CHAPTER II

ANALYTICAL PROCEDURES

Extraction of Flavonoids

Flavonoids were extracted by the method described by Ansari et al (1976). Dried plant materials were completely extracted with light petroleum (b.p 40 – 60°C). The treated material was dried and extracted with boiling acetone. The combined acetone extracts were concentrated at atmospheric pressure. This was extracted successively with light petroleum (b.p. 40 – 60°C), benzene and hot water to remove non-flavonoid and resinous matter. The residue was then refluxed with ethyl acetate for 10 hours and the mixture was filtered. The filtrate was evaporated to dryness and kept at 4°C until used for the experiment.

Estimation of flavonoids

Flavonoids were estimated by the method of Eskin et al (1978). To 10 ml diluted acetone solution of flavonoids 0.5 ml of titanium chloride – HCl (20 % TiCl₄ in concentrated HCl) reagent was added, mixed well and optical density was measured immediately at 405 - 480 nm using quercetin as standard.

Estimation of blood glucose

Blood glucose was analysed by the procedure of Asatoor and King (1954) with the modification that low alkaline copper reagent was used.
0.1 ml of the blood was pipetted out into 7.8 ml isotonic solvent contained (3.2 ml 3% Na2SO4 - 3 ml 7% CuSO4) in a centrifuge tube. Added 0.1 ml of sodium tungstate (10%). Mixed well and centrifuged. To 2 ml of the supernatant solution added 2 ml of low alkaline copper reagent and heated for 10 minutes in a boiling water bath. The tubes were then cooled and added 2 ml of arsenomolybdate reagent. Made up to 10 ml with water, kept for 15 minutes and read the optical density at 520 µ.

**Extraction of Serum And Tissues For Lipid Estimation**

**Extraction of serum**

Lipids were extracted from the serum by the procedure of Folch *et al* (1957). 1 ml of serum was added drop wise to 5 ml of methanol in a stoppered tube. Then 5 ml of chloroform was added and mixed. This mixture was incubated at 55°C for 15 minutes. At the end, another 5 ml of chloroform was added so that the proportion of chloroform to methanol was 2:1 (v/v). After filtration and washing the residue with chloroform: methanol (2:1) at least 3 times the combined filtrate was washed with 0.7% KCl solution (20% of the total volume of the extract). The aqueous upper phase was removed with a pasteur pipette and the lower layer was washed each time with 5 ml of chloroform: methanol: KCl solution (3: 48: 47 v/v). The washed lower layer of chloroform was evaporated to dryness and the residue redissolved in a known volume of chloroform. Aliquots were used for the estimation of lipids.

**Extraction of tissues**

Lipids were extracted from tissues by the method of Radin (1981). For each gram of tissue added 18 ml extraction solvent [Hexane: isopropanol (3: 2 v/v)] and homogenized thoroughly. After 30 - 60 sec of mixing,
centrifuged and transferred the supernatant into a 25 ml graduated flask. Resuspended the insoluble residue in 3 ml extraction solvent. Centrifuged after 5 minutes. Repeated these with another 3 ml of solvent and finally made up to 25 ml. Aliquots were used for the lipid estimation.

**Estimation of cholesterol**

Total cholesterol was estimated by the method of Abell *et al* (1952). An aliquot of the lipid extract was pipetted out into a glass stoppered centrifuge tube and was evaporated to dryness. 5.0 ml of ethanolic KOH (6 ml 33% KOH to 100 ml with absolute ethanol) was added, stoppered and shake well. It was then warmed in a water bath at 37-41°C for 55 minutes. After cooling to room temperature, 10.0 ml of petroleum ether (60-80°C) was added and mixed. 5.0 ml of water was then added to this and was shaken vigorously for 1 minute. It was then centrifuged at a low speed for 5 minutes. 4.0 ml of petroleum ether layer was pipetted out into a test tube and evaporated to dryness at 60°C. A standard was also treated in the same manner. 6.0 ml of colour reagent (20 ml acetic anhydride + 1 ml concentrated H₂SO₄ + 10 ml glacial acetic acid) was added to each tube and kept at 25°C after thorough shaking. 6.0 ml of colour reagent was taken as blank. After 30-35 minutes the optical density was read at 620 nm.

**Estimation of triglycerides**

Triglycerides were estimated by the method of Van Handel and Zilversmit (1957) with the modification that florisil was used to remove phospholipids. 2 g florisil was taken in a glass stoppered tube and 3 ml chloroform was added. An aliquot of the extract was layered on the top of the florisil and mixed. It was then stoppered and shaken intermittently for 10 minutes. It was then made up to 10 ml with chloroform and filtered. 1 ml of
the filtrate was pipetted out into 3 tubes. The solvent was evaporated at 60 - 70°C, 0.5 ml of ethanolic KOH (0.4 %) was then added to 2 out of 3 tubes (saponified sample) and 0.5 ml of ethanol was added to the third tube (unsaponified sample). The tubes are closed and kept at 60 - 70°C for 15 minutes. 0.5 ml of 0.2N H₂SO₄ was added to each tube and the tubes were then placed in a boiling water bath for 15 minutes to remove alcohol. They were then cooled to room temperature. 0.1 ml sodium metaperiodate (0.05M) was added to each tube and kept for 10 minutes. 0.1 ml of sodium arsenite (0.5M) solution was then added and kept for 10 minutes. A yellow colour of iodine appeared and vanished within a few minutes. 5 ml of chromotropic acid reagent (2g chromotropic acid in 1000 ml 70% concentrated H₂SO₄) was added to each tube and mixed. The tubes are then closed and heated in a boiling water bath for 30 minutes. They were then cooled and the absorbance was read at 570 nm.

**Estimation of phospholipids**

Phospholipids were estimated by the method of Zilversmit and Davis (1950). An aliquot of the extract was pipetted out into a kjeldahl flask and evaporated to dryness. 1 ml of 5N H₂SO₄ was added and digested in a digestion rack till it becomes light brown. It was then cooled to room temperature, one or two drops of 2N HNO₃ was added, digested again till it became colourless. The kjeldahl flask was cooled; 1 ml water was added and heated in a boiling water bath for 5 minutes. 1 ml of 2.5 % ammonium molybdate and 0.1 ml of ANSA (0.02g ANSA + 0.12g sodium bisulphate + 0.12g sodium sulphite dissolved in 10 ml water) were added and the volume made up to 10 ml with distilled water. The absorbance was measured at 660 nm within 10 minutes.
Estimation of free fatty acids

Free fatty acids were estimated according to the procedure of Falholt et al (1973). Lipid extract of the tissue and serum were used. About 50 μl of the lipid extract was evaporated to dryness at 40 – 45°C under nitrogen. 1 ml of 33 mM phosphate buffer (pH 6.4), 6 ml of extraction solvent (chloroform: n- heptane: methanol, 5:5:1 v/v/v) and 2.5 ml copper reagent (10 ml of 500 mM / litre of copper nitrate solution, 10 ml of triethanolamine [1 mole / litre] and 6 ml of 1 N NaOH were added and made up to 100 ml. 33 g NaCl was added and the pH adjusted to 8.1). After shaking vigorously for 90 seconds, it was kept for 15 minutes, and centrifuged at 4000 rpm for 5 minutes. The aliquot of the supernatant (1.0ml) was transferred to a tube containing 0.5 ml diphenyl carbazide (4g / litre in ethanol + 10 ml triethanolamine) solution. After mixing carefully, optical density was read after 15 minutes at 550 nm.

Assay of Aspartate Transaminase activity (GOT) (E.C.2.6.1.1)

GOT was assayed by the method described by King (1965). Pipetted out 0.5ml of substrate (200mM DL-aspartic acid and 2mM α-keto glutarate) solution to both test and control tubes and allowed to attain bath temperature at 37°C. Added 0.1ml of serum to the test and mixed by gentle shaking. Exactly 60 minutes after adding serum and without removing from the water bath, added 0.5 ml of colour reagent (1mM 2,4-dinitrophenyl hydrazine in 1N HCl) to both tubes to stop the reaction. 0.1 ml of serum was then added to the control tube. The tubes were then allowed to stand for 5 minutes, for complete decarboxylation, and leave in water bath for 20 minutes.

The standard tube contained 0.45 ml of substrate, 0.05 ml of oxalo acetic acid (2mM) and 0.1 ml of buffer (0.1M phosphate buffer pH 7.4). 0.6 ml of buffer was taken as blank. Incubate the tubes for 60 minutes at 37°C.
0.5 ml of colour reagent was then added, mixed well and incubated at 37°C for 20 minutes. Exactly after 20 minutes all the tubes were removed from the water bath and 5 ml of 0.4 N NaOH was added. Recorded the optical density of the solution at 520 nm after 1 minute (activity of GOT is expressed as μmoles of oxaloacetic acid liberated / ml / litre serum).

Assay of alanine transaminase activity (GPT) (E.C.2.6.1.2)

GPT was assayed by the method described by King (1965). Pipetted out 0.5 ml of buffered substrate (200mM DL-alanine and 2mM α-ketoglutaric acid in 0.1M phosphate buffer pH 7.4) into test and control tubes, allowed to attain bath temperature at 37°C. Added 0.1ml of serum to the test and mixed by gentle shaking. Exactly 30 minutes after adding serum and without removing from the water bath, added 0.5ml of colour reagent (1mM 2,4-dinitrophenyl hydrazine in 1N HCl) to both tubes to stop the reaction. 0.1ml of serum was then added to the control tube. Allowed to stand for 5 minutes, for complete decarboxylation, and leave in water bath for 20 minutes.

The standard tube contained 0.45 ml of substrate, 0.05 ml of pyruvate (2μM sodium pyruvate) and 0.1 ml of buffer. Blank contains 0.6 ml of buffer alone. Incubated the tubes for 30 minutes at 37°C. 0.5 ml of colour reagent was then added. Mixed well and incubated at 37°C for 20 minutes. Exactly after 20 minutes all the tubes were removed from the water bath and added 5 ml of 0.4 N NaOH. Read the optical density of the solution at 520 nm after 1 minute (activity of GPT is expressed as μmoles of pyruvate liberated / min / litre serum).

Serum Alkaline Phosphatase Assay (using Folin- Ciocalteu reagent)

Modifications of the King and Armstrong (1934) procedure was used for the assay (King 1946, 1965).
Pipetted out 2 ml of buffer (0.1M carbonate-bicarbonate buffer-pH 10) and 2 ml of substrate (0.1M phenyl phosphate) to test and control tubes and placed in 37°C water bath for a few minutes to attain bath temperature. Added 0.2 ml serum to the test, mixed and incubated for exactly 15 minutes. Added 1.8 ml of dilute Folin- Ciocalteu reagent (1: 2 with water) to both tubes, 0.2 ml serum to the control, mixed and centrifuged. Transferred 4 ml aliquots of the clear supernatants to fresh tubes, added 2 ml of 15 % carbonate solution and 4 ml of water, mixed and return to 37°C water bath for 10 minutes to permit maximum colour development. Recorded optical density using a red filter or transmission at 680 μm.

**Serum acid phosphatase assay (using Folin- Ciocalteu reagent)**

Assay was carried out by the method of Gutman and Gutman (1940) as described by King (1965). Into each of 2 tubes labeled test and control pipetted out 2 ml of buffer (0.2M citrate buffer pH 4.9), 2 ml of substrate (0.01M phenyl phosphate) and placed in 37°C water bath for a few minutes to attain bath temperature. Added 0.2 ml serum to the test, mixed and incubated for exactly 60 minutes. Added 1.8 ml of dilute Folin- Ciocalteu reagent to both tubes, 0.2 ml serum to the control, mixed and centrifuged. Transferred 4 ml aliquots of the clear supernatants to fresh tubes, added 2 ml of 15 % carbonate solution and 4 ml of water, mixed and return to 37°C water bath for 10 minutes to permit maximum colour development. Recorded optical density using a red filter or transmission at 680 μm.

**Activity of Acetyl Choline Esterase (EC 3.1.1.7)**

Acetyl Choline esterase was assayed by the method of Ellman et al (1961). Tissue was homogenized in sucrose buffer (0.25M), centrifuged and the supernatant was used for the assay. A blank reaction was set up to
estimate the non-enzymatic hydrolysis of the substrate. It was prepared by substituting phosphate buffer in place of samples. Pipetted successively into cuvette – 3 ml phosphate buffer (100mM / litre, pH 8.0), 0.02 ml acetylthiocholine iodide solution (75 mM / litre) and 0.1 ml DTNB (DTNB- 10 mM / litre in phosphate buffer pH 7.0 and NaHCO₃ 17.85 mM / litre) solution. Incubated at 25°C for 10 minutes. 0.02 ml enzyme was then added and mixed well. Read absorbance at intervals of 15 seconds for 2 minutes at 410 nm.

**Extraction and estimation of hepatic bile acids**

The procedure of Okishio *et al* (1967) was used for the extraction of bile acids from liver. The tissue was homogenized with 95 % (v / v) ethanol containing 0.1 % (v / v) of ammonium hydroxide (sp. gravity 0.88) and was refluxed for 30 minutes. After filtration, the residue was reextracted twice with the same volume of solvent and filtered. The combined filtrate was then concentrated in vacuum, made alkaline (pH 10) by the addition of sodium hydroxide. An equal volume of water was added and extracted twice with petroleum ether (40 – 60°C) to remove neutral sterols. The aqueous solution was acidified with HCl to pH 2.0 and extracted thrice with chloroform: methanol (1: 1). The chloroform layer was washed with a little amount of water and dried over anhydrous sodium sulphate. The bile acids were separated from free fatty acids by TLC over silica gel using n-hexane: ether: acetic acid (30: 6: 0.5 v / v) as the solvent system. The portion containing bile acids was scrapped out and extracted with chloroform and filtered. After filtration and evaporation, the bile acids were dissolved in a known volume of chloroform and aliquots were taken for the estimation of bile acids.

The bile acids were estimated by the enzymatic procedure described by Palmer (1967). An aliquot of the chloroform solution of the bile acid was evaporated to dryness and dissolved in the pyrophosphate buffer. The system consisted of 0.1 ml NAD⁺, 0.02 ml of the bile acid and 2.88 ml of
pyrophosphate buffer (0.1M, pH 9.5) in a 10 mm light path spectrophotometer cuvette. The reaction was started by adding 20μl of the enzyme solution and stirring rapidly. At 30 seconds and every 15 seconds thereafter, optical density measurements at 340 nm were taken against a control cuvette containing all components except the bile acid and compared with a standard of cholic acid. One unit of enzyme activity produces an increment in optical density of 0.001 per minute.

**Extraction & estimation of fecal bile acids and neutral sterols**

Fecal sterols and bile acids were extracted by the general procedure of Grundy *et al* (1965). 24-hour stool samples collected from the rats, in metabolic cages, were homogenized with equal weight of water and lyophilised to a fine powder. Approximately 600 mg of stool sample was extracted with 10 ml of 1N NaOH in 90% ethanol at 80°C for 2 hours. The mixture was cooled and centrifuged. The residue was again treated with 10 ml of 1N NaOH in 90% ethanol at 80°C for 2 hours. The combined extract was shaken with 1/3 volume of hexane to extract neutral sterols. The hexane layer was collected and washed with little amount of water. It was evaporated to dryness and redissolved in a known volume of chloroform for neutral sterol estimation. The solution left after extraction with hexane was then acidified to pH 2.0 and bile acids were extracted with ethyl acetate. The ethyl acetate layer was collected, washed with water. After extraction it was evaporated to dryness. The bile acids were re-dissolved in a known volume of ethyl acetate and aliquots were taken for the estimation of bile acids.

**Serum lipoprotein analysis**

Total cholesterol was estimated in the whole serum by the method of Abell *et al* (1952). The procedure described by Warnick and Albers (1978)
was used for the separation of HDL and LDL + VLDL. LDL + VLDL was precipitated from the serum by treating with heparin and manganese chloride (final concentration of heparin 0.144 % and MnCl₂ 0.091 M). After keeping at room temperature for 10 minutes, it was centrifuged for 30 minutes at 4000 rpm at 4°C. The supernatant, which contained HDL, was analyzed for cholesterol to obtain HDL cholesterol. The difference between total cholesterol and HDL cholesterol gives LDL cholesterol.

**Release of lipoproteins into circulation**

The procedure of Schurr et al (1972) was used to study the release of lipoproteins into circulation. 50 mg of triton WR 1339 / 100g body weight was administered intraperitoneally in normal saline to rats, which were deprived of food overnight, and four hours later blood was collected. Control animals received the same volume of normal saline as described above. Percentage change in serum cholesterol in the experimental animals given triton as compared to saline treated controls was taken as a measure of the release of lipoprotein into the circulation.

**Assay of β-hydroxy β-methyl glutaryl-CoA reductase (HMG-CoA reductase, EC 1.1.1.34)**

HMG CoA reductase activity in liver was estimated as described by Rao and Ramakrishnan (1975) by determining the ratio of HMG CoA to mevalonate. Equal volume of fresh 10% tissue homogenate (in saline arsenate) and dilute perchloric acid (50 ml made up to 1 litre) were mixed, kept for 5 minute and centrifuged at 2000 rpm for 10 minutes. To 1 ml supernatant, 0.5 ml of freshly prepared hydroxylamine reagent (alkaline hydroxylamine reagent in the case of HMG CoA) was added and mixed. After 5 minutes 1.5 ml of ferric chloride reagent (5.2 g TCA + 10 g FeCl₃ in 50 ml of 0.65N HCl and made up to 100 ml with the latter) was added. After shaking well, readings were taken after 10 minutes at 540 nm against a similarly treated saline arsenate blank. The ratio of HMG CoA to
mevalonate is taken as an index of activity of the enzyme which catalyses the conversion of β-hydroxy-β-methyl glutaryl-CoA to mevalonate. The lower the ratio the higher the activity.

**Lipoprotein lipase (LPL) activity of heart and adipose tissue (E.C. 3.1.1.3)**

Lipoprotein lipase activity of heart and adipose tissue was estimated according to the procedure of Krauss *et al* (1974). Acetone dry powder of the tissue was extracted with 0.025 M NH₄OH-NH₄Cl buffer, pH 8.6 containing 1 unit of heparin / ml and the extract was used as the enzyme source. Protamine inhibited activity was taken as a measure of lipoprotein lipase activity. The enzyme activity is expressed as μ moles of glycerol liberated per hour per gram protein.

**Assay of plasma lecithin:cholesterol acyl transferase (LCAT, EC 2.3.1.43)**

Blood was collected in heparinised tubes maintained at 0°C and centrifuged immediately at 0°C to separate the plasma. An aliquot was immediately extracted with acetone: ethanol. Another aliquot of plasma was incubated at 37°C for 3 hours, at the end of which lipid was extracted as above. Ester cholesterol and unesterified cholesterol were estimated in the lipid extract, by the procedure of Schoenheimer and Sperry (1934). The extend of increase in ester cholesterol / unesterified cholesterol ratio during incubation period was taken as a measure of LCAT activity.

**Activity of lipogenic enzymes**

**Glucose-6-phosphate dehydrogenase (Glucose-6-phosphate: NADP⁺ oxidoreductase, EC 1.1.1.49)**

The enzyme was assayed by the method of Kornberg and Horecker (1955). The chilled tissue was homogenized with 3 volumes of 0.04 M glycyl glycine buffer (pH 7.5). The homogenate was centrifuged at 2000 x g at 0°C for 10 minutes. The supernatant was used as the enzyme source.
To 1 ml of the substrate (0.02 M glucose-6-phosphate) in a quartz cell, 0.1 ml of NADP⁺ (0.0015M), 0.25 ml buffer (glycylglycine; 0.04M pH 7.5) and 0.2 ml MgCl₂ (0.1 M) were added. To this was added 0.05 ml of enzyme and the absorbance was read immediately at 340 nm at 1 minute intervals. One unit of enzyme activity is defined as that amount which causes an initial change in OD of 1.00/ minutes under the above conditions.

**Malic enzyme (L-malate: NADP⁺ oxidoreductase, EC 1.1.1.40)**

The activity of the enzyme was determined by the method of Ochoa (1955). The chilled tissue was homogenized with 3 volumes of 0.25 M glycyl glycine buffer (pH 7.4) at 0°C and the supernatant obtained by centrifuging at 2000 × g at 0°C for 10 minutes was used as the enzyme.

The reaction mixture in a quartz cell (d=1cm) consisted of 0.3 ml of 0.25 M glycyl glycine buffer (pH 7.4), 0.06 ml of 0.05 M MnCl₂ (3 μM), 0.2ml of NADP⁺ (0.135 μ moles), 0.05 ml of 0.03M L-malate, enzyme and water to a final volume of 3ml. The assay was carried out at room temperature (23 – 25°C). The reaction was started by the addition of either malate or enzyme and the optical density were taken, against a blank containing all components, except NADP⁺, at intervals of 15 seconds, for 1 to 2 minutes.

**Estimation of protein**

Protein was estimated in all enzyme extracts, after TCA (trichloroacetic acid) precipitation by the method of Lowry *et al* (1951). 0.1 ml of enzyme extract was made up to 1 ml with water and protein was precipitated by adding 1 ml of 10 % TCA. Centrifuged and the residue was dissolved in 1 ml of 0.1N NaOH. From this an aliquot was pipetted out and
made up to 1 ml with 0.1N NaOH. Standard albumin solution was also pipetted out and made up to 1 ml with 0.1N NaOH. Blank contains 1 ml of 0.1N NaOH. Added 5 ml alkaline copper reagent to all tubes and allowed to stand for 10 minutes. 0.5 ml Folin’s reagent was added to each tube and mixed well. The tubes were allowed to stand for 30 minutes at room temperature. Optical density was measured at 670 nm.

**In vivo incorporation of (1, 2-¹⁴C) acetate into lipids in liver.**

The rats deprived of food overnight for 16 hrs, were injected intraperitoneally with 0.5 ml solution of (1, 2-¹⁴C) sodium acetate (10 µci /100 g body weight). After 3 hours rats were sacrificed. The liver was quickly removed to ice cold containers, gently blotted and weighed.

The tissue lipids were extracted with chloroform: methanol according to the procedure of Folch et al (1957). Free cholesterol, ester cholesterol, triglycerides, and free fatty acids in the extract were separated by TLC over silica gel (silica gel G) using n–hexane: ether: acetic acid in the ratio of 30: 6: 0.5 (v/v/v) as solvent system. The activity was counted in Packard’s Priyas liquid scintillation counter. The scintillant fluid used was [6g 2,5-diphenyl oxazole (PPO) and 0.2 g 1, 4- bis [2- (5- phenyl oxazolyl) benzene (POPOP)] / litre toluene.

**Lipid Peroxidation & Antioxidant Status**

**Lipid peroxides in serum & tissues**

**Estimation of hydroperoxides**

Hydroperoxides were estimated by the method of Mair and Hall (1977). 1ml of the aqueous tissue homogenate (or 0.2ml serum + 0.8ml Tris-HCl buffer pH 7.5) was mixed thoroughly with 5 ml of chloroform:
methanol (2:1) followed by centrifugation at 1000 g for 5 minutes to separate the phases. The lower chloroform layer was evaporated to dryness under a stream of nitrogen at 45°C. 1 ml of acetic acid: chloroform (3: 2) mixture and 0.05 ml of KI (6g KI / 5 ml H2O) were quickly added to the evaporated sample and the test tube was stoppered and mixed. The tubes were placed in dark at room temperature for exactly 5 minutes followed by the addition of 3 ml of cadmium acetate (0.5%). The solution was mixed and centrifuged at 1000xg for 10 minutes. The absorbance of the upper layer was read at 353 nm against a blank containing the complete assay mixture without the tissue homogenate. Standardization of the reaction may be done using cumene hydroperoxide as the peroxide standard. The molar extinction coefficient of cumene hydroperoxide is $1.73 \times 10^4 \, \text{M}^{-1} \, \text{cm}^{-1}$.

**Estimation of conjugated dienes**

Conjugated diene was estimated by the method of Recknagel and Ghoshal (1966). The tissue extract (or serum) in chloroform was prepared and evaporated to dryness as described in the estimation procedure for hydroperoxides. The lipid residue was dissolved in 1.5 ml of cyclohexane, and the absorbance at 233 nm was determined against a cyclohexane blank. The amount of conjugated dienes produced can be calculated using a molar extinction coefficient of $2.52 \times 10^4 \, \text{M}^{-1} \, \text{cm}^{-1}$.

**Estimation of malondialdehyde (Thiobarbituric acid-reacting substances)**

MDA (TBA-RS) was estimated by the method of Nichans and Samuelson (1968). Tissue was homogenised with 0.1 M phosphate buffer (pH 7.0). 2 ml of TCA-TBA-HCl reagent (15% w/v TCA and 0.37 % w/v 2-thiobarbituric acid in 0.25 N HCl) was added to 1 ml of the tissue homogenate (or 0.2ml serum + 0.8ml buffer) and mixed thoroughly. The contents were heated in a boiling water bath for 15 minutes. After cooling,
the flocculent precipitate was removed by centrifugation at 1000xg for 10 minutes. The supernatant was shaken with n-butanol to extract the coloured complex into the organic phase. The absorbance of the butanol layer was read at 535 nm against a blank that does not contain the sample. The concentration of MDA can be calculated using an extinction coefficient of $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$.

**Antioxidants in blood & tissues**

**Estimation of reduced glutathione content**

The glutathione content was determined as described by the improved method of Benkey and Cheever (1974). 0.2 ml of tissue homogenate (or 0.2 ml blood) was mixed with 1.8 ml distilled water and 3 ml of the precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g of EDTA and 30 g of NaCl per 100 ml of distilled water). After mixing, the solution was allowed to stand for 5 minutes and filtered. 2.0ml of the filtrate was added to 8 ml of phosphate solution (0.3 M Na$_2$HPO$_4$ in distilled water) followed by 1 ml of DTNB (dithio bis nitro benzoic acid) solution. The optical density was measured at 412 nm. Blank was prepared by substituting the sample with water and following the entire procedure as for test. The quantity of reduced glutathione was expressed in mg / g tissue or mg / dl blood.

**Estimation of $\alpha$-tocopherol and retinol**

Estimation of vitamin E ($\alpha$-tocopherol) and retinol were carried out by the procedure of Catiganin (1986). All the reagents used were of HPLC grade. Taken 500 mg liver and grind in methanol for 2 minutes then added 5 ml hexane. In the case of serum 100 $\mu$l of plasma was taken in a 10 x 75 mm tube and added 100 $\mu$l ethanol, vortexed for 30 seconds then added 200 $\mu$l
hexane, capped and vortexed for 45 seconds. It was then centrifuged for 5 minutes at 2000 rpm. Drawn the upper hexane layer and then reextracted with hexane. The hexane layer was then evaporated under a stream of nitrogen and solubilized immediately in methanol (100 µl in the case of serum and 200 µl for liver) and an aliquot was injected into HPLC column. Column used was 4.6 mm x 15 mm reverse phase C-18 column, 5 µm Shimadzu at 296 nm. 100 % methanol was used as the solvent system at the flow rate of 2 ml / minute. Retinol elutes at about 2.5 and α- tocopherol at 6.8 minutes by using respective standards.

Assay of antioxidant enzymes

Superoxide dismutase (EC 1.15.1.1)

Activity of superoxide dismutase was determined by Kakkar et al (1984). Assay mixture contained 1.2 ml sodium pyrophosphate buffer (0.052 M, pH 8.3), 0.1ml 186 mM phenazine methosulphate, 0.3 ml 300 µM nitro blue tetrazolium, 0.2 ml NADH (780 µM), appropriately diluted enzyme preparation and water in a total volume of 3 ml. Reaction was started by the addition of NADH. After incubation at 30°C for 90 seconds the reaction was stopped by the addition of 1ml glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4 ml of n-butanol. The mixture was allowed to stand for 10 minutes, centrifuged and butanol layer was taken out. Colour intensity of the chromogen in the butanol was measured at 560 nm against butanol. A system devoid of enzyme served as control.

One unit of enzyme activity is defined as the enzyme concentration required to inhibit the optical density at 560 nm of chromogen production by 50% in 1 minute under the assay condition and expressed as specific activity in milli units / mg protein.
Catalase (EC 1.11.1.6)

Catalase activity was measured by the method of Maechly and Chance (1954). The estimation was done spectrophotometrically following the decrease in absorbance at 230 nm. The reaction mixture contained 0.01 M phosphate buffer pH 7.0, 2mM H₂O₂ and the enzyme extract prepared by homogenising the tissue in 10mM phosphate buffer (pH-7) and centrifuging at 5000 rpm. The specific activity is expressed in terms of units / mg protein.

It is calculated using the formula:

\[
\text{Activity} = \frac{2.303}{60} \times \log \frac{\text{OD at zero time}}{\text{OD at 60 sec.}}
\]

Specific activity = Activity / mg protein

Glutathione peroxidase (EC 1.11.1.9)

Glutathione peroxidase activity was determined by the method of Paglia and Valentine (1967) as modified by Lawrence and Burk (1976).

The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7), 1 mM EDTA, 1 mM NaN₃, 0.2 mM NADPH, 1.0 enzyme unit / ml glutathione reductase, 1 mM glutathione, 0.25 mM H₂O₂ in a total volume of 2 ml. All ingredients except the enzyme source and peroxide were combined at the beginning of the experiment. To 1.6 ml of the above mixture, 0.2 ml of the enzyme source was added and the mixture was allowed to incubate at room temperature for 5 minutes before the initiation of the reaction by the addition of 0.2 ml of peroxide solution. The oxidation of NADPH was followed at room temperature using a spectrophotometer for the next 5 minutes at 340 nm. Blank reaction with enzyme source replaced by distilled water was subtracted from each assay.
Enzyme activity was expressed as units/mg protein. (One unit is defined as the difference in optical density/minute)

**Glutathione reductase (EC 1.6.4.2)**

Glutathione reductase activity was determined by the procedure of David and Richard (1983). The assay system contained 1 ml of 0.12 M potassium phosphate buffer, pH 7.2, 0.1 ml of 15 mM EDTA, 0.1 ml of 10 mM sodium azide, 0.1 ml of 6.3 mM oxidized glutathione and 0.1 ml of enzyme source and water in a final volume of 2 ml. Kept for 3 minutes. Then 0.1 ml of NADPH (9.6 mM/L) was added. The absorbance at 340 nm was recorded at an interval of 15 seconds for 2-3 minutes. For each series of measurements, controls were done that containing water instead of oxidised glutathione. The enzyme activity was expressed as µmoles of NADPH oxidized/minute/mg protein.

**Glutathione - S - transferase (EC 2.5.1.18)**

Glutathione - S - transferase was assayed by the method of Habig et al (1974). Tissue was homogenized in 0.2 M sodium phosphate buffer pH 6.5 centrifuged at 20,000 g for 20' and the supernatant at 10,000 g for one minute. Cytosolic fraction was filtered through glass wool and used for the assay. The assay mixture contained sodium phosphate buffer (0.2M), glutathione (30mM), CDNB (30mM), enzyme and water. The increment of absorbance was measured at 340 nm due to the formation of 2,4 - dinitrophenyl –S- glutathione from CDNB and GSH.

**Determination of Superoxide Production**

Superoxide production was determined by the method of Winterbourn et al (1975). The assay tubes contain test sample + 0.2 ml
EDTA (0.1 M containing 1.5 mg NaCN / 100ml) + 0.1 ml NBT (1.5 mM) + 0.05 ml riboflavin (0.12 mM) + 2.55 ml phosphate buffer (M/ 15, pH 7.8). Control = 0.1ml DMSO + 0.2ml EDTA + 0.1ml NBT + 0.05 ml riboflavin + 2.55 ml phosphate buffer.

All the tubes were vortexed and measured the initial optical density at 560nm. After that these tubes were placed in an area where they received uniform illumination for 15 minutes. Again optical density was measured at 560nm. The difference in optical density before and after illumination is the quantum of superoxide production and the percentage of superoxide inhibition by the test sample was calculated by comparing with the optical density of control.

Serum Oxidation

Serum oxidation was done by the method described by Hodgson et al (1999). This method provides an indication of diene formation in lipoprotein fatty acids present in serum exposed to Cu$^{2+}$, assessed by measuring the change in absorbance at 234 nm. Serum was diluted to 0.67 % (final concentration) in phosphate buffered saline (138 mM NaCl, 27 mM KCl, 10.1 mM Na$_2$HPO$_4$, 1.8 mM KH$_2$PO$_4$). The appropriate dilution of the test sample was added. Control experiments consisted of identical assay conditions but without the sample. Oxidation was initiated immediately after addition of sample by the addition of 12µM final concentration of Cu$^{2+}$ added as CuSO$_4$.5H$_2$O dissolved in deionised distilled water. Oxidation was determined by measuring the absorbance at 234nm using a UV- Visible Spectrophotometer (Shimadzu). Absorbance were taken every 20 minutes over 240 minutes at 37°C. The lag time to lipoprotein diene formation was measured from the plot of absorbance against time. The lag time was defined as the intercept between the tangent of the absorbance curve during the propagation phase and the baseline.
Antiradical activity

Scavenging free radical potentialities are tested against a methanolic solution of DPPH as described by Joyeux et al (1995). The degree of decolouration indicates the scavenging efficiency of the added substances. For each compound 750μl of a 10^{-3} M or 10^{-4} M were added to 1.5 ml DPPH solution (20 mg / L). 5 minutes later, the absorbance was measured at 517 nm. A blank is realized in the same condition with 750μl of deionised water. The percentage of DPPH decolouration is calculated as

Decolouration percentage \(= 1 - \frac{\text{absorbance with compound}}{\text{absorbance of the blank}} \times 100.\)

Haematological Studies

The rats were anesthetised using ether and blood was collected by cardiac puncture into tubes containing EDTA for haemoglobin, red blood cell count, white blood cell count and ammonium oxalate for platelet count (Lamberg and Rothstein, 1978)

Blood cell count

Red blood cells and white blood cells were estimated with a hemocytometer.

Packed red cell volume (PCV)

PCV (hematocrit) was estimated by the Wintrobe method (Gradwohl’s Chemical Laboratory methods and Diagnosis, 1980) by centrifugation of anticoagulated whole blood to separate cells from the plasma. PCV was read directly of the calibration at the right side of the Wintrobe tube.
Haemoglobin

Haemoglobin was estimated by the following procedure (Hawke, 1954). Filled the Sahli Hellidge haemometer tube to the level of lowest graduation up to 2 mark with standard N / 10 HCl. Pipette 20 mm$^3$ of blood to this tube using a capillary pipette. Place the haemometer tube on the comparator for half an hour. Then diluted with N / 10 HCl or distilled water and mixed. Continue the dilution with mixing until the colour matched with the standard colour. Take out the sample holder and reading was noted. This gives the Hb content of blood in absolute terms.

Determination of serum urea

Urea was estimated according to the procedure of Marsh et al (1965). Add 1ml water and 1 ml TCA (10 %) to 0.2 ml serum. Mixed well and centrifuged, take 0.2 ml supernatant and added 3ml colour reagent. A reagent blank and an appropriate standard (10 mmol / L) were prepared by replacing the serum with water or the standard for the first step above. Heated in a boiling water bath for 20 minutes. Cooled to room temperature and read test and standard against blank at 520 nm within 15 minutes.

Determination of serum electrolytes

Serum electrolytes Na$^+$ and K$^+$ were determined by flame photometric methods (Wootton, 1964).

Emission flame photometry

Incorporate an internal standard, after lithium, with simultaneous measurement of Na$^+$ and K$^+$ by the electronic amplification of the flame signal. This permits greater dilution of the sample allowing a smaller volume
to be used and also reduces the effect of protein on the viscosity of diluted sample.

**Fibrin and Fibrinogen**

Fibrin and Fibrinogen in the plasma were determined by the procedure described by King and Wooton (1959).

To 0.05 ml plasma diluted with 2 ml of isotonic sodium chloride 0.2ml of calcium chloride solution was added. The mixture was kept at 37°C, until clotting occurs, preferably overnight. The fibrin was carefully collected on a thin glass rode and the liquid poured off, the elute is pressed on the sides of the tube to remove liquid and washed with water. 0.2 ml perchloric acid is added for digestion. Then colorimetric estimation is carried out.

\[ F = \frac{R(T)}{R(\text{std})} \times 0.25 \text{ g} / 100\text{ml plasma} \]

**Fibrinogen**

0.2 ml plasma was rinsed into 3.8 ml of fresh 12.5 % sodium sulphite (Na\(_2\)SO\(_3\)) solution. The mixture was shaken, allowed to stand for exactly 10 minutes and shaken again. The turbidity was read against the artificial protein standards.

\[ \text{Fibrinogen} = \text{mg albumin} \times \frac{(10/1000 \text{ g})}{100\text{ml plasma}} \]

**Estimation of albumin / globulin ratio**

0.2 ml plasma is added to and mixed by inversion with 5.8 ml 28 % sodium sulphate in a glass stoppered tube. 1 ml of ether span reagent is added and the stoppered tube gently inverted 20 times, and centrifuged for
10 minutes. The globulin forms a button at the ether-water interface. A pipette was inserted and 3 ml of centrifugate was withdrawn and added to 3 ml of Biuret reagent, after mixing, kept at 37°C for 10 minutes. The solution is cooled and colour compared at 540 nm (King and Wootton, 1959).

\[
\text{Globulin} = \text{Total protein} - \text{Albumin}
\]

\[
\text{A / G ratio} = \frac{\text{Albumin reading}}{(\text{Total protein} - \text{Albumin}) \times 2}
\]

**Assay of Protein Kinase C (PKC) activity**

Protein kinase C activity was measured in tissues by the method described by Devito *et al* (1991). PKC was assayed in a total volume of 250 µl reaction mixture containing 25 mM Tris buffer pH 7.4, 1.05 mM CaCl₂, 10 mM EGTA, 25 µg/ml phosphatidyl serine (PS), 2.5 µg/ml diolein (DG), 50 µg histone, 25 µl enzyme extract, 10 µM ATP \([10^6 \text{ cpm (}^{32}\text{P})]\), 10 mM MgCl₂, 10mM dithiothreitol with or without CaCl₂, PS and DG. Incubation was carried out at 37°C for 3 minutes and terminated by the addition of 1 ml of ice cold 10% TCA. The precipitate was collected by filtration on 2.4 cm glass fibre filters (GF / C, Whatman) and washed twice with ice cold TCA. Protein Kinase C activity was determined by subtracting the amount of \(^{32}\text{P}\) incorporation to histone noted in the absence of lipids and calcium chloride from the amount of \(^{32}\text{P}\) incorporation with histone noted in the presence of lipids and calcium chloride. Total PKC activity was expressed as pico moles of \(^{32}\text{P}\) incorporated / min / mg protein.

**Histopathological examination of tissues**

The tissues were fixed in 10% neutral formalin, processed and stained with haematoxylin and eosin (Lamberg and Rothstein, 1978).
Statistical analysis

The data given in tables and figures are the average of the values from the number of animals specified in the respective tables and figures ± SEM. Statistical significance was calculated using student's 't' test (Bennet and Franklin, 1967).