenic diet.

The second phase of the experiment lasted for six weeks. In the second phase of the study, group I was fed on a normal diet, consisting of skimmed milk powder. Group II,III and IV were also fed on the same diet but, in addition, it contained Locust Bean Gum at 10 per cent, 20 per cent and 30 per cent levels respectively. During this period, the food intake values was higher than those obtained in the atherogenic phase. A higher mean food intake was recorded by rats on the normal diet (11.75G) than the rats on the Locust Bean Gum diet. The mean food intake of rats on the 10 per cent, 20 per cent and 30 per cent Locust Bean Gum were 11.07g., 10.50g and 10.38g respectively. Figure 2 represents the food intake of the rats during the two phases.

TABLE II

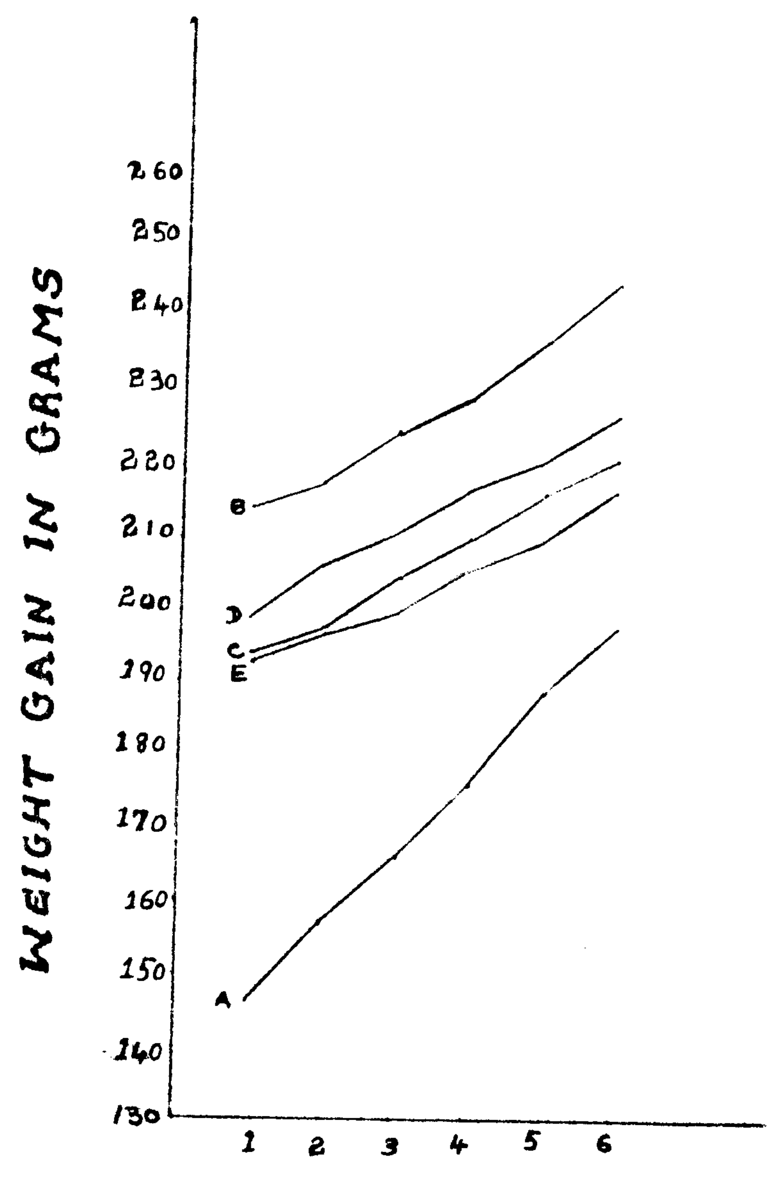
MEAN FOOD INTAKE OF THE RATS ON ATHEROGENIC AND EXPERIMENTAL DIETS

Group	Phase		Rat 1	Rate 2	Rate 3	Rat 4	Rat 5	Rat 6	Average
			Food Intake in grams						
I	First	W+	45.32	55.06	54.81	55.57	55.77	54.33	53.47
		D+	6.47	7.87	7.83	7.94	7.98	7.76	7.63
II	Second	W+	—	82.37	82.01	82.44	82.73	81.43	82.19
		D+	—	11.77	11.72	11.78	11.82	11.63	11.74
II	First	W+	53.59	51.30	50.82	52.05	50.71	50.58	51.51
		D+	7.66	7.33	7.26	7.44	7.24	7.23	7.36
	Second	W+	79.66	77.54	74.74	77.73	77.64	77.38	77.45
		D+	11.38	11.08	10.68	11.10	11.10	11.05	11.07
III	First	W+	51.33	51.47	51.58	52.37	51.13	53.19	51.83
		D+	7.33	7.35	7.37	7.48	7.30	7.60	7.41
	Second	W+	72.98	74.12	73.74	75.02	73.48	74.18	73.92
		D+	10.43	10.59	10.53	10.72	10.50	10.60	10.56
IV	First	W+	51.43	51.22	51.78	51.85	50.80	51.26	51.39
		D+	7.35	7.32	7.40	7.41	7.26	7.32	7.34
	Second	W+	—	72.20	72.69	73.85	72.40	73.03	72.83
		D+	—	10.31	10.38	10.55	10.34	10.43	10.40

W+ - Average food intake per week
D+ - Average food intake per day

A ATHROGENIC DIET
B NORMAL DIET
C 10 PER CENT LOCUST BEAN GUM DIET
D 20 PER CENT LOCUST BEAN GUM DIET
E 30 PER CENT LOCUST BEAN GUM DIET

PATTERN OF WEIGHT GAIN



PERIOD IN WEEKS

FIGURE III

B. Weight gain

Table III depicts the total weight gains of the rats in different groups and in the two phases of the study. Though there were variations in the weight of rats, due to variation in weight within the group, the graph drawn does not show much fluctuations. Weekly gain in weight of individual rats is given in appendix V.

TABLE III

TOTAL WEIGHT GAIN (GRAMS) OF THE RATS IN THE TWO PHASES OF THE EXPERIMENT

Group	Phase	Weight gain in grams						Average weight
		rat 1	rat 2	rat 3	rat 4	rat 5	rat 6	gain/week
I	First	71.6	69.2	71.0	64.5	71.3	55.1	11.18
	Second	-	31.6	32.3	33.3	33.1	40.7	5.7
II	First	39.3	27.6	48.4	51.9	64.6	61.7	8.15
	Second	33.5	34.6	27.0	29.8	31.5	23.1	4.99
III	First	70.1	44.1	52.7	48.7	44.7	66.6	9.08
	Second	25.8	27.3	26.8	26.0	16.1	25.4	4.09
IV	First	64.0	23.3	63.7	38.9	53.9	55.9	8.33
	Second	-	25.2	24.5	24.7	24.1	24.2	4.09

In the first phase of the study during which the rats

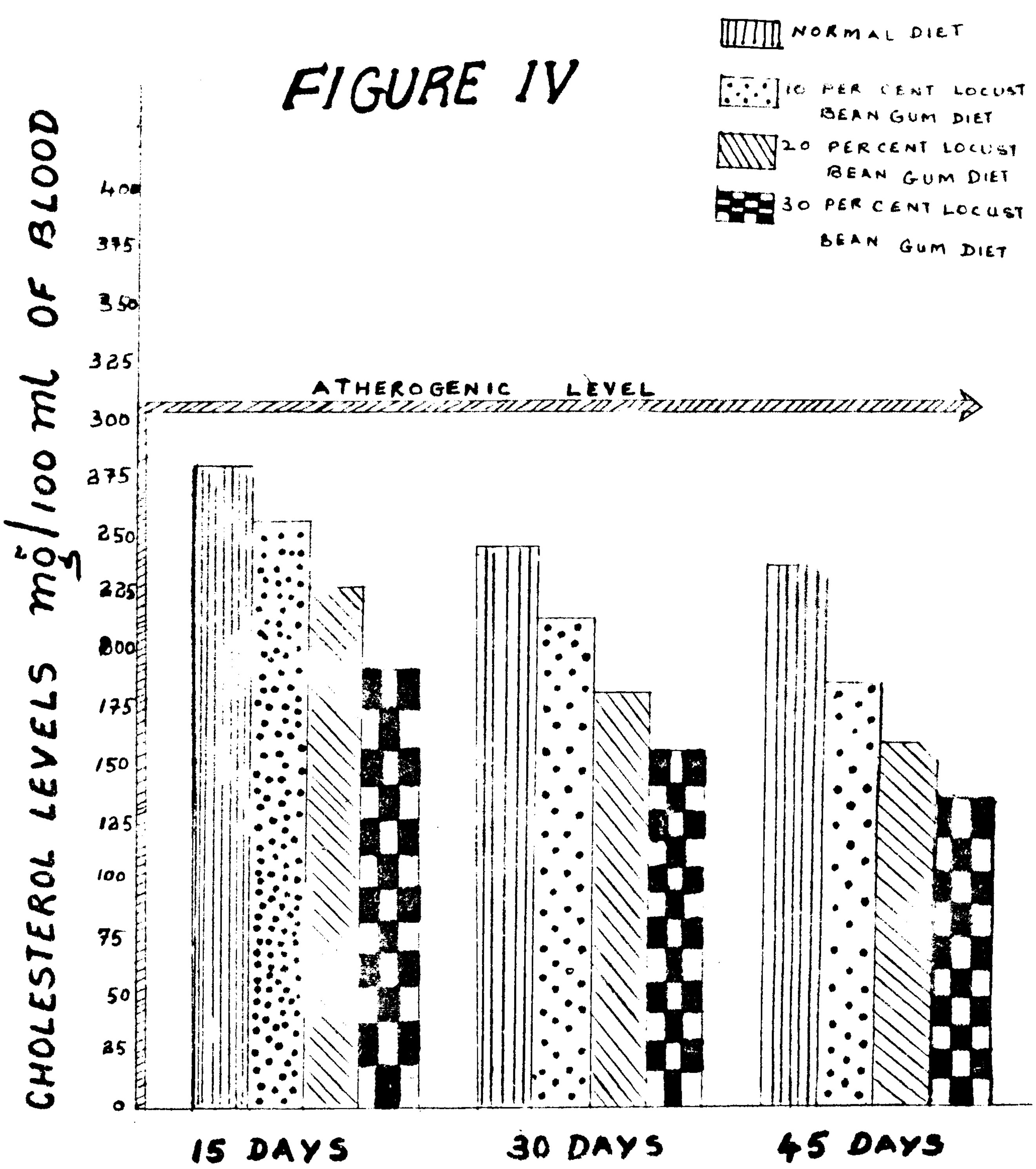
were maintained on an atherogenic diet, the animals in group I,II,III and IV registered an average weight gain of 11.18 g., 8.15g., 9.08g., and 8.3 g. per week respectively. It was found that group I registered the maximum gain in body weight.

It was found that as the food intake increased upto the sixth week, the body weight also increased showing a positive correlation (r = +0.7089).

In the second phase the average weight gain for groups I,II,III and IV was 5.7 g., 4.99 g., 4.09g., and 4.09 g. per week respectively. Figure III represents the pattern of weight gain in the two phases. Soon after the shift over from the first phase, the food intake of the animals in all the groups increased as also the weight again indicating a positive correlation (r = +0.965 for rats fed on control diet, r = +0.754 for rats fed on 10 per cent Locust Bean Gum diet, r = +0.9380 for rats fed on 20 per cent Locust Bean gum diet, and r = +0.973 for rats fed on 30 per cent Locust Bean Gum diet for all the diets.

C. Cholesterol levels

The cholesterol levels in all the four groups of rats were measured at the start of the experiment and then on



HYPOCHOLESTEROLEMIC EFFECT OF LOCUST BEAN GUM

the 30th and 45th day respectively, during phase 1. Then onwards, the cholesterol levels were measured every fortnight in phase II. The mean initial cholesterol levels of the rats are presented in Table IV. The details of the cholesterol values for individual rats appears in appendix VI

TABLE IV

MEAN CHOLESTEROL LEVELS IN mg/100ml OF BLOOD IN THE TWO PHASES.

Group	Atherogenic		Experimental		
	35 days	45 days	15 days	30 days	45 days
I	200	310	277.5	244.5	235
II	217.08	307.42	256.25	214.17	136.25
III	240	321.88	226.17	183.25	162.5
IV	211.42	317.71	192.5	155.4	134.5

*mean initial level = 86.5 mg/100 ml of Blood.

The serum cholesterol levels of the individual rats during the atherogenic diet phase was seen to range from 157.5 mg/100 ml to 265 mg/100 ml of blood, in the first month and 290 mg/100 ml to 347.5 mg/100 ml of blood at the end of the next 15 days. On an average during the first 30 days, the serum cholesterol levels in group I, II, III and IV were 200 mg/100 ml, 217.08 mg/100 ml, 240 mg/100 ml and 211.42 mg/100 ml respectively. At the end of phase I (45 days) it further increased to 310 mg/100 ml, 307.42 mg/ 100 ml, 321.88 mg/100 ml and 317.71 mg/100 ml of blood respectively for the four groups.

In the second phase of the study, rats in group I were placed on a normal diet of skim milk powder, fat, starch, vitamin and mineral mixture. Groups II , III and IV were placed on a diet containing 10 per cent, 20 per cent and 30 per cent Locust Bean Gum respectively. The diet was fed for a period of 45 days. Once in a fortnight the serum cholesterol levels were analysed.

The average cholesterol levels in the animals of group I decreased, but not to the extent found in group II, III and IV. The cholesterol levels of group I had fallen from 310 mg/100 ml to 277.5 mg/100 ml, group II from 307.42 mg/100 ml and group IV from 317.71 mg/100 ml to 192.5 mg/ 100 ml of blood, at the end of the first 15 days. After 30 days the cholesterol levels had further decreased to

244.5 mg/100 ml in group I, 214.17 mg/100 ml in group II, 183.25 mg/100 ml in group III and 155.4 mg/100 ml in group IV. At the end of phase II (45 days) the cholesterol levels further, decreased to 235 mg/100 ml for group I, 186.25 mg/100 ml for group II, 162.5 mg/100 ml for group III and 134.3 mg/100 ml of blood for group IV. These details are depicted in Figure 4.

The statistical comparison of cholesterollevels between the atherogenic phase and experimental phase in groups I, II, III and IV were done using paired 't' test. Comparison in cholesterol values between the normal diet, 10 per cent, 20 per cent, 30 per cent Locust Bean Gum diets and between 15th, 30th and 45th days in phase II were also made, using 't' test. The data is presented in Table V.

It is evident that there is a highly significant ($P < 0.01$) difference for all three levels of the Locust Bean Gum diets, in the cholesterol levels, when compared to the atherogenic phase of the same animals.

When the Locust Bean Gum was fed to rats incorporated at the different levels, and samples compared for the cholesterol lowering effect it is evident that there is a highly significant difference between the groups during the first 15 days of phase II ($P < 0.01$). However, by the 30th day and 45th day, the statistical analysis between the experimental groups was significant and the cholesterol levels of rats fed on the Locust Bean Gum diet showed reduced levels of cholesterol than the animals on normal diet. There was also significant difference in the cholesterol levels between the atherogenic and the experimental groups.

When days were compared for all the diets fed as the periodicity increased, there was significant difference in blood cholesterol levels. These results are in Table VI.

TABLE VI

STATISTICAL COMPARISON OF CHOLESTEROL LEVELS BETWEEN NUMBER OF DAYS

Source of Comparison	't' values
Between the days:	
Normal diet:	
15 days Vs 30 days	6.265**
15 days Vs 45 days	4.8871**
30 days Vs 45 days	1.5861
10 per cent Locust Bean Gum Diet:	
15 days Vs 30 days	8.1867**
15 days Vs 45 days	14.0005**
30 days Vs 45 days	6.2153**
20 per cent Locust Bean Gum Diet:	
15 days Vs 30 days	7.2416**
15 days Vs 45 days	18.8553**
30 days Vs 45 days	4.975**
30 per cent Locust Bean Gum Diet:	
15 days Vs 30 days	10.5967**
15 days Vs 45 days	13.5307**
30 days Vs 45 days	9.5205**

** Significant at one per cent level

* Significant at five per cent level

These results are in tune with the results reported in the literature (Fahrenbach et al. 1966) and Leveille, 1977).

Chen and Anderson (1979), Nisha and Sharma (1980) and Zavarol et al. (1981) have suggested that the cholesterol lowering effect of pectin, guar gum and similar products may be due to the soluble plant fibres, which bind bile acids (Story and Kritchevsky, 1976), thereby preventing their absorption and hence removing their inhibitory effect on cholesterol conversion to bile acids. When the food intake and cholesterol levels of the animals were compared, it was found that in the atherogenic phase, as the food intake increased, the cholesterol level also increased, showing a positive correlation ($r = +0.9693$). In the experimental phase, as the ~~wheef~~ food intake increased, the cholesterol level came down as shown by the negative correlation ($r = -0.9513$ for normal diet, $r = -0.9895$ for 10 per cent Locust Bean Gum diet $r = -0.9759$ for 20 per cent Locust Bean Gum diet and $r = -0.9847$ for 30 per cent Locust Bean Gum diet), thus showing the effectiveness of Locust Bean Gum on serum cholesterol levels of blood. Table VII shows the relationship between food intake, weight gain and cholesterol level in both the phase.

TABLE VII

STATISTICAL COMPARISON OF GROUPS FOR FOOD INTAKE, WEIGHT GAIN AND CHOLESTEROL LEVELS

Groups Compared	Correlation Coefficient 'r'
Food Intake Vs Cholesterol Levels:	
Atherogenic Diet	+ 0.9693
Normal diet	− 0.9513
Diet containing 10 per cent Locust Bean Gum	− 0.9895
Diet containing 20 per cent Locust Bean Gum	− 0.9759
Diet containing 30 per cent Locust Bean Gum	− 0.9847
Weight Gain Vs Cholesterol Levels:	
Ahterogenic Diet	+ 0.8880
Normal Diet	− 0.9970
Diet containing 10 per cent Locust Bean Gum	−0.8776
Diet containing 20 per cent Locust Bean Gum	− 0.9898
Diet containing 30 per cent Locust Bean Gum	− 0.9802

The relationship between weight gain and cholesterol was also calculated. The experimental groups showed negative correlation emphasizing the hypocholesterolemic effect of locust Bean gum.

D. Triglyceride Levels:

Elevated fasting plasma concentration of triglycerides is an independent and important risk factor than cholesterol in myocardial infarction (JADA, 1974). Serum triglyceride are biologically related to HDL-cholesterol(Lancet, 1979). Hence in the present study it was also of interest to evaluate the triglyceride levels of randomly selected rats. The data obtained for all the groups is given in Table VIII.

TABLE VIII

TRIGLYCERIDE LEVELS OF THE SELECTED RATS

S.No.	Diet	Triglyceride level mg/100 ml of blood
1.	Stock diet	68
2.	Atherogenic diet	290
3.	Normal diet	220
4.	10 per cent Locust Bean Gum diet	180
5.	20 per cent Locust Bean Gum diet	110
6.	30 per cent Locust Bean Gum diet	84

Triglyceride levels showed a similar trend like cholesterol. Among the experimental diets, the 30 per cent Locust Bean Gum diet showed triglyceride level almost on par with the normal dlevel. All the triglyceride values obtained after feeding the experimental diet were much lower than the atherogenic diet phase.

E. Histopathological Changes of the Selected Tissues:

The liver, heart and aorta of the animals sacrificed at the end of the first and second phases respectively were subject to histopathological examination.

The findings of the study is discussed below:

Phase I:

In the first phase of the experimental period, all the animals were maintained on an atherogenic diet. Microscopic examination of the heart muscle with adjoining root of the aorta (Figure 5) show that the endothelium and subendothelial layers are thickened with deposits of lipid and cholesterol. There is damage to the subendothelial elastic laminae with myxomatous damage, with small round cell infiltration and capillary ingrowth indicating athromatous degeneration.

The cardiac muscles show typical myocarditis, and are at places replaced by hyaline and fibrous matrix with diffuse lymphatic and plasma cell infiltration. The coronary arteries

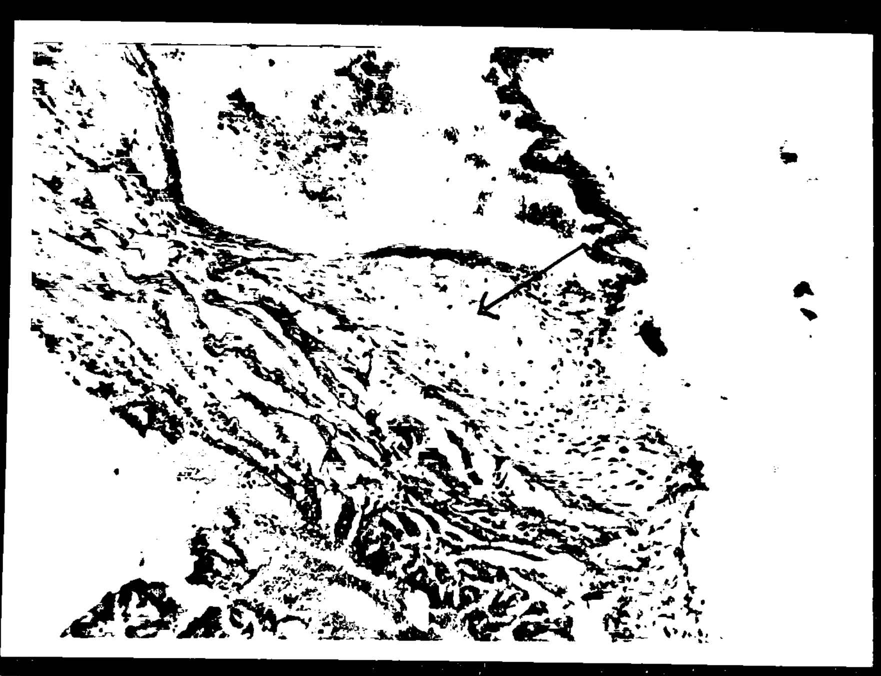


Figure 5: ROOT OF THE GREAT VESSELS SHOWING ATHEROSCLEROSIS AND CARTILAGENOUS METAPLACIA OF THE VALVES

Figure 6: THE ADJOINING GREAT VESSEL(AORTA) SHOWING GRADE I ATHEROSCLEROSIS AND EARLY [illegible] CHANGES

show thickened endothelium with subendothelial accumulation of fat cells, and clear spaces with lipid.

Microscopic section of the aorta, at its base shows plagues of atherosclerosis. Thickening of the intima, at places, ruptrued and protruding into the lumen. Beneath the affected intima, there are vaculated spaces inbetween the internal elastic laemnas. These spaces are suggestive of accumulation of lipid materials. There is also clear areas of cartilaginous metaplacia of the valve at the root of the aorta. There are areas of focal accumulation of lymphocitic cell infiltration and new capillary formation around the athromatous plagues. This is suggestive of grade I atherosclerosis. Figure 6 depicts the details of the finding.

This is indicative of the fact that a high coconut oil diet of the order of 20 per cent given for a period of 60 days is capable of inducing plague formation in the arteries of the rat. This is in line with the results of Marita <u>et al</u> (1979) who have indicated that a dietary regimen containing cholesterol 3 per cent, coconut oil 1.5 per cent and thiouracil 25 mg/100 gm body weight provided for a period of two months induced sclerotic plague formation in the aorta and also raised cholesterol levels to the order of 250 - 300 mg/100 ml from an initial level of 96.37 $\pm$ 5.02 mg/100 ml.

In the first phase all the rats showed high increments in cholesterol levels (325 mg/100 ml) which might be one of the reasons why plague formation had developed in the aorta.

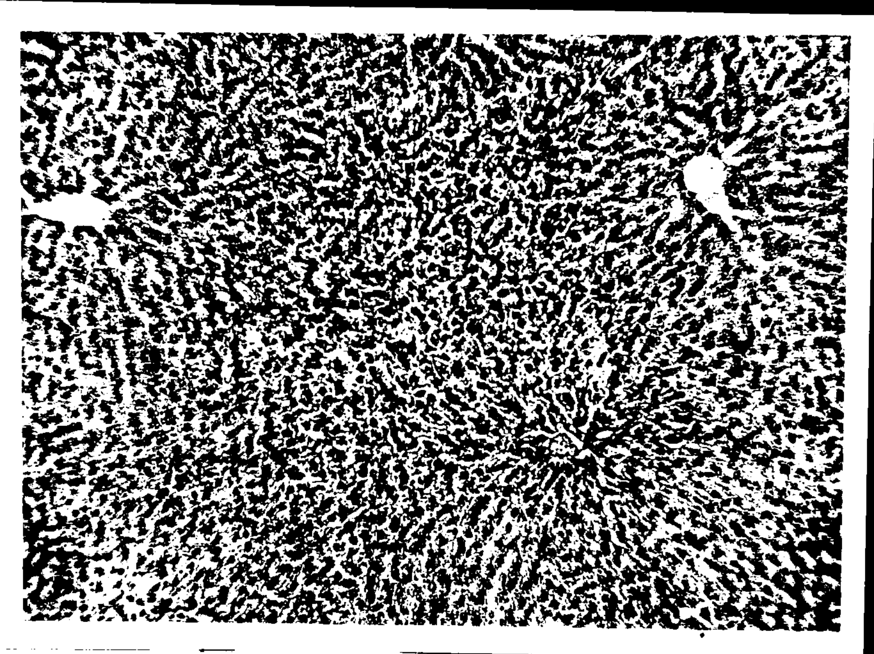


Figure 7: LIVER SHOWING DILATED INTER-LOBULAR VEIN AND NARROWED SINUSOIDAL SPACES SHOWING MILD HEPATITIS

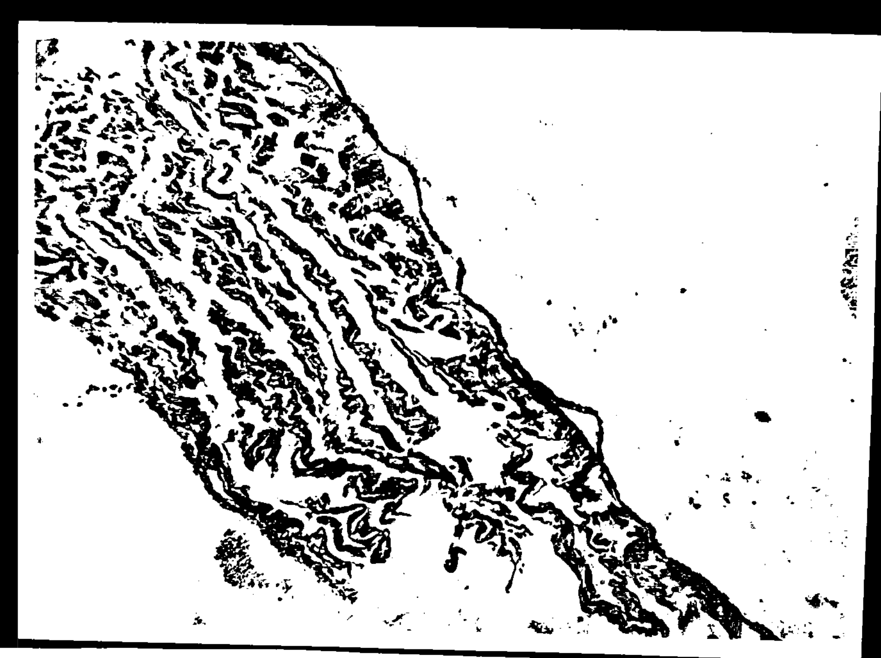


Figure 8: BASE OF AORTA SHOWING LOCAL THICKENING AND BULGING INTO LUMEN

The liver tissues in this group differed from the normal in that it showed very much dilated inter-lobular vein but the sinusoidal spaces are narrowed by the swollen parenchymal liver cells and showing atrophy. At places the atrophied liver cells are replaced by globular fat cells, showing a generalized picture of fatty infiltration of liver. Figure 7 indicates the fatty degeneration of the liver with mild hepatitis.

Phase II:

Group I:

Animals in group I were placed on an atherogenic diet in the first phase of the study and subsequently placed on a normal diet.

Microscopic examination of the longitudinal section of the base of the aorta showing local thickening, and bulging into the lumen, with vaculated spaces in the sub-endothelial elastic laminae. These spaces are probably due to accumulation of lipid. Tunica intima is filled with fatty cells. There is a mild lymphocitic infiltration at places (Figure 8).

The microscopic picture of the heart muscles shows a marked infiltration and accumulation of lymphocytes and plasma cells at places beneath the endothelial lining of the

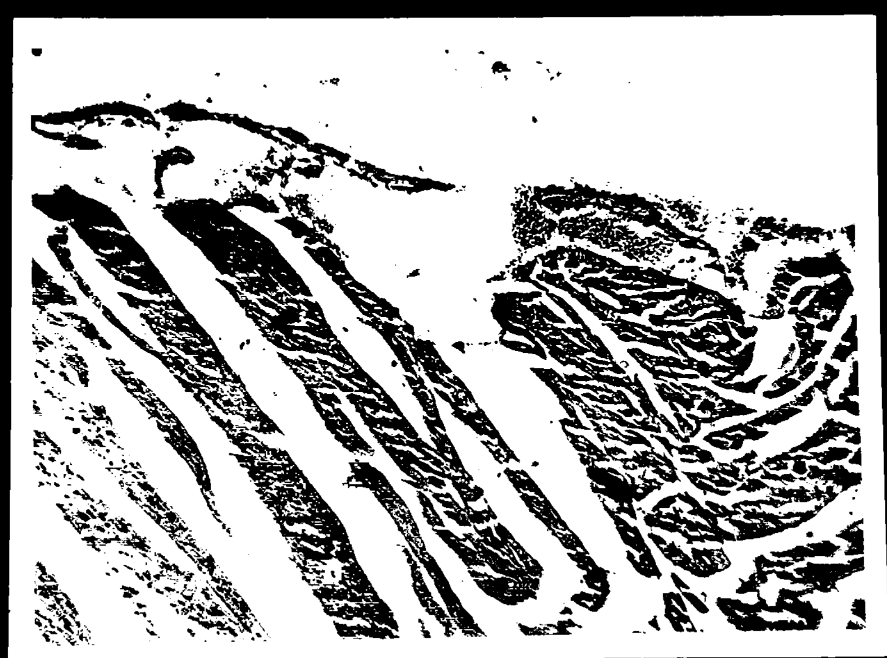


Figure 9: MILD MYOCARDITIS

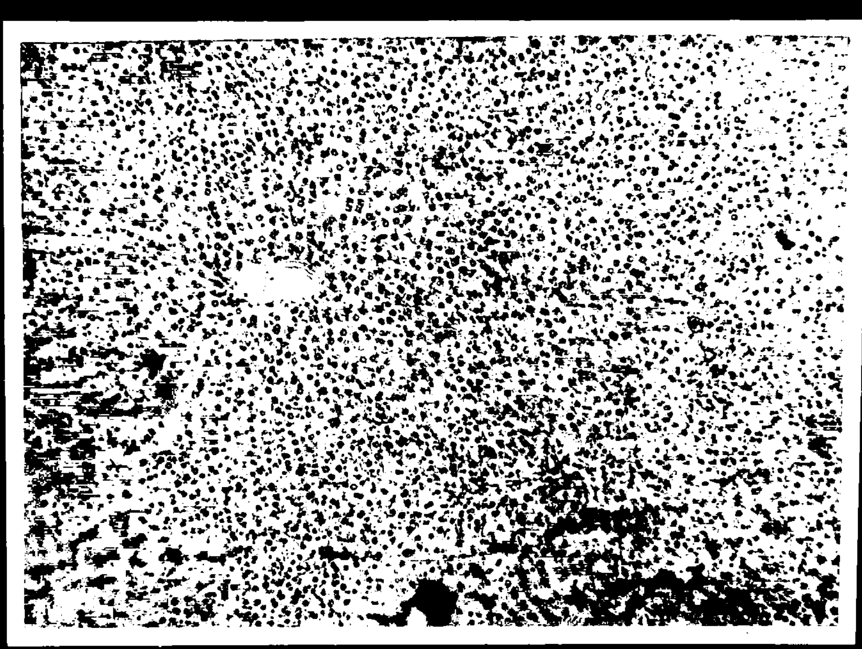


Figure 10: [illegible] MILD FATTY DEGENERATION AND [illegible] CONGESTION

Figure 11: HEART MUSCLE WITH NORMAL HISTOLOGY

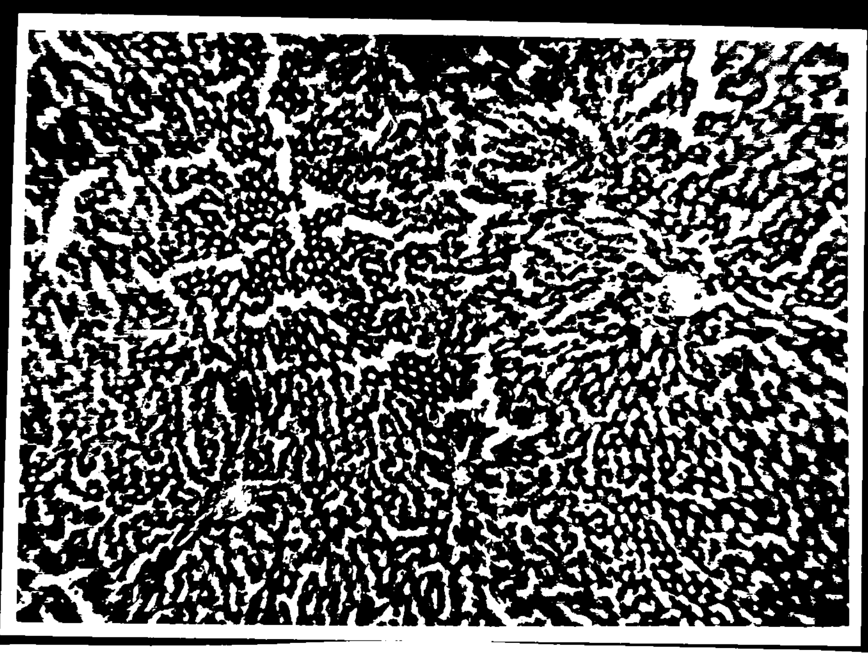


Figure 12: LIVER SHOWING CONJUSTION, WITH SINUSOIDAL DILATATION

lumen, indicating mild myocarditis. Sub-endothelial and myocardial focus of fibrosis are seen with capillary vessels transversing the area, replacing the part of myocardium. The lumen of the coronary arteries are irregular showing protrusion of the atheromatus plagues into the lumen. The subendothelial elastic tissues is infiltered with eccentric nucleated fat cell and empty spaces with cholesterol and lipids (Figure 9).

The liver of this group revealed enlarged sinusoidial speces. Parenchymal cells are grouped into columns with no morphalogical changes. The inter lobular veins are filled with red blood cells, showing a generalised congested liver (Figure 10). At places, fatty vacuolation and degeneration of the liver cells are seen, indicative of mild fatty degeneration.

Group II:

Rats in this group were fed on an atherogenic diet for 45 days and then subsequently fed on a 10 per cent Locust Bean Gum diet.

All the sections through the heart appeared to be normal. The coronary vessels showed normal histology with no fat cells or interstetial vacuolation in the artery walls (Figure 11).

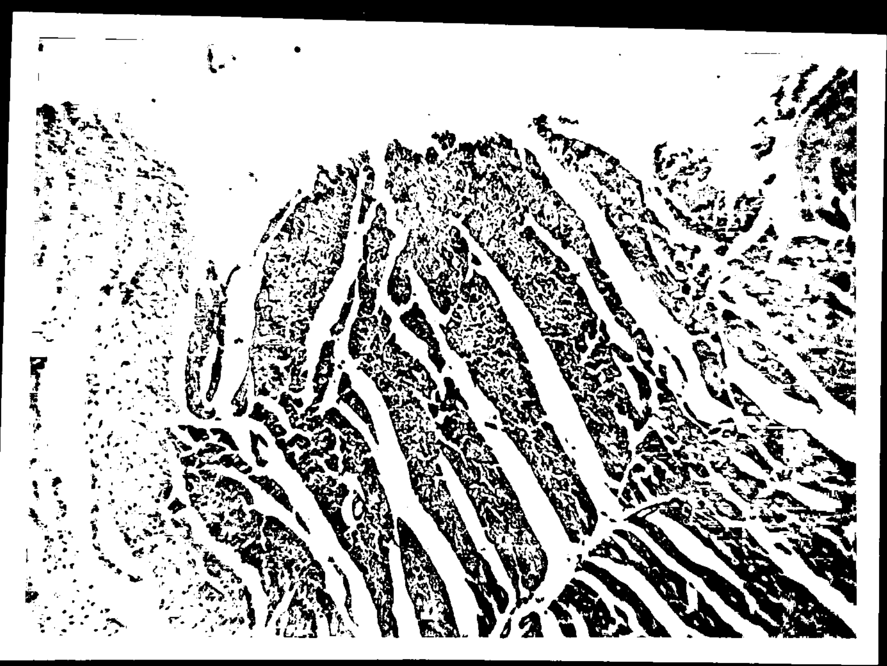


Figure 13: NORMAL HEART MUSCLE

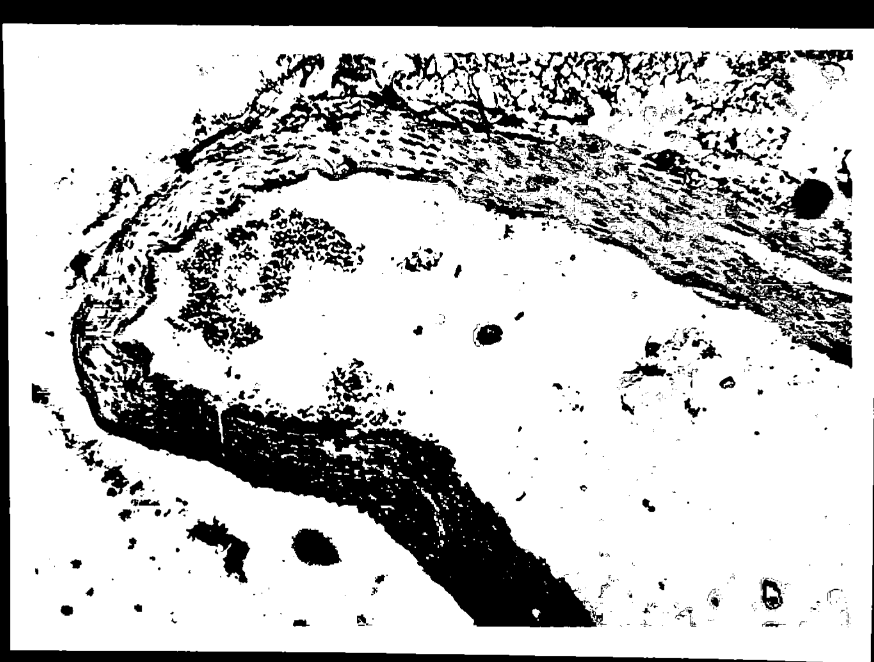


Figure 14: NORMAL AORTA

In the liver enlarged sinusoidal spaces were seen. Comparatively less fat cells were seen. Though the parenchymal cells were grouped into columns, there were no morphological changes. The liver showed slight congestion (Figure 12).

Group III and IV:

Rats in group III and IV were initially fed on an athrogenic diet for 45 days; and subsequently placed on a 20 per cent and 30 per cent Locust Bean Gum diet respectively.

All the sections through the heart appeared to be normal. Microscopic sections of the base of the aorta with adjoining left ventricle, the endothelial lining and the endocardial layers are normal. The intima and media of the aorta and the coronary vessels and the muscles of the myocardium of the heart also appeared normal (Figure 13 and 14). The coronary vessels showed normal histology with no fat cells or interstetial vacuolation in the artery walls.

The microscopic picture of the liver of group III, showed mild congestion (Figure 15). But the liver in group IV appeared to be normal (Figure 16).

The above observations and discussions brings out the fact that Locust Bean Gum (a legume) is indeed effective in bringing down the cholesterol level, which is one of the risk factors in coronary heart disease.

The histopathological pictures revealed that from a very

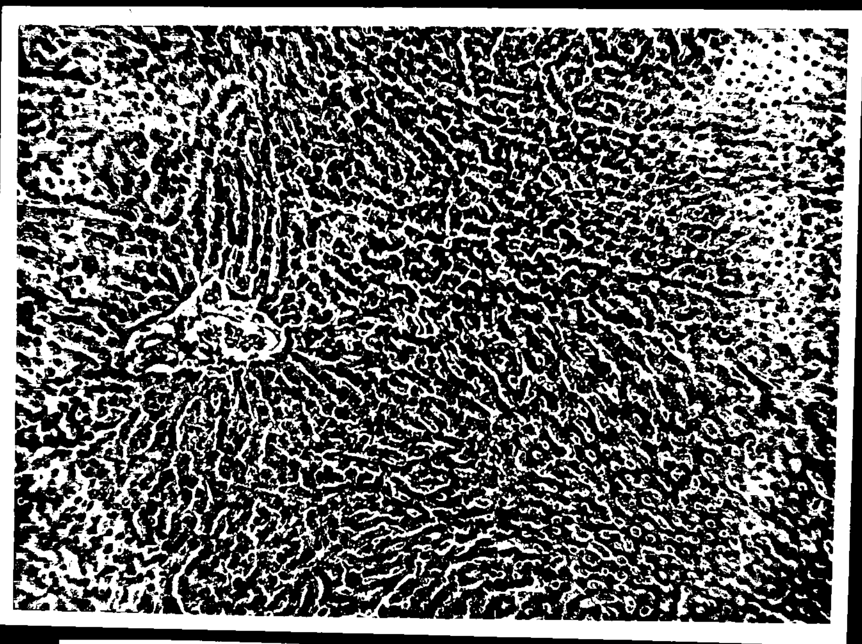


Figure 15: LIVER SHOWING MILD CONJUSTION

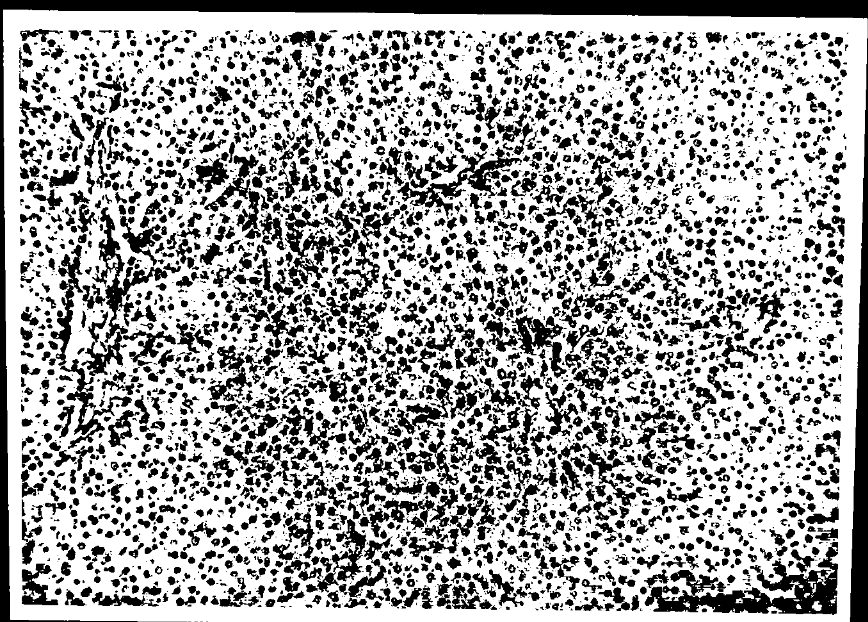


Figure 16: NORMAL LIVER

atherogenic picture, the Locust Bean Gum, restored the heart, liver and aorta to a normal histology. It significantly modified the fatty infiltration in the liver cells. It brings out the role of Locust Bean Gum as hypocholesterolemic agent. Locust Bean Gum is not very popularly used, but being such a potential hypocholesterolemic agent, should be promoted for popular use in various food items. In both home-made and commercial food items, it can be incorporated at higher levels in items like ice-cream mixes and thus enabling consumption of all such banned foods even by high risk populations.

V. SUMMARY AND CONCLUSION

This study investigated on the effect of Locust Bean Gum on Serum Cholesterol Levels, Triglyceride Levels and Histopathological Alterations in the heart, Aorta and Liver of Alibino rats.

Twenty four albino rats were selected weighing 130 - 150 grams and were grouped into four different groups, and designated as groups I, II, III and IV. All the rats were administered atherogeni diet for a period of 45 days, during which time the food intake and weight gain was recorded. Immediately after the completion of the first phase, two rats were sacrificed and the heart, aorta and liver were removed and preserved for histopathological analysis.

In the second phase of the group I was fed on a normal diet. Groups II, III and IV were fed on a diet containing 10 per cent, 20 per cent and 30 per cent Locust Bean Gum respectively. This phase lasted for 45 days.

The serum cholesterol levels were analysed every month during the atherogenic phase and every fortnight during the second phase. One rat in each group was randomly selected and serum triglyceride level was estimated at the end of each phase.

The animals were sacrificed at the end of the study and

liver, heart, and aorta were removed for histopathological study.

The results of this study revealed that

1. The average daily food intake for group I was 8.91 g., group II was 8.61 g., group III was 8.64 g., and group IV was 8.56 g., during the first phase. In the second phase the average food intake was 11.75 for group I, 11.07 g for group II, 10.50 g for group III, and 10.38 g for group IV.

2. During the first phase, the rats in groups I, II, II and IV registered an average weight gain of 11.18 g., 8.15 g., 9.08 g. and 8.33 g respectively and in the second phase 5.7 g., 4.99 g., 40.[illegible]9 g., and 4.09 g. respectively, showing a positive correlation between food intake and weight gain in all the groups in both the phases.

3. At the end of phase I, the cholesterol level raised to 310 mg/100 ml., 307.42 mg/100 ml, 321.88 mg/100 ml, and 217.71 mg/100 ml for groups I, II, III and IV respectively, showing the role of coconut oil, in raising the blood cholesterol level.

4. At the end of the second phase the cholesterol level came down to 235 mg/100 ml, 186.25 mg/100 ml, 162.5 mg/100 ml, and 134.5 mg1100 ml for groups I, II, III and IV respectively. The fall in serum cholesterol

levels was significant ($P < 0.01$) for all groups.

5. When the days were compared for all diets (ie. normal diet, 10 per cent, 20 per cent, 30 per cent Locust Bean Gum diet), as the periodicity increased there was statistically significant difference in the cholesterol levels within the sample.

6. As the percentage of Locust Bean Gum in the diet increased, the triglyceride level in the blood decreased (220 mg/100 ml for normal diet, 180 mg/100 ml, 110 mg/100 ml and 84 mg/100 ml for rats fed on 10 per cent, 20 per cent, 30 per cent Locust Bean Gum diet respectively).

7. Feeding coconut oil for a period of 45 days induced plagues formation, with thickening of endothelial and subendothelial layers. There was accumulation of fat cells in the intimal lining of the aorta, and vaculated spaces between the internal elastic leamnae, suggestive of fat accumulation. The liver tissue showed a dilated inter-lobular vein, with narrowing of the sinusodial spaces and atrophied liver cells, replaced by globular fat cells.

8. During the second phase group I rats on normal diet showed the same picture as the athrogenic group in their histophathology but to a lesser degree. The liver of this group showed mild fatty degeneration.

9. The histopathological studies of group II rats fed 10 per cent Locust Bean Gum diet showed that the sections through the heart appeared to be normal. The coronary vessels were also normal. But the liver showed enlarged sinusoidal spaces but comparatively less fat cells with slight

The forgoing discussions indicate the beneficial effect of Locust Bean Gum in bringing down the serum cholesterol and triglyceride levels and restoration of normal histology of the organelles like liver, heart and aorta. Hence, it is recommended that the not so commonly used legumes like locust bean gum may be popularised and the incorporated in as many home-made and commercial food items, was to enable even the high risk population groups to consume a variety of food items. Further, studies on higher levels of incorporation of the legume and evaluation of incorporated food products for their hypocholesterolamic effect is recommended.

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A P P E N D I C E S

APPENDIX I

COMPOSITION OF VITAMIN AND MINERAL MIXTURES

Vitamin mixture		Per 1 gm	Mineral mixture	Per 100 gms.
Vitamin A	..	100 I.U	Sodium chloride	13.93 g
Vitamin D	..	100 I.U	Potassium dihydrogen Ortho Phosphate	38.9 g
Vitamin E	..	10 I.U	Calcium Carbonate	38.14 g
Vitamin K	..	.50 mg	Magnesium Sulphate	5.73 g
Thiamine	..	.50 mg	Ferrous Sulphate	2.7 g
Riboflavin	..	1.0 mg	Manganese Sulphate	.401 g
Pyridoxine	..	.40 mg	Potassium iodide	.070 g
Pantothenic acid	..	4.0 mg	Copper Sulphate	.0477 g
Niacin	..	4.0 mg	Zinc Sulphate	.054 g
Choline	..	200 mg	Cobalts Choloride	.023 g
Inositol	..	25 mg		
Para amino ebnzoic acid	.	10 mg		
Vitamin B_{12}	..	2.0 mg		
Biotin	..	0.02 mg		
Folic acid	..	0.2 mg		

APPENDIX II

ZAK'S METHOD FOR THE ESTIMATION OF CHOLESTEROL

Aim:

To estimate the amount of cholesterol present in the serum.

Principle:

Cholesterol reacts with ferric chloride in the presence of concentrated sulphuric acid to give a pink colour. The intensity of the colour developed is directly proportional to the amount of cholesterol present and is read at 540 mµ in a colorimeter.

Reagents:

1. Stock ferric chloride reagent:

840 mg of pure dry ferric chloride was weighed and dissolved in 100 ml of glacial acetic acid.

2. Ferric chloride precipitating reagent:

10 mg of the stock ferric chloride reagent in a 100 ml standard flask and made up to the mark with pure glacial acetic acid.

3. Ferric chloride diluting reagent:

8.5 ml of stock ferric chloride is diluted to 100ml with pure glacial acetic acid in a 100 ml standard flask.

4. Standard cholesterol solution:

100 mg of pure dry cholesterol is placed in a clean dry 100 ml standard flask, dissolved in glacial acetic acid, then made upto the mark with pure glacial acetic acid.

5. Working Standard:

10.0 ml of the stock standard was placed in 100 ml standard flask containing 0.85 ml of ferric chloride stock regent and made upto the mark with pure glacial acetic acid. 100 ml of this solution contains 100 mg of cholesterol.

Procedure:

0.5 to 2.5 ml of the working cholesterol standard solution were pipetted out into a clean dry test-tube. The total of each tube was made upto 5.0 ml with ferric chloride diluting agent.

To 0.1 ml of the serum added 4.9 ml of ferric chloride precipitating reagent and mixed well, Allowed to stand for a while and centrifuged. Transferred 2.5 ml of the clean suppernatent into a clean dry test tube and added 2.5 ml of ferric chloride diluting agent, mixed well. The test tubes were allowed to come to the room temperature. A blank was also simultaneously prepared by taking 5.0 ml of the diluting reagent. Add 4.0 ml of concentrated sulphuric acid. After 30 minutes the intensity of the colour developed was read at 540 mg using the control as balank.

Solution vòlume in ml	Concentration in	Volume of $FeCl_3$ dil reagent ml.	Volume of conc. H_2SO_4 ml	Klett reading
Blank	—	5.0	4.0	0
Standard				
0.5	50	4.5	4.0	
1.0	100	4.0	4.0	
1.1.5	150	3.5	4.0	
2.0	200	3.0	4.0	
2.5	250	2.5	4.0	
Serum				
2.5		2.5	4.0	
2.5		2.5	4.0	

Calculation:

Klett x corresponds to Y of cholesterol 2.5 ml of diluted serum contains Y of cholesterol.

2.5 ml of the supernatant contains 0.01 ml of serum.

(ie) 0.01 ml of serum contains Y of cholesterol

.·. 100 ml of serum contains $\frac{Y \times 100}{0.01 \times 1000}$

mg/ml of cholesterol.

APPENDIX III

HISTOPATHOLOGICAL TECHNIQUES

The general preparation of tissue consists of : (1) fixation (2) Dehydration (3) cleaning (4) Infiltration and impregnation (5) Embedding or casting or blocking.

(1) Fixation:

It is essential that tissues to fixed as soon as possible after death or removal from the body to prevent putrifaction and autolysis. Fixing also helps to preserve, harden, solidify colloid material and helps in optical differentiation of tissue components.

Usually 10 per cent formalin or slightly higher concentrations are used to preserve specimens.

Preparation of 10 per cent formalin:

Formalin (40 per cent formaldehyde)	10ml
Distilled water	90ml

Tissues should be completely immersed in the fixative solution.

(2) Dehydration:

After fixation delicate tissues need to be dehydrated slowly, starting in 50 per cent ethyl alcohol. The tissues are then placed in 70 per cent, 96 per cent and 100 per cent alcohol for 2 - 4 hours in each solution. The volume of the reagent should be 50 - 100 times the bulk of the specimen. Beofre transferring to the next concentration of alcohol, the tissue is laid on a piece of filter paper and lightly blotted to remove excess fluid.

(3) Clearing of tissue:

The most common clearing agents in use are chloroform, benzene, xylene (xylol), toluene, carbon tetrachloride and ceder wood oil.

Small pieces of tissue are cleared within 2 - 4 hours of immersing in xylene. The tissue becomes clearer as the alcohol is replaced owing to the difference in refractive index.

Tissues for clearing should be lightly blotted during transfer from one reagent to the next. The volume of clearing in xylene should be 50 - 100 times that of the tissues. Tissues cleared in xylene should be given one change after 30 - 60 minutes and transferred to wax when they are seen to be clear (translucent).

(4) Impregnation with wax:

Impregnation with wax (paraffin) takes place in an oven heated to 54 - 60°C. The temperature of the paraffin wax must be maintained at 58 - 60°C.

After blotting with filter paper, the tissue is transferred from the clearing agent to molten paraffin wax. The volume of wax should be about 25 - 30 times the volume of the tissue and must be changed at least once during impregnation. The change is effected by simply lifting the tissue from one pot of wax to the next with warmed forceps.

Casting and blocking:

Tissue is blocked by transferring it from the final wax bath to a mould filled with molten wax, inverting the tissue

to free the surface to be cut from air bubbles and oriented so that this surface rests on the base of the mould. The block is then quickly cooled.

Cutting of the paraffin wax embedded sections.

Trimming the block:

When cutting sections on microtomes, blocks must first be trimmed and fixed tp wooden billets. Wax is then removed with a sharp knife until 1/8 inch remains on all sides of the tissues. Great care is necessary to avoid exposing the tissue at any point, and the edges of the block should be made paralled so that an average ribbon of sections result. The surface of the block which is to be cut should be trimmed on the microtome during the preliminary cutting.

Only small flakes of wax should be trimmed at a time; trimming large pieces can lead to splitting of the block and exposure of the tissue; this will entail reblocking in fresh wax.

Fixing the block on the microtome:

When using a microtome with a flact metal stage on which the block can be fixed directly, a wooden handle spatuala, or a metal scalped which has been inserted into a wooden handle is heated in a bunsen burner. When hot, but not red hot this is applied to the underside of the block and to a layer of wax on the stage until the two wax surfaces melt, when they are quickly and firmly pressed together.

The surface of the wooden fillet is first serrated with a saw or a scalpel and coated with not wax. The block of the tissue is then attached to the wood in a manner described earlie

The wooden fillet is then mounted in a clamp type holder on a microtome.

Cutting Technique:

(1) Fix the block in the block holder on the microtome in such a position that it will be clear of the knife when this is in position.

(2) Turn back the feed mechanism on the microtome almost as far as it will go.

(3) Insert the appropriate knife holder and screw it tightly in position, check that the tilt of the knife is set at a correct angle (if this is adjustable). If it is intended to use the knife obliquely, the movable knife holder should be adjusted in the desired position.

(4) Move the block holder forward or upward, or adjust the feed mechanism until the wax block is almost touching the knife. Ensure that the whole surface of the block will move parallel to the edge of the knife, and that the leading edge of the block is a,most parallel to the edge of the knife in order to ensure a straight ribbon of sections.

(5) Tighten all the adjusting screws on the microtome. Faults in section cutting are most frequently due to the looseness of the block or the knife in the early stages of section cutting than to any other reason.

(6) To trim the block set the section thickness gauge to about 15 microns and with the rough knife - or one end of a large knife

which is kept for trimming - in position,.operate the microtome until complete sections of the tissues are being cut.

(7) Replace the rough kinfe by a sharp one, or move the knife to a new position, check that it is screwed tightly in position, Back the feed mechanism block a little to allow for slight differences which always exist in different knives, and even in different parts of the same knife. Apply it to the surface of the block and wipe the surface free of water; this is optimal, but makes flat even sections easier to cut.

(8) Set the thickness gauge:

For routine work 6 microns will give moderable thin sections with ease of cutting.

(9) The microtome is now operated until complete sections are being cut.

(10) Routine sections may be laid in small cardboard boxes or directly foloated on to a slide. Small cardboard boxes on which the number or the name of the tissue is marked in pencil are useful

(11) Cutting is normally only continued until sufficient perfect sections have been produced. The free end of each ribbon is supported either with fine forceps or with the fingers until it is about 18 inches in length; the ribbon is then freed from the knife by bringing a dissecting needle up under the last section. It must be allowed to touch the cutting edge of the knife. The sections are then laid on back paper and should

be fixed in position by gentle pressure with a ginger at each end of the ribbon to avoid them being scattered by stray draughts.

Fixing the sections or slides:

During cutting, paraffin wax embedded sections become slightly creased. Before being attached to slides these creases must be removed and the section flattened, and this is achieved by floating them on warm water (50-60°C), by one of the following three methods:

Water bath method:

Thermostatically controlled baths with inside coloured block are maintained at a temperature 5 - 6 degrees below the melting point of the paraffin wax, or an enamel or glass bowl filled to the brim with a correct temperature may be used. Air bubbles forming on the bottom of the dish must be dislodged with fingers or by vibration.

Sections are divided with a scalpel into lengths convenient to go one slide, usually single sections or 3-4 small sections. Room must be left at each end of the slide, for a label or one side, and space on the other side to prevent any difficulty when examining them under the microscope. It is perferable to leave 3/4 inch for the label and 1/2 inch at the other end.

The sections or sections are lifted off the bench and on to the surfaces of the water by inserting the point of a dissectin needle into the wax at one end, care being taken not to damage the tissues during the peeparation.

If creases in the section do not disappear immediately after contact with water, dissecting needles should be used to tease them out by applying one to each side of a crease with a gentle pressure.

A clear or albuminised (the albumin is prepared by mixing albumin of the eff with glycerine, and this is applied as a very thin coat on to the slides and allowed to dry). Slide is half submerged in the water, and with a dissecting needle the section in brought into contact with it. With the needle the section is then oriented on the slide by withdrawing the slide and bringing the flattened section with it. It is important that only the wax edge be touched with the needle and that this operation be carried out while there is still water on the slides. The slide is then set in an upright position to drain the mounted sections may then be left in the incubator at a temperature of 37°C overnight to dry and are ready for staining.

Hot stage method:

Hot stages are available commercially with a specially shaped metal top which is heated and maintained at 45-50°C. A clear or albuminized slide is laid on the hot stage, flooded with distilled water and the section or sections are laid on the surface of the water. Creases should be teased out with needles or forceps and the slides left for a few moments to get warm. The crease will flatten out due to the heat, When completely flat, the slide is removed from

the hot plate, the excess water is drained off and the section oriented into the correct position. It is then returned to the stage, section downwards to prevent dust from settling on it and left for 30 minutes. The section is then ready for staining.

Warmed slide method:

A clean or albuminated slide is flooded with distilled water, sections are laid on the surface and with the major creases removed the slide is held over a bunsen burner for a second or two to slowly warm the water. If necessary a second heating is given but the wax must not be melted during this operation. The creases will flatten out during this process, following which the excess water is drained off and the slide put into the oven at a temperature of 37°C or 56°C to dry. The section is ready for staining.

Stages in staining and mounting paraffin sections:

(1) Removal of wax with xylal (2) Hydration with graded clcohols (3) Staining (4) Dehydration through graded alcolols (5) Clearing with xylol (6) mounting under a cover slip.

Technique:

(1) Removal of wax:

Sections are placed in xylol for 1-2 minutes to dissolve the wax

(2) Hydration:

The section is taken out of xylol (it should

appear quite clear) and it is transferred to absolute alcohol for 1 minute, when it will become opaque.

The section is removed from absolute alcohol drained and placed in 90 per cent alcohol for 1 minute.

Sections fixed in formation should be removed from alcohol, placed in picric alcohol (Saturated solution of picric acid in alcohol) for 5-10 minutes, followed by the washing in the slide tray for 10 minutes. The sections are then transferred to 90 per cent alcohol of 2 minutes.

(3) <u>Staining:</u>

(a) Slides are transferred from 90 per cent alcohol after hydration to haematoxylin and left for 10-40 minutes. (b) Slides after draining off excess haematoxylin, are transferre to the slide washing they and washed until the sections are blue (when first removed from the haematoxylin they are pink). This takes about 10 minutes in tap water and is known as "bluing sections". (c) Sections are dipped into acid alcohol where they are agitated for a few seconds and returned to th slide washing tray until blue again. They should be observed under low power of the staining microscope to ensure that they are sufficiently differentiated. (d) If the sections are under differentiated it is again returned to the acid alcohol for a short period; if over differentiated (nuclei too pale) the section is rinsed in distilled water and returned to the haematoxylin for 10-15 minutes. (e) Sections which are differentiated and blued are transferred to 1 per cent

eosin for 2-4 minutes to counterstain them.

(f) Sections are transferred from eosin to the slide washing tray for 3-4 minutes, this will differentiated the eosin.

(4) Dehydration:

(g) after draining, sections are transferred from the slide washing tray to 90 per cent alcohol where they are agitated for 10-15 seconds.

(h) from 90 per cent alcohol they are transferred to absolute alcohol I where they are agitated for 10-15 seconds.

(i) They are then taken from absolute alcohol I to absolute alcohol II for 30 seconds.

(5) Clearing:

(j) Sections should be transferaed from absolute alcohol II to xylol I and left until completely clear for 15 seconds.

(k) Sections are then trasferred to xylol II, when clear, from which they may be mounted.

(6) Mounting:

Mountants:

Histologival sections which need to be examined for any length of time or to be stored, must be mounted under a cover slip. There are generally two main classes.

(1) Agueous media (ii) Resinous media. Canada Balsan (refractive index 1.52) a resinous media is a commonly used mountant. It is obtained from Canadian fir trees. Canada Balsaw is ground in a mortar and pestile until free of lamps and dissolved in xylol to 55 - 70 per cent by weight.

(l) Rectangular cover slips are wiped with soft, fluffless glass cotton.

(m) A cover slip is laid on a blotting paper; the section is removed from this xylol, the surplus xylol is removed by wiping the back of the slide and around the section, leaving a margin of about 1/8 of an inch; this stage should be quickly completed to avoid the section from drying.

(n) One or two drops of Canada Balsam, (depending on the size of the cover slip) ar placed on the section;.being laid along the middle of the section to reduce the likelihood of trapping air bubbles.

(o) The slide is quickly inverted over the cover slip; one end is placed over the blotting paper and the other end slowly lowered until the balsam touches the cover slip. The balsam quickly spreads under the cover slip and the slide, with the cover slip attached it is again quickly inverted and the cover slip guided into place with a dissecting needle.

An alternative method is to put the mountant on the section as described, place one end of the cover slip on the slide (Class of the section) and with the aid of a dissecting needle slowly lower the cover slip in position.

Air bubbles:

Air bubbles may be expressed by gentle pressure on the cover slip with a dissecting needle. When there is more than one air bubble in the mountant it is easier to put the slide back into xylol (which will remove the cover slip) and remove the section.

Routine stainings:

Hematoxylin and eosin is the most popular routine stain and the one devised by Ehrlich is the most popular because of i s durability, easy differentiation and comparative permanency.

As a counter stain 0.5 - 1.0 per cent aqueous solution of water soluble eocin is generally preferred to give a more informative picture.

Reagents required for Ehrulich's Alum Haematoxylin stain:

Staining time	30-40 minutes
Haematoxylin	6 g
Absolute alcohol	300 ml.
Distilled water	300 ml.
Glycerol	300 ml.
Glacial aceentic acid	300 ml.
PPotassium alum	in excess.

Preparation:

Haematoxylin should be dissolved in alcohol and the other components added in the order given. Finally potassium alum is added while the solution is shakon until there is a deposit of alum crystals at the stock container. Glycerol is said to give more even previse staining and to stabilise the stain againt over oxidation and evaporation.

Ripening:

After preparation of the stain it must be kept in a loosely plu ged bottle in a warm and well a lit place (window sill) until the oxidation of the haematocylin to haematein had taken place (this process is known as ripening). This will take 1-2 months.

APPENDIX IV

FOOD INTAKE (IN GRAMS) OF THE INDIVIDUAL RATS ON THE ATHEROGENIC DIET

Rat No.		FOOD INTAKE IN GRAMS							
		I Week	II Week	III Week	IV Week	V Week	VI Week	Total	Mean
Group I	1.	47.49	51.33	54.37	59.25	59.07	62.92	334.43	55.74
2	2.	45.85	50.41	54.43	58.77	60.15	63.02	333.13	55.52
	3.	47.42	50.61	53.75	56.77	59.21	62.60	380.36	55.06
	4.	47.26	51.29	54.48	59.26	59.78	63.01	335.08	55.85
	5.	48.43	50.27	54.85	58.48	60.28	68.08	335.39	55.90
	6.	48.24	49.86	52.59	56.18	58.49	61.89	327.22	54.54
GroupII	1.	47.33	49.00	51.34	55.30	58.63	61.08	322.68	53.78
	2.	45.86	47.50	49.73	52.88	54.84	57.84	308.65	51.44
	3.	45.87	47.38	48.70	52.54	54.75	56.97	306.23	51.05
	4.	46.40	48.92	50.94	56.14	55.38	57.95	315.73	52.62
	5.	45.24	46.74	48.71	52.62	54.08	57.85	335.27	55.88
	66.	45.14	47.38	48.88	52.68	53.99	57.61	305.68	50.95
GroupIII	1.	45.51	47.83	49.89	52.21	54.84	57.98	308.26	51.38
	2.	46.11	47.33	49.40	52.84	55.04	58.72	309.44	51.57
	3.	46.52	48.23	49.97	52.35	54.84	57.37	309.78	51.63
	4.	46.60	49.38	50.36	53.16	55.90	59.12	314.52	52.42
	5.	45.49	48.06	49.02	52.92	54.36	58.50	308.35	51.39
	6.	48.05	50.28	51.21	54.07	56.87	[illegible]	353.65	53.28
GroupIV	1.	46.03	49.03	49.94	52.25	54.11	58.18	309.54	51.59
	2.	45.47	48.20	50.29	53.12	54.01	57.55	308.64	51.44
	3.	45.82	48.52	50.65	52.73	54.87	58.91	311.5	51.92
	4.	47.89	49.88	50.75	52.68	54.22	58.16	313.58	52.26
	5.	44.86	47.01	49.42	52.18	54.45	59.52	307.44	51.24
	6.	44.86	46.22	49.29	52.54	55.49	59.03	308.37	51.40

FOOD INTAKE (IN GRAMS) OF THE INDIVIDUAL RATS ON THE EXPERIMENTAL DIET

Groups	Rat No.	Food intake in grams							
		I week	II week	III week	IV week	V week	VI week	Total	Mean
Group I	1.	-	-	-	-	-	-	-	-
	2	67.72	72.75	77.18	84.09	92.82	99.39	493.95	82.33
	3.	66.43	72.33	77.94	84.36	92.05	98.67	491.98	82.00
	4.	66.95	72.76	78.00	85.35	92.52	98.62	494.2	82.37
	5.	67.11	73.43	79.14	84.92	92.38	99.30	496.28	82.71
	6.	65.05	70.46	77.85	91.54	98.10	88.10	4[illegible]8.23	81.37
Group II	1.	64.62	71.11	76.92	82.08	88.58	94.69	478	79.67
	2.	62.05	67.36	72.91	80.52	86.83	93.91	463.58	77.26
	3.	59.95	66.79	72.19	79.72	86.13	92.78	457.46	76.24
	4.	61.24	67.80	72.11	80.52	87.71	94.06	463.44	77.24
	5.	61.55	76.62	73.13	81.28	85.99	94.16	464.73	77.46
	6.	61.00	67.27	72.66	80.81	87.44	93.81	462.99	77.17
Group III	1.	60.90	64.5	68.88	75.31	80.45	86.49	436.53	72.76
	2.	61.68	65.65	70.25	82.03	87.22	76.43	443.31	73.87
	3.	61.70	65.54	69.73	75.38	81.50	86.78	440.63	73.44
	4.	62.26	65.56	71.38	77.77	82.97	87.86	447.8	74.63
	5.	60.03	65.25	69.82	75.90	80.71	86.86	485.7	73. 10
	6.	61.72	65.42	70.28	77.16	81.46	86.55	442.59	73.77
Group IV	1.	-	-	-	-	-	-	-	-
	2.	58.72	64.85	69.56	74.27	79.55	84.68	431.6[illegible]	71.94
	3.	60.01	64.99	68.90	74.60	80.42	86.[illegible]7	435.29	72.55
	4.	59.35	65.07	70.83	76.91	82.30	87.46	441.92	73.65
	5.	59.89	64.43	68.86	74.93	79.61	85.92	433.64	72.27
	6.	59.94	64.99	70.13	75.59	80.10	85.10	436.7	72.78

WEIGHT GAIN (IN GRAMS) OF THE INDIVIDUAL RATS ON THE EXPERIMENTAL DIETS.

Rat No.		Weight gain in grams: I week	II week	III week	IV week	V week	VI week	Total	Mean
Group I	1.	-	-	-	-	-	-	-	-
	2.	2.9	4.1	5.3	6.9	4.7	7.7	31.6	5.27
	3.	2.3	4.5	5.2	5.7	6.7	7.9	32.3	5.38
	4.	2.7	4.2	6.0	6.1	6.5	7.8	33.3	5.55
	5.	4.2	4.1	5.7	5.9	4.6	8.6	33.1	5.51
	6.	3.0	4.4	4.9	10.2	11.3	6.9	40.7	6.78
Group II	1.	5.5	3.8	7.3	4.8	6.3	5.8	32.5	5.58
	2.	3.7	4.6	3.6	7.5	9.3	6.9	34.6	5.77
	3.	4.1	3.3	7.2	4.7	3.9	3.8	27.0	4.50
	4.	1.7	3.8	5.5	8.2	4.4	6.2	29.8	4.97
	5.	2.7	4.1	6.3	6.6	7.2	4.6	31.5	5.25
	6.	2.5	2.9	[illegible]	3.9	4.6	4.9	23.1	3.85
Group III	1.	2.9	3.1	4.2	4.7	5.6	5.3	25.8	4.30
	2.	4.3	3.2	4.1	4.9	5.7	5.6	27.3	4.55
	3.	2.4	3.3	3.9	4.4	4.8	5.9	26.8	4.47
	4.	3.8	1.3	4.1	5.9	4.9	4.1	26.0	4.33
	5.	1.9	3.2	2.1	2.1	1.9	4.9	16.1	2.68
	6.	2.6	4.0	5.1	4.1	5.7	3.9	15.4	4.23
Group IV	1.	-	-	-	-	-	-	-	-
	2.	2.0	3.1	3.9	4.2	5.2	6.8	25.2	4.20
	3.	2.3	3.3	4.1	3.8	6.4	4.4	24.5	4.08
	4.	6.2	2.8	3.4	3.1	5.1	4.1	24.1	4.12
	5.	1.5	2.8	3.4	5.7	5.6	15.6	24.1	4.02
	6.	2.8	3.9	3.5	4.7	4.1	5.2	24.2	4.03

APPENDIX VI

SERUM CHOLESTEROL LEVELS OF THE INDIVIDUAL RATS IN THE TWO PHASES OF THE EXPERIMENT (mg/100 ml OF BLOOD)

Group		Atherogenic		Experimental		
		30 days	45 days	15 days	30 days	45 days
I	1	212.5	290	—	—	—
	2	207.5	322.5	287.5	240.0	220.0
	3	180.0	308.75	280.0	246.5	240.0
	4	2?7.5	325.0	270.0	252.5	232.5
	5	157.2	313.75	272.5	245.0	257.5
	6	21.50	300.0	277.5	237.5	225.0
II	1	175.0	297.5	247.5	220.0	177.5
	2	212.5	320.0	257.5	217.5	180.0
	3	242.5	292.5	250.0	200.0	182.5
	4	215.0	305.0	265.0	207.5	182.5
	5	260.0	317.5	255.5	227.5	205.5
	6	197.5	312.5	262.5	212.5	190.0
III	1	255.0	322.5	232.5	197.5	162.5
	2	232.5	313.75	220.0	185.0	165.0
	3	225.0	305.0	217.5	177.0	157.5
	4	265.0	320.0	430.0	180.0	170.0
	5	235.0	322.5	207.5	185.0	155.0
	6	227.5	347.5	237.5	175.0	165.0
IV	1	265.0	325.0	—	—	—
	2	226.0	330.0	200.0	152.5	130.0
	3	177.5	325.0	197.5	165.0	135.0
	4	197.5	295.0	190.0	160.0	145.0
	5	212.5	308.75	180.0	142.5	120.0
	6	190.0	322.5	195.0	157.0	142.5