MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 Population investigated and materials used
2.2 Sources of fine chemicals and reagents used
2.3 Sampling and analysis made
2.4 Plasma lipoprotein and lipid methodology
2.5 Serum enzymes
2.6 Immunoglobulins and other plasma proteins
2.7 Miscellaneous assays in blood and serum
2.8 Studies made on erythrocytes
2.9 Lipid peroxidation and antioxidant assays
2.10 Leucocyte enzymes and lipid assays
2.11 Statistical analysis
2. MATERIALS AND METHODS

2.1 POPULATION INVESTIGATED AND MATERIALS USED

2.1.1 The survey included 190 male and 43 female patients between 30 and 70 years, admitted to the Intensive Coronary Care unit, Cardiology block, Kilpauk Medical College Hospital, Madras, with episodes of acute myocardial infarction (AMI).

2.1.1.1 Acute myocardial infarction in the patients was diagnosed by clinical examination and confirmed by ECG and serum CPK levels by three specialists in cardiology headed by Dr. S. Thanikachalam, M.D., D.M. (Cardiology), Professor of Cardiology, Kilpauk Medical College Hospital, Kilpauk, Madras - 10. For comparison, 151 healthy volunteers between age 34 and 60 years were included. They were employees in the administrative wing of the University of Madras and their relatives.

2.1.1.2 Blood sampling was made within 24 hours of admission in 49 cases, on the second day in 44 cases, third day in 30 cases, fourth day in 33 cases and after 4 days in 74 cases. Case history obtained included family and personal history of myocardial infarction, hypertension and diabetes mellitus. The patients' history also included earlier reports of ischaemia and hypertension, cigarette smoking, alcohol consumption, intake of meat and eggs and physical activity. The drugs used such as beta blockers, calcium channel blockers, antihypertensives, antiarrhythmics and other lipid lowering agents were categorically entered in the data sheets (a copy of which is given in Fig. 2.1). 75% of the patients studied after the episode of AMI, had prior history of IHD, and were on low fat (less than 10% calories) diet, and exercise
<table>
<thead>
<tr>
<th>Serial No.</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Name</td>
<td>Place</td>
</tr>
<tr>
<td>Sex</td>
<td>Age</td>
</tr>
<tr>
<td>Height</td>
<td></td>
</tr>
<tr>
<td>Occupation</td>
<td></td>
</tr>
<tr>
<td>Monthly Income</td>
<td></td>
</tr>
<tr>
<td>History of</td>
<td></td>
</tr>
<tr>
<td>Personal</td>
<td>Family</td>
</tr>
<tr>
<td>IHD</td>
<td></td>
</tr>
<tr>
<td>HBP</td>
<td></td>
</tr>
<tr>
<td>MI</td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>Yes/No</td>
</tr>
<tr>
<td>Diet</td>
<td>Veg/N.V.</td>
</tr>
<tr>
<td>Physical Examination</td>
<td>Pulse</td>
</tr>
<tr>
<td>ECG Changes</td>
<td>Arrhythmia/Ischemic</td>
</tr>
<tr>
<td>Peripheral vessels</td>
<td></td>
</tr>
<tr>
<td>Drugs taken</td>
<td></td>
</tr>
<tr>
<td>Beta blocker</td>
<td></td>
</tr>
<tr>
<td>Calcium channel blocker</td>
<td></td>
</tr>
<tr>
<td>Antihypertensive drugs</td>
<td></td>
</tr>
<tr>
<td>Antiarrhythmic drugs</td>
<td></td>
</tr>
<tr>
<td>Lipid lowering</td>
<td></td>
</tr>
<tr>
<td>Address:</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
regimen (walking 30 mts or jogging) for not less than two years before the onset of AMI. Nearly 50% had cut down on smoking in the interim period.

2.1.1.3 In the intensive coronary care unit, the ECG was monitored, oxygen supply was provided to give relief from dyspnoea. In the first stage heparin was administered with glucose-saline infusion. The prescriptions for the different patients were dictated to their immediate requirements. They included antihypertensive medications like propranolol, atenolol, nifedipine, verapamil, antianginal drugs (mostly isosorbide) and the diabetics had insulin therapy. In case of associated lung-infection, antibiotics (ampicillin or garamycin) were prescribed. Phenergon, benzdiazipam and ranitidine were also included in patients according to their requirements. All the patients came from urban literate families well above the poverty line.

2.1.1.4 Earlier surveys made in our laboratory have shown that the diet of the subject groups (i.e., from Madras Urban Middle class family) provided sufficient calories out of which 65% to 75% was derived from carbohydrates, 10 to 15% from proteins and 15% to 25% from fats, which had 70% unsaturation and P/S ratio below 1.0. (Shanmugasundaram et al, 1986).

2.1.1.5 The patients were already on low fat diets (less than 10% fat calories). Butter and other saturated fats did not form part of their diets. Intake of simple sugars never exceed 25g/day.

2.1.2 The part II of the study was on 50 (including 9 women) subjects between 35-65 years with Ischaemic Heart Disease on modified Anna Pavala
Sindhooram therapy (APSm) for control of lipids and improvement of coronary artery disease. 16 out of 50 had diabetes, 19 had already suffered myocardial infarction and 7 had undergone coronary bypass surgery, subsequent to earlier myocardial infarction. 15 males were executives, 17 were businessmen, 2 were agriculturists (landlords), and 7 were professionals. Among the females, 5 were housewives while 4 were professionals or executives. Government officers and heads of departments are also classified as executives.

2.1.2.1 Their total cholesterol was $269 \pm 40$ (mean $\pm$ S.D.) mg/dl, HDL cholesterol $47.1 \pm 8.5$ mg/dl and triglycerides $165 \pm 30$ mg/dl. 2 out of 50 patients had very high triglycerides (above 800mg/dl) at the time of inclusion in the study. The coronary risk index (Total/HDL cholesterol) was $5.82 \pm 1.06$. 7/50 showed characterise of Type IIa hyperlipoproteinaemia, 21 showed Type IIb and 11 Type IV HLP. 9/50 did not show either hypercholesterolemia or hypertriglyceridemia. Criteria for HLP classification used:

- **Type IIa**
  \[
  \text{Chol} > 265 \text{ or LDL} > 175 \text{ and TG} < 150\text{mg/dl}
  \]

- **Type IIb**
  \[
  \text{Chol} > 265 \text{ or LDL} > 175 \text{ and TG} > 150\text{mg/dl}
  \]

- **Type IV**
  \[
  \text{Chol} < 265 \text{ and LDL} < 175 \text{ and TG} > 150\text{mg/dl}
  \]

All 50 were followed up for a period of 6 months of APSm therapy, 41 continued for 12 months, 27 for 18 months, 18 for 24 months and 12 for 36 months at the time of completion of the study. The subjects in the clinical trial were inducted after informed consent, and were the private patients of Dr.S.Thanikachalam or Dr.J.R.Shankaran, Professor of Medicine (Retired), Madras Medical College, Madras-3.

2.1.2.2 APSm in soft gelatine capsules (100mg/each) was administered at 300mg/day in three divided doses after food. The patients continued other drugs according to the individual needs (as prescribed by the Physician), and their earlier diets, while no further changes in the consumption of tobacco, exercise and life style were recommended.
2.2 SOURCES OF FINE CHEMICALS AND REAGENTS USED

2.2.1 Cholesterol, digitonin, cholesterol oleate, sodium taurocholate, heparin, Bovine serum albumin, nicotinamide adenine dinucleotide phosphate (NADP, NADPH), dithionitrobenzoic acid (DTNB), 2-thiobarbituric acid, epinephrine and uric acid were obtained from Sigma Chemical Company, St. Louis, U.S.A.

2.2.2 Ammonium molybdate and sodium dodecylsulphate were obtained from KOCH - Light Laboratories Limited, Colnbrook, Bucks, England.

2.2.3 Chromotropic acid, tripalmitin, Sodium metaarsenite and tris (hydroxy methyl aminomethane) were from E. Merck, Darmstadt, West Germany.

2.2.4 Acetylacetone, glyceroltrioleate, diethyldithiocarbamate, silicic acid and silica gel-G were from Glaxo Laboratories, BDH Division, Poole, England.

2.2.5 Dextran sulphate was from Sochibo, France, while sodium pyruvate used was obtained from Boehringer Ingelheim, West Germany.

2.2.6 Ascorbic acid and 1-amino-2-naphthol-4-sulphonic acid (ANSA) were obtained from Sisco research laboratories Pvt. Ltd., Bombay, India.

2.2.7 Antisera for IgG, IgM and IgA along with reference standards were obtained from Immunodiagnostics Ltd., New Delhi.

2.2.7.1 Lyophilised thromboplastin (calcium impregnated) was obtained from Glaxo Laboratories, BDH Division (Bombay).
2.2.8 Modified Anna Pavala Sindhooram (APSm), used in the present study is a herbomineral Indian medicine described by Shanmugasundaram et al (1983) and prepared after some modifications. The efficacy of APSm has already been confirmed in animal models (Dhandapani et al, 1984) and a clinical trial on a double blind cross over design has been completed earlier (Shanmugasundaram et al, 1991). The drug was prepared by Dr.E.R.B. Shanmugasundaram, Senior Professor of Biochemistry (retired) University of Madras, and was his gift.

2.2.8.1 Anna Pavala Sindhooram is a Siddha drug (or health food supplement) to prevent vascular hardening and regression of atherosclerotic lesions and is aimed to increase the life span. Indian medicinal system considers cardiovascular degeneration as a significant age-related change and together with cancer is the main cause for reducing the life span. Vascular degeneration characterised by arterial hardening is understood to originate by the accumulation of waste materials, especially fat. Together with other cellular constituents, they deposit on the blood vessels, making the lumen narrow, and the artery hard and susceptible to occlusion and haemorrhage. Prevention of arterial degeneration is recommended by a combination of dieting, physical exercise, and therapy using minerals and herbals with expectorant, laxative, diuretic, liver stimulant, cholesectic (stimulating bile secretion), haematinic, tonic and cardiotonic properties - APS is designed on the basis with green vitrol (FeSO₄ 7H₂O), and coral reef (consists of CaCO₃) and herbs having the desirable pharmacological effects as described in Indian Native Materia Medica (Shanmugasundaran et al, 1983).

2.2.8.2 Earlier experiments on several models of atherosclerosis and hypercholesterolemia in animals had confirmed the action of APS as bringing down
serum cholesterol (Shanmugasundaram et al., 1983b) regression of atherosclerosis
(Marita and Shanmugasundaram, 1982), and cholesterol removal with faeces
(Dhandapani et al., 1984). Clinical trials in IHD and hypercholesterolemic subjects
on a double blind cross-over basis (Shanmugasundaram et al., 1991), confirmed the
cholesterol lowering, HDL raising effect of APSm in men including in types IIa, IIb
and IV HLP and the absence of hepto and nephrotoxicity.

2.3 SAMPLING AND ANALYSIS MADE

2.3.1 Blood Sampling in AMI

2.3.1.1 Venous blood sample were drawn from all the subjects, in the morning (atleast
6 hours after the last glucose infusion in the AMI patients). Blood was correlated
into three tubes for each subject one with disodium salt of ethylene diamine tetra
acetic acid (EDTA) as anticoagulant, the second with sodium fluoride as
anticoagulant and the third with citrate as anticoagulant.

2.3.2 Blood sampling in healthy volunteers

2.3.2.1 Venous blood samples were collected from healthy volunteers between 8.00
AM and 9.30 AM after an overnight fast, lasting not less than 12 hours. Blood was
collected in three tubes (a) containing EDTA (b) with sodium fluoride and (c) with
citrate as anticoagulant.

Some of the analysis were made together with Mr.D. Sathiyaraj, whose work was
more concentrated with respect of erythrocytes and antioxidant activities.
2.3.3 Blood sampling in the clinical trial

2.3.3.1 Two venous blood samples were collected before the clinical trial, with at least 15 days interval and analysed. The plasma lipids and other values with two samples differed by less than 5%, and the values obtained at the second sampling was taken as the initial level.

2.3.3.2 Subsequently fasting blood samples were collected at the end of 2, 4, 6, 12, 18, 24 and 36 months. Anticoagulants used for blood sampling were the same as described earlier.

2.3.4 Processing of samples

2.3.4.1 The plasma was separated (from blood with EDTA) and used for assay of lipids, lipoprotein, leucocytes and erythrocytes were used subsequently for further analysis as described in later section.

2.3.4.2 Plasma was separated from blood collected with EDTA as anticoagulant and was also used for the assay of the following plasma enzymes. Creatine phosphokinase (CPK), lactate dehydrogenase (LDH), glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT), and gammaglutamyl transpeptidase (GGTP).

2.3.4.3 An aliquot of blood sample collected with sodium fluoride was used for the estimation of blood glucose and urea.

2.3.4.4 The sample collected with citrate as anticoagulant was used for the assay of fibrinogen, plateletcount, prothrombin time and immunoglobulins.
2.3.5 Lipids and Lipoprotein Profile

2.3.5.1 Cholesterol distribution in the plasma lipoproteins were studied to include HDL₂, HDL₃, LDL and VLDL by precipitation methods. Cholesterol was estimated in the fraction by the method of Parekh and Jung (1970). Coronary risk index (total cholesterol/HDL cholesterol ratio) and the ratio of HDL₂/HDL₃ were arrived at. Triglycerides, phospholipids and free fatty acids were also assayed in the plasma.

2.3.6 Immunoglobulins and other plasma protein

2.3.6.1 The three major classes of Immunoglobulin’s were assayed in plasma by the technique of single radial immunodiffusion. Total protein was also assayed in plasma.

2.3.6.2 An aliquot of blood collected using sodium citrate as anticoagulant for the assay of prothrombin time and fibrinogen. Platelet count was done from the blood samples collected with EDTA.

2.3.7 Lipid peroxidation

2.3.7.1 Malondialdehyde (MDA, the end product of lipid peroxidation) was assayed in the plasma, HDL fraction, erythrocyte membrane and in the whole erythrocytes. The activities of the enzymes, superoxide dismutase and glutathione peroxidase were assayed in the haemolysate, while the activity of catalase was assayed in the erythrocyte membrane.
23.8 Leucocyte Lipids and Enzymes

23.8.1 Leucocytes were separated from blood collected with EDTA as anticoagulant and the fraction was rich (upto 85%) in the lymphocytes. Lipids cholesterol, triglycerides and phospholipids were assayed in this fraction. The activities of the enzymes cholesterol ester synthetase (CES), and cholesterol ester hydrolase, also known as lysosomal acid lipase (LAL) were also assayed in the leucocyte homogenate.

23.9 Biochemical follow-up of APSm therapy

23.9.1 High risk group of 50 patients described in section 2.1.2 were put on APSm for periods upto 36 months to assess the long-term benefits of the drug administration. The clinical picture and related data are given in Table 3.11.

23.9.2 Biochemical monitoring consisted of (a) plasma lipids, lipoprotein profile, (b) fibrinogen, immunoglobulins and total proteins, (c) leucocyte cholesterol ester synthetase and cholesterol ester hydrolase activities; (d) malondialdehyde assayed in plasma, HDL, erythrocytes and in the erythrocyte membrane, (e) catalase, superoxide dismutase and glutathione peroxidase activities.

2.4 PLASMA LIPID AND LIPOPROTEIN METHODOLOGY

2.4.1 Fractional precipitation of lipoproteins

Lipoproteins were fractionated by a dual precipitation technique (Wilson and Spiger, 1973) with minor modifications. This method has been in use in our
laboratory since 1978, and had been found to be highly reproducible (error less than 5%).

2.4.1.1 Addition of heparin-manganese chloride solution to plasma caused precipitation of VLDL and LDL. The supernatant obtained after sedimentation represented the HDL fraction and cholesterol and MDA were measured in this fraction. HDL is expressed as HDL-cholesterol (HDLC) in mg/dl plasma.

2.4.1.2 In an aliquot of the supernatant (containing HDL) from section 2.4.1.1, HDL₂ was separated from HDL₃ by the method of Gidez et al (1982) using dextran sulphate.

2.4.1.3 In a second aliquot of plasma sodium dodecyl sulphate (SDS) was added which resulted in the aggregation of VLDL which flocculates on the top. The subnatant contained both HDL and LDL, and cholesterol content of this mixture was assayed (Burstein and Scholnick, 1972).

2.4.1.4 A third aliquot of plasma was assayed for total cholesterol. The cholesterol content of each fraction was arrived at in the following manner.

\[
\text{Total cholesterol} = \text{subnatant from SDS (containing HDL + LDL)} = \text{VLDL cholesterol}
\]

\[
\text{Subnatant from SDS} = \text{Heparin Mn}^{2+} \text{ supernatant (HDL}_c\text{)} = \text{LDL cholesterol}
\]

\[
\text{HDL}_c - \text{HDL}_3c = \text{HDL}_2c
\]

The flow sheet of the lipoprotein fraction is shown in Fig.2.2.
Fig. 2.2

FLOW SHEET FOR THE DUAL PRECIPITATION METHOD OF LIPOPROTEIN ANALYSIS (WILSON AND SPIGER, 1973) AND HDL - SUBFRACTIONS (GIDEZ et al., 1979) FOR THE STUDY OF CHOLESTEROL DISTRIBUTION IN PLASMA LIPOPROTEINS

Whole plasma

- Total cholesterol measured

Sodium dodecyl sulphate

- Centrifuge
  - Pellicle (VLDL)
  - Subnatant (LDL + HDL) Cholesterol measured

Heparin manganese chloride

- Centrifuge
  - Residue
  - Supernatant
    - Dextran sulphate
      - Centrifuged
        - HDL Cholesterol measured
        - HDL3 Cholesterol measured
        - Precipitate HDL2
2.4.2 HDL and its subfractions

2.4.2.1 Reagents

1. Heparin - Manganese chloride reagent

3.167g of manganese chloride was added to 1.0 ml solution of heparin containing 20,000 units. This mixture was made up to 8.0 ml with distilled water.

2. Dextran sulphate.

121 mg of dextran sulphate (mol. wt. 15,000) was dissolved in 10 mg of 0.89% saline.

2.4.2.2 Procedure

2.0 ml of serum was added to 0.18 ml of heparin - manganese chloride reagent and mixed well. The solution was allowed to stand at 4°C for 30 minutes and then centrifuged in a refrigerated centrifuge at 2,500rpm maintained at 10°C for 30 minutes. The supernatant contained the HDL fraction. 1.0 ml of this was used for the fractionation of HDL subfractions HDL$_2$ and HDL$_3$. The remaining supernatant was taken for the estimation of cholesterol by the method of Parekh and Jung (1970) to be described in section 2.4.5.2.

2.4.3 HDL$_3$ assay

2.4.3.1 1.0 ml of the clear total HDL supernatant from section 2.4.2 was mixed with 0.1 ml of dextran sulphate and allowed to stand at room temperature for 20 minutes to precipitate HDL$_2$. The supernatant represented the HDL$_3$ subfraction. Cholesterol was assayed in the HDL$_3$ fraction.
2.4.4 LDL and VLDL

2.4.4.1 Reagents

Sodium dodecyl sulphate (SDS) - 10% solution in 0.15 M sodium chloride pH 9.0.

2.4.4.2 Procedure

2.0 ml of plasma was added to 0.15 ml of sodium dodecyl sulphate. The contents were mixed well and incubated at 37°C for 2 hours. The contents were centrifuged in a refrigerated centrifuge at 10,000 rpm for 15 minutes. VLDL aggregated as a pellicle at the top. The supernatant contained the HDL and LDL fractions. Cholesterol was estimated from this fraction.

2.4.5 Cholesterol

Cholesterol was estimated in the plasma and the fractions of lipoproteins obtained by precipitation methods described in the proceeding sections (2.4.2 and 2.4.3) by the method of Parekh and Jung (1970). This method was found to be more reliable than the Zak's method (Zak, 1957) and similar in sensitivity to O-phthalaldehyde method of Rudel and Morris (1973) as observed by earlier scholars in our laboratory (Shanmugasundaram et al, 1985).
2.4.5.1 Reagents

1. Ferric acetate - uranyl acetate reagent:

   500 mg of ferric chloride was dissolved in 10 ml of water and 3.0 ml of ammonia was added and shaken well. The precipitate was washed several times with distilled water, dissolved in glacial acetic acid and made up to a litre with acetic acid. 100 mg of uranyl acetate was added and the contents were shaken well and left overnight. The reagent was stored in a brown bottle.

2. Sulphuric acid - ferrous sulphate reagent:

   100 mg of anhydrous ferrous sulphate was partially dissolved in 100 mL of glacial acetic acid, followed by 100 ml of concentrated sulphuric acid with stirring. After cooling to room temperature, the solution was made up to one litre with concentrated sulphuric acid.

3. Standard Cholesterol

   Standard cholesterol solution was prepared by dissolving recrystallised cholesterol in glacial acetic acid (AR) at 2 mg/ml.

2.4.5.2 Procedure

   0.05 ml of plasma (or) 0.1 ml of the lipoprotein subfractions were mixed with 10 ml of ferric acetate-uranyl acetate reagent. The contents were mixed well and allowed to stand for 15 minutes. The precipitated proteins were sedimented by centrifugation. 3.0 ml of the clear supernatant was mixed with 2.0 ml of sulphuric acid
ferrous sulphate reagent. In case of standard, 0.05ml was mixed with 10 ml uranyl acetate reagent and 3 ml aliquots were treated with 2.0 ml of the acid - reagent. Blank comprised of 3.0 ml of ferric acetate-uranyl acetate reagent. The colour developed was read at 530 nm after 20 minutes.

**Standard cholesterol solutions in the range of 15 to 60µg were also treated as above.**

Cholesterol levels are expressed as mg/dl plasma for both total and the different lipoprotein classes.

### 2.4.6 Phospholipids

Phospholipids in plasma was estimated by the method of Rouser *et al* (1970).

#### 2.4.6.1 Reagents

1. Trichloroacetic acid 10%
2. Ammonium molybdate 3%
3. Ascorbic acid 3%
4. **Standard phosphate** was an aqueous solution of potassium dihydrogen phosphate containing 80µg of phosphorous/ml **working standard solution** had a concentration of 8µg phosphorous/ml.
2.4.6.2 Procedure

0.1 ml of plasma was diluted to 2.0 ml with distilled water and 2.0 ml of 10% TCA was added. The precipitated proteins were sedimented by centrifugation. The supernatant was discarded. 1.0 ml of perchloric acid (70%) was added to the residue and digested on a sand bath till the solution became colourless. After cooling, the solution was made upto 5.0 ml with distilled water. Standard phosphate solutions and blank containing water were mixed with 0.8 ml of perchloric acid and the final volume was made upto 5 ml with distilled water. 0.5 ml of each ammonium molybdate and ascorbic acid were added and the mixture was kept in a boiling water bath for 6 minutes. The blue colour developed was read at 710 nm.

Phospholipids are expressed as mg/dl plasma after multiplying by a factor 25 to give the phospholipid content according to Scheig (1974).

2.4.7 Triglycerides

Triglycerides were estimated in plasma by the method of Rice (1970) based on the method of Van Handel (1961).

Lipids were extracted with chloroform methanol mixture (2:1, v/v). Phospholipids were adsorbed on to silicic acid and the triglycerides remaining in solution were saponified with alcoholic potassium hydroxide. The liberated glycerol was oxidised by periodate to formaldehyde and the excess oxidising agent was destroyed by reaction with sodium arsenite. The formaldehyde formed was determined by the chromotropic acid colour reaction.
2.4.7.1 Reagents

2. Sodium chloride - saturated
3. Activated silicic acid
4. Alcoholic potassium hydroxide 0.4%
5. Sulphuric acid 0.2 N
6. Sodium metaperiodate 0.5%
7. Sodium arsenite 0.5 M
8. Chromotropic acid reagent.

1.14 g of disodium salt of chromotropic acid was dissolved in 100 ml of distilled water. Then 450 ml of dilute acid solution (prepared by mixing concentrated sulphuric acid and distilled water in the ratio 2:1, v/v) was added and stirred well.

9. Tripalmitin standard solution was made in chloroform. The working standard contained 0.1 mg tripalmitin/ml

2.4.7.2 Procedure

0.2 ml of plasma was mixed with chloroform - methanol mixture and left aside for 30 minutes before centrifugation. After centrifugation, 4.0 ml of the lipid extract was added to tubes containing 8.0 ml of saturated sodium chloride and shaken vigorously. The contents were allowed to settle for one hour and then centrifuged. The supernatant saline - methanol phase was discarded. The washed chloroform phase was filtered into a dry tube. 200 mg of activated silicic acid was added to chloroform phase, shaken vigorously and allowed to stand for 30 minutes. After
centrifugation, 0.5 ml of the supernatant as well as tripalmitin standards were evaporated to dryness. Then to the test, standard and blank tubes, 0.5 ml of alcoholic potassium hydroxide solution was added and the mixture was saponified in a 60-70°C water bath for 20 minutes. 0.5 ml of 0.2N sulphuric acid was added and heated in a boiling water bath for 10 minutes. After cooling the tubes, 0.1 ml of sodium metaperiodate was added and allowed to stand for 10 minutes. The excess periodate was reduced by the addition of 0.1 ml of sodium metaarsenite. 5.0 ml of chromatropic acid reagent was added, mixed thoroughly and kept in a boiling water bath for 30 minutes. After cooling, 0.5 ml of thiourea solution was added. The colour developed was measured at 540 nm.

Triglycerides are expressed as mg/dl plasma.

2.4.8 Free fatty acids (FFA) in plasma

Free fatty acids in plasma was estimated by the method of Hron and Menahan (1981).

2.4.8.1 Reagents

1. Chloroform - heptane - methanol solvent 200: 150: 7 (v/v)

2. Copper nitrate - triethanolamine (Cu - TEA) solution.
   500 ml of 0.1m copper nitrate and 50 ml of 0.2M triethanolamine were mixed with 33 g of sodium chloride. The pH was adjusted to 8%.

3. Colour reagent: 0.1% diethylidithio carbamate in n-butanol.

4. Activated silicie acid.

5. Standard palmic acid contained 200 µg/ml dissolved in chlorotorm.
2.4.8.2 Procedure

An aliquot of 0.2 ml plasma was mixed with 6.0 ml of chloroform heptane-methanol solvent and was shaken vigorously. 200 mg of activated silicic acid was added, shaken and left aside for 30 minutes. The solution was then centrifuged and the supernatant was transferred to tubes containing 2.0 ml of Cu-TEA solution. Blank contained only the solvent while standards with different concentrations were made upto a known volume with solvent and 2.0 ml of copper nitrate - triethanolamine solution was added. The contents were agitated using a mechanical shaker for 20 minutes. The mixture was separated into two phases by centrifugation. 2.0 ml of the upper layer was mixed with 1.0 ml of the colour developing reagent. The colour developed was read at 430 nm.

Plasma free fatty acids are expressed as mg/dl.

2.5 PROCEDURES USED IN SERUM ENZYME ASSAYS

2.5.1 Lactate dehydrogenase (L-lactate: NAD oxidoreductase, E.C. 1.1.1.27)

The enzyme was assayed according to the method described by King (1965) with some modifications. The enzyme catalyse the reaction.

\[
\text{LDH} \quad \text{Pyruvate} + \text{NADH} + H^+ \quad \text{-----} \rightarrow \text{Lactate} + \text{NAD}
\]
2.5.1.1 Reagents

1. Glycine buffer; 0.1 M containing 0.1m NaCl.
2. Buffered substrate: 4.0 gm of lithium lactate was dissolved in 125 ml of glycine buffer and 75ml of 0.1 N sodium hydroxide. The pH was adjusted to 10.0.
3. Cofactor: 5.0 mg of nicotinamide adenine dinucleotide (NAD) was dissolved in 1.0 ml of water.
4. Standard solution: 11 mg of sodium pyruvate was dissolved in 100 ml of buffered substrate.
5. Sodium hydroxide 0.4 N
6. Reduced nicotinamide adenine dinucleotide (NADH) solution.

8.5 mg of NADH was dissolved in 10 ml of buffered substrate.

2.5.1.2 Procedure

1.0 ml of buffered substrate, 0.2 ml of NAD\(^+\) solution and 0.1 ml of serum were mixed and incubated at 37°C for 30 minutes. The reaction was terminated by the addition of 1.0 ml of DNPH and the tubes were further incubated for 20 minutes, 10 ml of 0.4N sodium hydroxide was then added and the colour developed was read at 440 nm. Control tubes did not receive the coenzyme solution and standard curve was prepared with aliquots of pyruvate solution, substrate, NAD\(^+\) and NADH as per test.

The activity of serum lactate dehydrogenase is expressed as IU/l. One unit is the amount of enzyme that catalyses the formation of one \(\mu\) mole of pyruvate/minute.
2.5.2 Creatine phosphokinase (ATP: Creatine Phosphotransferase, E.C.2.7.2.3)

The reaction catalysed by this enzyme is as follows:

\[
\text{CPK} \\
\text{Creatine + ATP} \quad \longleftrightarrow \quad \text{Creatine phosphate + ADP.}
\]

The enzyme was assayed by the method of Okinaka et al (1961).

2.5.2.1 Reagents

1. Tris - HCl buffer 0.1 M pH 9.0
2. ATP solution: 112 mg of ATP was dissolved in 10 ml of Tris-HCl buffer.
3. Cysteine - hydrochloride solution: 59 mg of magnesium sulphate and 15.7 mg of cysteine hydrochloride was dissolved in 10 ml of distilled water.
4. Substrate: 240 mM creatine solution.
5. Trichloroacetic acid (TCA) 10%.
6. Ammonium molybdate 2.5% in 3N H\textsubscript{2}SO\textsubscript{4}.
7. 1-Amino-2-naphthol-4-sulphonic acid (ANSA)

500 mg of 1-amino-2-naphthol-4-sulphonic acid was dissolved in 195 ml of 15% sodium bisulphite and 5 ml of 20% sodium sulphite. The solution was filtered and stored in a brown bottle.
8. Standard: 35.1 mg of pure potassium dihydrogen phosphate was dissolved in 100 ml of water to give a concentration of 80\mu g/ml.
2.5.2.2 Procedure

The reaction mixture contained 0.05 ml of serum, 0.1 ml of substrate, 0.1 ml of ATP solution and 0.1 ml of cysteine hydrochloride solution. The final volume was made upto 2.0 ml and incubated at 37°C for 30 minutes. The reaction was arrested by the addition of 1.0 ml of TCA and the contents subjected to centrifugation. An aliquot of the supernatant was taken and the phosphorus content was measured by the method of Fiske and Subbarow (1925).

The enzyme activity of serum creatine phosphokinase is expressed as IU/l.

2.5.3 Serum glutamate oxaloacetate transaminase

(SGOT; L-aspartate-2-oxoglutarate aminotransferase, (E.C. 2.6.1.1). This enzyme was assayed according to the method of Bergmeyer and Bernt (1974).

The reaction catalysed by this enzyme is as follows:

\[
\text{GOT} \\
\text{L-Asp} + \text{2-oxoglutarate} \rightarrow \text{Oxaloacetate} + \text{L-glutamate}
\]

2.5.3.1 Reagents

1. Buffered Substrate: Dissolved 15 gm dipotassium hydrogen phosphate, 2.0 gm potassium dihydrogen phosphate and 300mg 2-oxoglutarate in 700 to 800 ml distilled water and 13.2 gm L-aspartic acid was added. The pH of the solution was adjusted to 7.4 with sodium hydroxide and made upto a litre with water.
2. 2,4-Dinitrophenyl hydrazine (DNPH) 1 mmol (200 mg/l in 1 mol/l dihydrochloric acid.
3. Sodium hydroxide 0.4 N
4. Standard pyruvate solution: 1μ mol pyruvate/ml

2.5.3.2 Procedure

1.0 ml of the substrate was incubated for 10 minutes at 37°C. Then 0.2 ml of serum was added and the incubation was continued for an hour. To the control tubes, serum was added after the reaction was arrested by the addition of 1.0 ml of dinitrophenyl hydrazine reagent. The tubes were kept at room temperature for 20 minutes. Then, 10 ml of 0.4N sodium hydroxide was added. A set of standard pyruvate was also treated in a similar manner. The colour developed was read at 540nm. The enzyme activity is expressed as IU/l.

2.5.4 Serum glutamate pyruvate transaminase (L-alanine: 2-oxoglutarate aminotransferase, E.C.2.6.1.2)

The enzyme catalyses the reaction as:

L - alanine + 2-oxoglutarate → pyruvate + L-glutamate

Its activity was measured by the method of Bergmeyer and Bernt (1974).

2.5.4.1 Reagents

1. Phosphate buffer 0.1 M, pH 7.5.
2. Substrate: 1.78g of DL-alanine and 30 mg of α-ketoglutarate were dissolved in 20 ml of buffer. 0.5 ml of 1N sodium hydroxide was added and made up to 100 ml with buffer.
3. 2,4-dinitrophenyl hydrazine-0.02% in HCl.
4. Sodium hydroxide-0.4N
5. Standard pyruvate: 1μ mol pyruvate/ml

2.5.4.2 Procedure

1 ml of the substrate was incubated for 10 minutes at 37°C. Then 0.2 ml serum was added. The tubes were incubated for 30 minutes at 37°C. To the control tubes, serum was added after arresting the reaction. After the incubation period, 1.0 ml of dinitrophenyl hydrazine was added to arrest the reaction. The tubes were kept at room temperature for 20 minutes. Then 10 ml of 0.4 N sodium hydroxide was added. A set of standard pyruvate was also treated in a similar way. The colour developed was read at 540 nm. The enzyme activity is expressed as IU/l.

2.5.5 Gamma-glutamyl transpeptidase (GGT) (γ-glutamyl peptide: amino acid-glutamyl transferase, E.C.2.3.2.2)

Gamma glutamyl transpeptidase is a plasma membrane bound enzyme which catalyses the reversible transfer of glutamyl groups from gamma glutamyl donors to aminoacids of peptides or to water. The enzyme was assayed according to the method of Orlowski and Meister (1965) as modified by Rosalki and Rau (1971).

2.5.5.1 Reagents

1. Substrate: 30.3 mg of L-gamma-glutamyl p-nitroanilide/10 ml. The substrate was sparingly soluble and was dissolved by warming to 50-60°C. The substrate solution was used within two hours of its preparation.
2. Tris-HCl buffer 0.1M, pH 8.2

3. Glycylglycine (1.32 mg/ml). This was used as a second substrate.

4. Acetic acid 10%

5. Standard 13.8 mg of p-nitroanilide (recrystallized) per 100 ml distilled water.

2.5.5.2 Procedure

0.5 ml of serum was added to the incubation mixture containing 0.5 ml gamma glutamyl p-nitroanilide, 2.2 ml glycyl glycine and 1.0 ml buffer. After incubation for 30 minutes at 37°C the reaction was arrested by the addition of 1.0 ml of 10% acetic acid.

The amount of p-nitroanilide liberated in the supernatant was measured as the difference in optical density at 410 nm between samples, with and without substrate. The substrate incubated in the absence of serum under the same conditions was used as a reference blank. Optical density of solutions of p-nitroanilide in the range of 15-75µg served as standard curve for arriving at the amount of product formed.

Enzyme activity is expressed as IU/l of serum, where one unit is defined as the amount of enzyme required to release one µmole of p-nitroaniline/minute under incubation conditions.
2.6 IMMUNOGLOBULINS AND OTHER PLASMA PROTEIN STUDIES

2.6.1 Immunoglobulins G, A and M in serum

Quantitation of IgG, IgA and IgM was attained by single radial immunodiffusion technique by the method of Mancini et al (1965).

2.6.1.1. Reagents

1. Agarose-3% in phosphate buffered saline (PBS), pH 7.4.
2. Antisera for IgG, IgA and IgM obtained from Immunodiagnostics, Ltd., New Delhi.
3. Antisera-agarose mixture: Equal volumes of antisera after required dilution and 3% molten agarose were mixed so as to get a final concentration of 1.5% agarose containing antiserum.
4. Phosphate buffered saline, pH 7.4.

2.6.1.2 Procedure

The antibody (antisera) agarose mixture was used to obtain plates of 74 x 74 mm and allowed to set at 4°C in the cold room for 2-3 hours. Then the gel plate was placed on top of a template and wells of 1.5mm diameter were punched at regular intervals of 1.5 cm. For IgG estimation serum was diluted 5-times with PBS, for IgA 2 times and for IgM, undiluted plasma were used. 10 microlitres of this antigenic solution (serum) was delivered into the well using a microsyringe. Graded amounts of standard immunoglobulins solutions diluted in PBS were loaded in some of the wells. After filling the wells, the plates were kept in a humid chamber at a perfectly horizontal position at room temperature for three days. After equilibrium
has been attained as a result of antigen-antibody reactions, the diameter (d) of the precipitation ring was measured by means of an immuno-measure scale. A standard curve was constructed by plotting the diameter (d, in centimeters) of precipitation rings against the log concentrations of the standards. The concentration of unknown samples were read from the curve.

Immunoglobulin values were are expressed as mg/ml serum.

2.6.2 Fibrinogen in plasma

Fibrinogen in plasma was estimated by the method of Ratnoff and Menzie (1951) using the biuret colour reaction.

2.6.2.1 Reagents

1. Sodium citrate 3 18%
2. Calcium chloride 0 025M
3. Saline 0 89%
4. Stock biuret reagent: 45g of Rochelle salt was dissolved in 400 ml of 0 2N sodium hydroxide to which was added 15g of copper sulphate stirring continuously until the solution is complete. To this solution, 5g of potassium iodide was added and made up to a litre with 0 2N sodium hydroxide.
5. Working biuret solution
   200 ml of the stock reagent was made up to 1 litre with 0 2N sodium hydroxide containing 5g potassium iodide/litre.
6. Standard BSA. 6% aqueous solution of BSA was used as the standard.
2.6.2.2 Procedure

1.6 ml of blood was collected into a graduated tube containing 0.4 ml of 3.18% sodium citrate, shaken and centrifuged to separate plasma. To 0.5 ml plasma, 4 ml distilled water and 3 ml calcium chloride were added, mixed and the tube incubated at 37°C for 15 minutes. The tube was taken outside and centrifuged for 5 minutes. The fibrin clot shranked and it was held at the bottom of the tube. The supernatant was discarded and the fibrin clot was washed thrice with 5 ml volumes of saline. The fibrin clot in the bottom was dissolved in 5 ml of working biuret reagent by warming in a boiling water bath carefully. After the biuret colour formation, the tube was removed from the water bath and 3 ml of saline was added to the tube and the optical density measured at 540 nm. Graded concentrations of BSA standard solution ranging from 60 to 240 mg were taken. The final volume was made upto 3 ml with saline. To these tubes 5 ml of biuret reagent was added and read at 540 nm.

Fibrinogen contents are expressed as mg/dl plasma.

2.6.3 Prothrombin time

Prothrombin time was done by the Quick's one stage method (Dacie and Lewis, 1977).

Principle

A potent preparation of rabbit brain emulsion impregnated with calcium is added to citrated plasma and the clotting time estimated.
2.6.3.1 Reagents

1. Thromboplastin with calcium: This was prepared from a commercially available lyophilised rabbit brain preparation. It was obtained from Bio Merieux, Laboratory Reagents and Products, France (REF.68801).

2. Trisodium citrate - 3.18%

2.6.3.2 Procedure

1.6 ml of blood was collected into a graduated tube containing 0.4 ml of 3.18% trisodium citrate, shaken and centrifuged to separate the plasma. The plasma was kept at room temperature until the time of test. The test was performed within 4 hours of blood collection. 0.1 ml of plasma was delivered into the bottom of a short 5 ml test tube (75 mm x 10 mm) and it was placed in the water bath at 37°C. To this tube 0.2 ml of thromboplastin was added by forcibly blowing from a pipette as quickly as possible, and a stop watch started simultaneously. The tube was held with its lower end submerged in water (37°C) and gently tilted back and forth until the first strand of fibrin appeared. Tilting was done by gently inclining the tube from the vertical to just short of the horizontal so that the fluid content (plasma) could be observed. The stop watch was stopped at the time of appearance of the clot and the time noted. This test was repeated once for each specimen and the mean time was recorded. A normal (control) plasma was included with every batch of test samples.

Prothrombin time was expressed in seconds.
2.6.4 Platelet count

Platelets in blood were counted using the improved Neubauer counting chamber by the method of Dacie and Lewis (1977).

2.6.4.1 Reagents

Dacie’s fluid: This was prepared by dissolving 5.0g of sodium citrate and 1 ml of 40% formaldehyde and making up to 100 ml with distilled water. To 19 ml of this solution 1 ml of 0.2% brilliant cresol blue solution was added just before use. This solution was filtered and used.

2.6.4.2 Procedure

Venous blood collected with EDTA was used for platelet count. 0.05 ml blood was diluted with 0.95 ml of the Dacie’s fluid and mixed well using a narrow bore pasteur pipette, the counting chamber was filled with the diluted blood. The cells were allowed to settle to the bottom of the chamber for 15 minutes. To prevent from drying, the chamber was placed in a petridish, which contained a piece of wet filter paper.

Using the 40 x objective with reduced condenser aperture, the platelets were counted in 1/5 sq.mm 5 of the small squares of the large centre square. From this the number of platelets in 1 cubic millimeter of blood was calculated as

\[
\text{Cells} \times \text{blood dilution} \times \text{chamber depth} \div \text{area of chamber counted}
\]

Platelet count is expressed as number of cells \(10^5/\text{mm}^3\)
2.6.5 **Plasma Proteins**

The protein content of plasma was estimated by the method of Lowry *et al* (1951).

2.6.5.1 **Reagents**

1. **Alkaline copper reagent:**
   
   Solution A: Sodium carbonate : 2% in 0.1N sodium hydroxide.
   
   Solution B: Copper sulphate : 0.5% in 1% sodium potassium tartarate.
   
   50 volumes of solution A and 1 volume of solution B were mixed just before use.

2. **Folin's-phenol reagent:** This was prepared according to the procedure of Folin-Ciocalteau (1927). This was diluted with distilled water in the ratio 1:2 just before use.

3. **Standard BSA** contained a concentration of 100μg/ml.

2.6.5.2 **Procedure**

Serum was diluted 1:100 with distilled water. From the diluted serum 0.1 ml was taken, made upto 1.0 ml with water and was mixed with 5.0 ml of alkaline copper reagent. The contents were allowed to stand at room temperature for 10 minutes. Then 0.5 ml of Folin's-phenol reagent was added and mixed well. Standards and blank containing distilled water (1 ml) were made upto 6.0 ml with alkaline copper reagent and were processed similarly. The blue colour developed was read at 620 nm after 20 minutes.

Protein content of plasma was expressed as g/dl.
2.7 MISCELLANEOUS ASSAY IN BLOOD AND SERUM

2.7.1 Blood glucose

Blood glucose was estimated by the O-toluidine colour reaction of Dubowski (1962) modified by Sasaki and Matsui (1972).

2.7.1.1 Reagents

1. Trichloroacetic acid - 10%
2. O-toluidine - boric acid reagent: 2.4g of boric acid and 2.5g thiourea were dissolved in 100 ml of a solution containing distilled water, glacial acetic acid and freshly distilled O-toluidine in the ratio of 10:75:15 (v/v).
3. Standard glucose was prepared in distilled water and had a concentration of 100 µg glucose/ml.

2.7.1.2 Procedure

0.1 ml of blood collected with sodium fluoride was mixed with 3 ml of 10% TCA to precipitate the proteins. The suspension was centrifuged to sediment the precipitated proteins. 1.0 ml of the supernatant was mixed with 4.0 ml of O-toluidine reagent. Standard glucose solutions containing 25 to 100µg and a blank containing 1.0ml of distilled water were also processed in the same manner and heated in a boiling water bath for 8 minutes. The colour developed was read at 640 nm.

Blood glucose values are expressed as mg/dl.
2.7.2 Blood urea

Blood urea was estimated according to the method of Marsh et al (1965).

2.7.2.1 Reagents

1. Sodium tungstate-10%
2. Sulphuric acid-2/3 N
3. Diacetyl monoxime - thiosemicarbazide (DAM - TSC) reagent: 1.0g of diacetyl monoxime was dissolved in 500 ml of water. 200 mg of thiosemicarbazide was then added to it followed by 9g of sodium chloride. The solution was finally made upto a litre.
4. Acid reagent: 10ml of o-phosphorlic acid and 60 ml of sulphuric acid were added slowly to 800 ml of distilled water. After cooling, 1 ml of 10% ferric chloride was added and made upto a litre.
5. Standard urea containing 100µg/ml was prepared.

2.7.2.2 Procedure

0.2 ml of whole blood was diluted with 3.2 ml of distilled water, 0.3 ml of 10% sodium tungstate and 0.3 ml of 2/3 N sulphuric acid. The precipitated proteins were then sedimented by centrifugation. 1.0 ml of the clear supernatant was mixed with 1.5 ml of DAM-TSC reagent and 1.5 ml of the acid reagent. Blank containing distilled water and standard solutions were treated in a similar manner. The tubes were kept in a boiling water bath for 15 minutes. The pink colour developed was read at 530 nm.

Blood urea levels were expressed as mg/dl.
2.7.3 Haemoglobin

Haemoglobin in blood was measured by the method of Drabkin and Austin (1932).

The basis of the method is dilution of blood in a solution containing potassium cyanide and potassium ferric cyanide during which haemoglobin is converted to cyanomethemooglobin. The absorbance of the solution was then measured at 540 nm.

2.7.3.1 Reagents

1. Diluent: Dissolved 200 mg of potassium ferricyanide and 1.0 g of sodium bicarbonate in distilled water and made up to a litre. This was stored in a brown bottle.

2. Cyanomethemooglobin standard: This was obtained commercially and had a concentration of 16 g/ml.

2.7.3.2 Procedure

0.02 ml of blood was diluted with 5.0 ml of the diluent. The tube containing the solution was stoppered with a rubber band and inverted several times. After allowing to stand at room temperature for 10 minutes to ensure the completion of the reaction, the absorbance was read at 540 nm together with the standard of cyanomethemooglobin. Reagent blank was used to set the optical density to zero.

Blood haemoglobin is expressed as g/dl.
2.7.4 Uric acid

Uric acid in plasma was estimated according to the procedure of Caraway (1963)

2.7.4.1 Reagents

1. Phosphotungstic acid reagent: 30g of sodium tungstate was dissolved in 300 ml of water. 3.2 ml of 85% o-phosphoric acid was added to it, and refluxed for 2 hours under a low flame. After cooling to room temperature, 18g of lithium sulphate was added and the solution was made up to a litre.

2. Sodium carbonate 14%

3. Sulphuric acid 2/3 N

4. Sodium tungstate 10%

5. Standard uric acid: 100 mg of uric acid and 60 mg of lithium carbonate were dissolved in about 50 ml of distilled water with slight heating. After cooling to room temperature, the solution was made up to 100 ml. This was used as the stock.

Working standard contained 100 μg of uric acid/ml

2.7.4.2 Procedure

0.5 ml of serum was mixed with 3.5 ml of water, 0.5 ml of 10% sodium tungstate and 0.5 ml of 2/3 N sulphuric acid. The precipitated proteins were separated by centrifugation. 30 ml of the clear supernatant was mixed with 10 ml of
phosphotungstic acid and 1 ml of 10% sodium carbonate. Standard solutions in the range of 10 to 50 μg and a blank containing only water were treated similarly. The blue colour developed was read at 680 nm after 10 minutes.

The serum uric acid levels are expressed as mg/dl.

2.8 STUDIES MADE ON THE ERYTHROCYTES

2.8.1 Isolation of erythrocytes and their analysis

Blood collected with EDTA as anticoagulant was used. Plasma was separated by centrifugation at 3,000 rpm for 20 minutes. The packed cells were washed thrice with physiological saline and the plasma free red cells were used for the analysis.

2.8.2 Isolation of erythrocyte membrane

Erythrocyte membrane was isolated according to the method of Dodge et al (1963) with a change in buffer according to Quist (1980).

2.8.2.1 Reagents

1. Saline-0.89%
2. Tris-HCl buffer 0.31 M, pH 7.4
3. Tris-HCl buffer pH 7.2, 0.015M.
2.8.2.2 Procedure

Packed cells remaining after the removal of plasma was washed with isotonic saline to remove the buffy coat. 4.0 ml of packed cells were then washed three times with isotonic Tris-HCl buffer pH 7.4. Hemolysis was performed by pipetting out the washed red blood cell suspension into polypropylene centrifuge tubes which contained hypotonic buffer (pH 7.2). Erythrocyte ghosts were sedimented in a high speed refrigerated centrifuge at 20,000 x g for 40 minutes. The supernatant (hemolysate) was decanted carefully and used for analysis. The ghost sedimented at the bottom was resuspended by swirling with sufficient buffer of the same strength to reconstitute to the original volume. The ratio of the cells to washing solution is approximately 1:3 by volume. The ghosts of cell membrane were washed three times subsequent to hemolysis. The supernatant after the last wash was either pale pink or colourless.

The pellet of the erythrocyte membrane was resuspended in 10 ml of Tris-HCl buffer pH 7.4. Aliquots of this reconstituted membrane preparation was taken for the estimation of protein and other analysis.

2.8.3 Assay of proteins in the membrane

Total proteins was estimated in an aliquot (0.1 ml of 1 in 100 diluted membrane preparation) by the method of Lowry et al (1951) described in section 2.6.5.
2.8.4 Catalase activity in erythrocyte membrane (E.C 1.11.1.6)

The activity of catalase in the erythrocyte membrane suspension was assayed by the method of Sinha (1972).

Catalase causes the rapid decomposition of hydrogen peroxide to water

$$2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$$

2.8.4.1 Reagents

1. Dichromate-acetic acid reagent
   5% potassium dichromate was prepared with dilute (1.3 v/v) glacial acetic acid
2. Hydrogen peroxide, 0.2 M
3. Phosphate buffer - 0.01M, pH 7.0

2.8.4.2 Procedure

0.1 ml of erythrocyte membrane preparation from section 2.8.2 was taken to which 1.0 ml each of phosphate buffer and hydrogen peroxide were added and a timer started. The reaction was arrested with 2.0 ml potassium dichromate-acetic acid reagent. Standard \(\text{H}_2\text{O}_2\) in the range of 4 to 20 micromoles were taken and treated similarly. The contents were heated in a boiling water bath for 10 minutes. The green colour developed was read at 570 nm.

Catalase activity is expressed as \(\mu\text{moles of }\text{H}_2\text{O}_2\) consumed/minute/mg protein.
2.8.5 Hemolysate

The supernatant hemolysate collected after the first spin from section 2.8.2 was used for the assay of haemoglobin. The activities of glutathione peroxidase and superoxide dismutase were also measured.

2.8.5.1 Haemoglobin

Haemoglobin was assayed in 0.02 ml of washed erythrocytes as well as hemolysate by the method of Drabkin and Austin (1932) as described in section 2.7.3.

2.8.6 Superoxide dismutase (E.C.1.15.1.1)

Superoxide dismutase in the hemolysate was assayed by the method of Misra and Fridovich (1972) based on the oxidation of epinephrine - adenochrome transition by the enzyme.

Superoxide dismutase catalyses the dismutation of superoxide anions ($O_2^-$) to hydrogen peroxide and molecular oxygen in the following manner:

$$H_2O + 2O_2^- \rightarrow H_2O_2 + O_2$$

2.8.6.1 Reagents

1. Sodium Carbonate-bicarbonate buffer, pH 10.2
2. Ethylene diamine tetracetic acid-0.49 mM
3. Epinephrine - 3 mM
2.8.6.2 Procedure

0.5 ml of hemolysate was diluted with 0.5 ml of water. To this, 0.25 ml of ethanol and 0.15 ml of chloroform (all reagents chilled) were added. The mixture was shaken for 1 minute at 4°C and centrifuged. The enzyme activity in the supernatant was determined.

To 0.5 ml of the supernantant, 1.5 ml of buffer was added. The reaction was initiated by the addition of 0.4 ml of epinephrine and change in optical density per minute was measured at 480 nm.

Change in optical density per minute measured at 50% inhibition of epinephrine to adenosine transition by the enzyme is taken as one enzyme unit.

SOD activity is expressed as enzyme units/mg hemoglobin.

2.8.7 Glutathione peroxidase (Glutathione hydrogen peroxide oxidoreductase (E.C.1.11.1.9))

Glutathione peroxidase (GPx) catalyses the following reaction

\[
\text{GPx} \quad \text{ROOH} + 2\text{GSH} \quad \rightarrow \quad \text{GSSG} + \text{ROH} + \text{H}_2\text{O}
\]

The activity of glutathione peroxidase in the hemolysate was assayed by the method of Rotruck et al (1973).
2.8.7.1 Reagents

1. Sodium phosphate buffer 0.32 M, pH 7.0
2. Ethylenediaminetetraacetate 0.8 mM
3. Sodium azide 10 mM
4. Reduced glutathione 4 mM
5. Hydrogen peroxide 2.5 mM
6. Trichloroacetic acid 10%
7. Phosphate solution 0.3 M disodium hydrogen phosphate
8. DTNB - 40 mg of 5'-5'dithio bis (2-nitro-benzoic acid) was dissolved in 100 ml of 1% sodium citrate.
9. Reduced glutathione standard 0.02%.

2.8.7.2 Procedure

0.2 ml each of EDTA, sodium azide, glutathione, \( \text{H}_2\text{O}_2 \) together with 0.4 ml of buffer and 0.1 ml of enzyme (hemolysate) were mixed and incubated at 37°C for 10 minutes. The reaction was arrested by the addition of 0.5 ml of TCA and the tubes were centrifuged. To 0.5 ml of the supernatant 3.0 ml of disodium hydrogen phosphate and 1.0 ml of DTNB were added and the colour developed was read at 412 nm immediately. Graded concentrations of the standard were also treated similarly.

Glutathione peroxidase activity is expressed as \( \mu \text{g glutathione utilised/mg hemoglobin/minute} \).
2.9 LIPID PEROXIDATION AND ANTIOXIDANT ASSAYS

2.9.1 Assay of plasma lipid peroxide

Lipid peroxide levels in plasma was determined by the method of Yagi (1976) using the thiobarbituric acid (TBA) reaction. In this method, the elimination of water soluble substances that react with TBA was obtained by precipitating lipid peroxides along with plasma proteins in a phosphotungstic acid system. The interference from sialic acid and bilirubin, both of which react with TBA, was avoided by performing the TBA reaction in an acetic acid solution (Yagi, 1982).

2.9.1.1 Reagents

1. Sulphuric acid-N/12
2. Phosphotungstic acid-10%
3. Thiobarbituric acid reagent - equal volumes of aqueous 0.67% thiobarbituric acid and glacial acetic acid were mixed.
4. n-Butanol
5. Standard malondialdehyde (MDA)

A stock standard solution of malondialdehyde was prepared in distilled water using 1,1,3,3-tetraethoxy propane. This was stored at 4°C and diluted just before use such that the working standard contains 50 n moles/ml.

2.9.1.2 Procedure

To 0.2ml of plasma, 4.0 ml of N/12 sulphuric acid was added, mixed well and 0.5 ml of 10% phosphotungstic acid was added. The contents were centrifuged
and supernatant discarded. The sediment was mixed with 2.0 ml of sulphuric acid and 0.3 ml of phosphotungstic acid. The mixture was centrifuged and the sediment was dissolved in 4.0 ml of distilled water, and 1.0 ml of TBA reagent and the contents were heated in a boiling water bath for 60 minutes. After cooling, 5 ml of n-butanol was added and the contents were shaken vigorously, centrifuged for 20 minutes and the supernatant read at 515 nm.

Standard graph was obtained taking 2.10 nmoles of tetraethoxypropane mixed with TBA reagent and processed in the same manner.

MDA values are expressed as nmoles/l plasma.

2.9.2 Lipid peroxide in HDL

Lipid peroxide level in HDL from section 2.4.1.1 was assayed by the TBA reaction described in section 2.8.1. 50μl of the HDL fraction was mixed with 0.5 ml of 10% phosphotungstic acid and 3.5 ml of water. (Nishigaki et al (1981) and TBA reaction was carried out in the mixture.

2.9.3 Lipid peroxidation in Erythrocytes

Erythrocyte lipid peroxidation was determined by the method of Cynamon et al (1985).

2.9.3.1 Reagents

1. Isotonic phosphate buffered saline pH 7.4, 0.1M
2. Sodium azide - 0.026% in isotonic phosphate buffered saline.
3. Hydrogen peroxide - 0.75%, 3%
4. Trichloroacetic acid - 40%
5. Thiobarbituric acid - 1%
6. Standard malondialdehyde was prepared with a concentration of 50 nmoles/ml.

2.9.3.2 Procedure

0.2 ml of the washed erythrocytes from section 2.8.1 was suspended in 3.8 ml of phosphate buffered saline. After incubation at 37° for hour, 10 ml of 40% TCA was added, and centrifuged to sediment the proteins. To the supernatant, 1.0 ml of 1% thiobarbituric acid was added. The contents were boiled for 20 minutes, cooled and read at 535 nm.

Lipid peroxidation in erythrocytes expressed as nmoles MDA/mg hemoglobin.

2.9.4 Erythrocyte membrane lipid peroxidation

1 ml of the membrane preparation from section 2.8.2 was used for lipid peroxide assay as described in section 2.9.3.
2.10 LEUCOCYTE CHOLESTEROL ESTER SYNTHETASE AND HYDROLASE

2.10.1 Isolation of leucocytes

Leucocytes were isolated from whole blood by the method of Yeh et al (1971).

2.10.1.1 Procedure

2.0 ml of venous blood collected in 0.1% EDTA was transferred to polycarbonate centrifuge tubes containing 4.0 ml of 0.89% sodium chloride. After thorough mixing with a glass rod, the mixture was centrifuged for 5 minutes at 400g.

The supernatant was separated and the erythrocyte present along with sedimented leucocytes were lysed by adding 8.0 ml of ice-cold distilled water. The hypotonic suspension was stirred with a glass rod and then 0.8 ml of ice-cold 0.89% sodium chloride was added to bring the leucocyte back to an isotonic environment. The contents were then centrifuged for 10 minutes at 1,120 x g and the clear supernatant as well as loosely packed layer of erythrocyte ghosts were carefully removed and discarded. The leucocyte pellet was transferred completely to a centrifuge tube with an aliquot of 0.89% sodium chloride and centrifugation at 400 x g for 4 minutes. The supernatant was again discarded. The washings were repeated twice and the leucocyte pellet obtained was uniformly suspended in 0.34M sucrose containing 0.1% Triton X-100 (Patsch et al, 1980). An aliquot of this suspension was used in making a smear on a glass slide and stained with Leishmann's stain for
differential counts. The isolated leucocytes were predominantly lymphocytes (PMNL-75 to 85%).

2.10.2 Protein content was determined in an aliquot of leucocyte suspension according to the procedure described in section 2.6.5.

2.10.3 Aliquots of the leucocyte suspension were used for assaying cholesterol esterifying enzymes, namely, cholesterol ester hydrolase (CEH), also called lysosomal acid lipase (LAL) and cholesterol ester synthetase (CES).

2.10.4 Lysosomal acid lipase (LAL) or cholesterol ester hydrolase (CEH) (E.C.3.1.1.13)

Lysosomal acid lipase or CEH was estimated in the leucocytes by the method of Kothari et al (1970; 1973) with slight modification by Kritchevsky and Kothari (1973).

2.10.4.1 Reagents

1. Acetate buffer - 0.1M, pH 4.0
2. Acetone - ethanol mixture 1:1 (v/v)
3. Acetone - ether mixture 1:2 (v/v)
4. Glacial acetic acid 10%
5. Digitonin 5 mg/ml in 50% ethanol
6. Substrate:

   - 1.54 μmoles of cholesterol oleate and 3.8 μmoles of lecithin were dissolved in 0.1 ml of ether. 3.75 μmoles of sodium taurocholate was
dissolved in 1.0 ml of 0.1M acetate buffer. They were mixed together and homogenised. After evaporation of the organic solvent the mixture was sonicated to near clarity.

2.10.4.2 Procedure

0.3 ml of acetate buffer was added to 0.4 ml of substrate and 0.2 ml of leucocyte suspension and mixed well. This was incubated at 37°C for 6 hours with occasional shaking. The reaction was stopped by the addition of 5.0 ml of acetone - ethanol (1:1, v/v) mixture. The precipitated proteins were sedimented by centrifugation.

The free cholesterol liberated from cholesterol oleate was precipitated by the addition of 1.0 ml of digitonin followed by two drops of 10% glacial acetic acid. The contents were mixed well. The tubes were securely closed and kept in a dark chamber for 16 hours. The precipitated cholesteryl digitonide was sedimented by centrifugation at 1000 x g for 15 minutes and the upper phase was carefully decanted and discarded. The precipitate was washed twice with acetone - ether mixture (1:2, v/v) and finally with dry ether.

Ether was evaporated and the tubes were heated in a sand bath at 110°C to 115°C for 30 minutes. The precipitate was then dissolved in 3.0 ml of uranyl acetate and cholesterol content was estimated according to the method of Parekh and Jung (1970) as described in section 2.4.5.

Lysosomal acid lipase (LAL) or (CEH) activities are expressed as nanomoles of cholesterol liberated/hour/mg protein at 37°C.
2.10.5 Cholesterol Ester synthetase (CES, Acyl cholesterol acyl transferase, ACAT, E.C.2.3.1.26)

This lysosomal enzyme has an acidic pH and was assayed by the method of Kothari et al (1973). It is a measure of the total esterifying activity in the cells.

2.10.5.1 Reagents

Reagents 1-5 as in section 2.10.4.1.

Substrate: 15.5 μmoles of pure cholesterol and 46.5 μmoles of oleic acid were dissolved in 0.3ml ether, 31 μmoles of sodium taurocholate and 100μmoles of ammonium chloride were dissolved in 1.5 ml of acetate buffer. This was forcibly injected into the tube containing cholesterol in ether. The contents were mixed well and homogenised and the ether phase was evaporated.

2.10.5.2 Procedure

The incubation mixture contained 0.5 ml of acetate buffer 0.2 ml of substrate and 0.2 ml of leucocyte suspension. Incubation was carried out at 37°C for 6 hours with occasional shaking. The reaction was arrested by the addition of 5.0 ml of acetone - ethanol mixture (1:1, v/v).

The precipitated proteins were sedimented by centrifugation. 1.0 ml of digitonin added to the supernatant followed by 2 drops of acetic acid. The contents were mixed well, securely closed and kept in a dark chamber for 16 hours. The cholesteryl digitonide which sedimented after centrifugation was washed twice with acetone-ether mixture (1:2, v/v) and finally with dry ether. The precipitated
cholesterol was estimated according to the method of Parekh and Jung as described in section 2.4.5.

Cholesterol ester synthetase activities are expressed as nanomoles of cholesterol esterified hour/mg protein at 37°C.

2.10.6 Extraction of lipids

Lipids were extracted from cell suspension in an aliquot immediately after the final washing as described in section 2.10.1. Extraction were performed with cold solvents at 2-5°C.

To 25 ml of the cell suspension was added 5.0 ml of methanol and stirred constantly for 15 minutes, 10 ml of chloroform was then added and the mixing was continued for a long time. The chloroform - methanol extracts were washed by the method of Folch et al (1957) with 0.1M potassium chloride in the upper phase. Partition between the water phase (upper) and the chloroform phase became evident after the extract was centrifuged for 15 minutes at 1,400 x g. The supernatant water phase was completely removed and discarded, the remaining chloroform extract was filtered through Whatman filter paper (No.3) previously washed with benzene and filter was rinsed twice with chloroform - methanol mixture (2:1, v/v). The extract was then evaporated and further dried. The residue was dissolved in a known volume of chloroform and the aliquots were used for the estimation of cholesterol, phospholipid and triglyceride in the leucocyte samples.
2.10.7 Leucocyte cholesterol

The cholesterol content in the lipid extract obtained from leucocytes (section 2.10.6) was determined according to the method of Parekh and Jung (1970) as detailed in the section 2.4.5.

Values are expressed as mg/mg protein.

2.10.8 Leucocyte phospholipids

A known volume of the lipid extract from section 2.10.6 was taken and the phospholipid was estimated by the method of Rouser et al (1970) as given in the section 2.4.6.

Values are expressed as mg/mg protein.

2.10.9 Leucocyte triglyceride

An aliquots of the lipid extract from section 2.10.6 was processed from the alcoholic potassium hydroxide hydrolysis step onwards as given in section 2.4.7.

Values are expressed as mg/mg protein.

2.11 STATISTICAL ANALYSIS

2.11.1 Plasma and cellular biochemical values are expressed as mean ± standard deviation for patient and healthy populations separately.
2.11.2 Students 't' test was used to arrive at the statistically significant differences between the blood values of the patients with AMI compared to the healthy volunteers.

2.11.3 In the clinical trials carried out, the change in plasma lipids, lipoproteins, fibrinogen, platelet count, immunoglobulins, prothrombin time and leucocyte enzymes etc. are studied statistically, using students 't' test. Significant alterations with \( \text{APS}_m \) therapy is noted by comparing with initial levels.