CHAPTER 2
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ISOLATION AND PURITY OF EMBELIN

Embelin, a plant derived compound (2,5-dihydroxy-3-undecyl-1,4 benzoquinone; Fig.1), was isolated from the sun-dried seeds of Embelia ribes with hot benzene in a Soxhlet's apparatus following the procedure of Kaul et al (1929). The solidified masses of minute yellow glistening crystals, embedded in a viscous brown mother liquor, were obtained through reduced pressure. Embelin was purified in the form of orange plates with metallic luster through crystallisations and repeated recrystalisations with cold alcohol. Embelin was also obtained commercially from Bio Organics Ltd., Madras.

Purity of embelin was tested by 1) thin layer chromatography (TLC) in at least two different solvent systems, chloroform:methanol (50:50, v/v) and benzene:methanol (50:50 v/v), 2) ultraviolet absorption (UV) spectroscopy in methanolic sol, 3) melting point determination, 4) $^1\text{H}$-nuclear magnetic resonance (NMR) spectroscopy in trifluoroacetic acid at 90 MHz in a EM 390, Varian (USA) instrument, using tetramethyl silane as the internal standard, 5) Chemical ionisation - mass spectral
(CI-MS) analysis and 6) infra-red (IR) absorption spectroscopy in a KBr pellet, using a Perkin-Elmer 137 instrument.

**DRUG TREATMENT**

Adult male rats, weighing 150-200 g of Porton strain were obtained from the animal house of the Central Research Institute, Kasauli (Himachal Pradesh State) and housed in individual cages under controlled light (7AM to 7PM) and temp conditions (20-22°C). They were provided with rat chow (Lipton India Ltd.) and water ad Libitum. All the animals were kept in quarantine for 20 days before the drug treatment.

For the selection of the optimal dose different concentrations of embelin viz. 10,20 and 30 mg/kg body weight, suspended in olive oil (0.2 ml vol) were administered to the rats subcutaneously for 15 and 30 days. The control animals were injected with 0.2 ml of olive oil. After 15 and 30 days of treatment the histology of the testis and accessory organs was studied and the optimum dose to produce histopathological changes was found to be 20 mg/kg body wt. the animals were divided into four groups as under:
Groups I and II: The animals were injected subcutaneously with embelin, 20 mg/kg body wt. suspended in olive oil (0.2 ml vol) for either 15 (Group I) or 30 days (Group II).

Group III and IV: Drug treatment was withdrawn in half of the treated animals in Group II and were kept for recovery for 15 and 30 days, which formed the Groups III and IV, respectively.

DRUG TREATMENT BY ORAL ROUTE

The animals were treated orally by tube feeding with embelin, 75 mg/kg body weight, suspended in 20% alcohol (2 ml vol) for either 15- (Group I) or 30-days (Group II).

Groups III and IV: Drug treatment was withdrawn in half of the treated animals in Group II and were kept for recovery for 15- and 30-days which formed the Groups III and IV, respectively. Normal controls were kept with each embelin treated groups of animals which strictly received the equal vol of the carrier of the drug.

After 24 hours of administration of the last dose, the overnight fasted animals were sacrificed under diethyl ether anaesthesia. For various histological and cytochemical studies, testis, epididymii, seminal vesicles, ventral prostates and intestine were taken out in 0.9% saline. Each of these tissues were weighed on a single pan analytical balance. Intestine was also measured. The tissues were fixed in the following fixatives (i) Zenker (12-24 h fixation)
(ii) Bouins, (24 h fixation), (iii) formaldehyde calcium (6-8 h fixation). The testis and epididymii were also fixed in 2.5% glutaraldehyde for ultrathin sections.

After fixation in the above fixatives, the tissues were processed carefully for paraffin wax (58°C-60°C) and gelatin embedding according to the standard techniques (Pearse, 1968). The paraffin sections were cut at 5-7μ whereas the gelatin sections were cut at 10μ thickness. These sections were later subjected to various histological and cytochemical techniques.

Testis and epididymii fixed in 3% glutaraldehyde prepared in 0.2M phosphate buffer pH 7.4 were processed for electron microscopy.

SPECIMEN BLOCK PREPARATION FOR TEM

Washing: Following fixation in the primary fixative, tissues were washed in 0.1M phosphate buffer (pH 7.6) and post fixed in 1% OsO₄ prepared in 1 M sodium cacodylate buffer for 2 h at 4°C. Dehydration of the tissues was done with the different grades of aqueous acetone and finally washed in absolute acetone. Clearing was done with xylene and finally the embedding of the tissues was carried out in the Araldite embedding medium using gelatin capsules. Trimming of the blocks was done with the help of a trimmer and then was fitted in the specimen block holder of the ultramicrotome to get the sections of about 600 Å thickness. To obtain a good contrast, a double staining method using
Uranyl acetate and lead citrate was routinely followed for ultrastructural studies. Uranyl acetate and lead citrate were prepared as follows:

**Uranyl acetate** saturated sol of uranyl acetate was prepared into 50% ethanol and stored at 4°C.

**Lead citrate** one half of a pellet of NaOH was dissolved in 12 ml of water and 50 mg of lead citrate was added to it. This was centrifuged and stored at 4°C.

**IN VITRO ADDITION OF DRUG**

To 1 ml of the epididymal sperm suspension in minimal capacitation medium (110 mM NaCl, 1 mM sodium pyruvate, 25 mM sodium bicarbonate and 1 mM CaCl₂, pH 7.4) containing 50x10⁶ cells, embelin was added in 0-5 mg concentrations in a vol of 0.1 ml in 20% alcohol and incubated at 37 °C upto 60 min. Aliquots of the sample in each tube were taken for the assays of spermatozoal morphology, motility and enzyme activities of carbohydrate metabolism. The Spermatozoal samples were sonicated in an ice water environment with probe type sonicator (B.Brown Instruments) thoroughly till an uniform suspension is obtained which is then centrifuged at 2000xg for ten min and the supernatant served as the source of enzyme activities.

**MOTILITY COUNT**

A known length of the vas-deferens was removed from the animal and sperms were squeezed out and diluted with minimal capacitation medium. The spermatozoal motility
was checked by progressively forward movements of the sperm as described by Seth et al. (1981). Aliquot of sperm suspension was filled up to the mark of 0.5 in a WBC pipette and diluted with 3.8% Na-Citrate sol upto mark eleven. Sperm count was done in a Neubauer's haemocytometer chamber in WBC squares. Sperm count/Cm\(^2\) was calculated as follows: \(X \times 20 \times 10^4 /\text{cm}^2/\text{ml}\), where \(X\) is the average mean of the spermatozoa of all four squares.

**MORPHOLOGICAL STUDIES OF SPERMATOZOA**

Spermatozoal smears were made on glass slides and stained by Delafield's haematoxylin/eosine and carbol fuchsin techniques for light microscopic observations (Pearse, 1968). For scanning electron microscopic studies, the technique used was that of Baccetti (1975). Sperm suspensions were centrifuged at 500 g for 10 min and the pellets were washed with 0.1 M phosphate buffer, pH 7.2 and fixed overnight in 2.5% glutaldehyde in the same buffer. They were washed and sputter-coated with gold pelladium and scanned under a Jeol, 2601 electron microscope.

**FERTILITY TEST**

The drug treated males were placed with 2 or 3 pro-oestrous females for mating for a test period of 2 weeks at a time, according to the method described by Ahsan et al. (1976). Mating was confirmed by finding spermatozoa in vaginal smears and by detection of vaginal plug and this day
was designated as day 1 of pregnancy. Females were allowed to complete pregnancy. Gestation period and litter size in control and drug treated groups were recorded.

**QUANTITATION OF EMBELIN IN TISSUE SAMPLES**

After 24 h of administration of the last dose, the overnight fasted animals were sacrificed and the different organs e.g. testis, epididymis, seminal vesicle, ventral prostates, kidneys, liver, intestine, brain, spleen, heart, and lungs were carefully removed, freed of extraneous materials and blood clots, weighed and 1 g tissue of each was homogenised in 5 ml of sol A (ethanol+0.2 ml glacial acetic acid/l) and 2 ml of ether. The homogenisation was done in a flask surrounded with ice water to prevent heating, filtered and thoroughly washed with sol B. (60% ethanol - water sol prepared by diluting 715 ml of 95% ethanol to 1 litre and added 200 ml of ethyl ether and 0.2 ml of glacial acetic acid). Total volume of the sol after washing was made to 13 ml with sol B. Transfer 1 ml aliquot in triplicate, one of which is used as the reference sol after diluting to the mark with sol B. To the other tubes added 4 ml of freshly distilled aniline, shaken for 10 min and allowed to stand for 10 min. The absorbance was determined at a wavelength of 445 nm.

Standard curve was prepared by dissolving 40 mg of pure embelin in 100 ml absolute alcohol. This served as the stock sol. Graded vol of the stock sol (1.0, 1.5, 2.0,
2.5, and 3.0) were taken in glass stoppered tubes and diluted to 6 ml with sol B, then 4 ml of aniline sol was added and the mixture was shaken for 10 to 15 sec and allowed to stand for 10 min. The colour was finally read at 445 nm.

Blood was collected from retro orbital plexuses at different time intervals after drug treatment, allowed to clot, serum separated by centrifugation and embelin estimated. Serum embelin concentration was graphically plotted versus time and fractional turnover rate constant (K) and biological half life (T½) were calculated. The first order rate equation was employed as follows:

\[ K = \frac{2.303 \log A_0 - A_{\infty}}{t \log A_t - A_{\infty}} \]

where, \( A_0, A_t, \) and \( A_{\infty} \) represented embelin concentration (absorption) at time \( 0, t, \) (time point) and (infinity), respectively. \( T_{\frac{1}{2}} \) was calculated by the equation \( T_{\frac{1}{2}} = \frac{0.693}{K} \) where 0.693 was the calculated value of \( \ln/2. \)

HISTOLOGICAL TECHNIQUES

For histological studies, Bouins' and Zenker fixed sections were stained with Heidenhain's iron haematoxylin and Delafield's haematoxylin/eosine techniques.

**Heidenhain's Iron Haematoxylin technique:** After removing paraffin and Zenker the sections were downgraded through the various grades of alcohol and brought finally to
water. These were then put in 4% iron alum sol for 10-15 min. After washing thoroughly in running water, these were stained in haematoxylin for 10 min and differentiated in 2% iron alum. They were washed again with water, followed by dehydration, clearing in xylene and mounting in DPX.

**Delafield haematoxylin/eosine technique**: The sections were down graded in alcohol brought to water and stained in Delafield's haematoxylin for 10-15 min. After rinsing in water, slides were differentiated in acid water (2 drops of Conc HCl and 50 ml distilled water) till nuclei became pink. They were then differentiated in ammonia water (2-3 drops aqueous ammonia in 50 ml distilled water) till nuclei became blue. After washing in water, slides were upgraded upto 90% alcohol. Dipped in 1% alcoholic eosine for 30 sec, dehydrated in absolute alcohol, cleared in xylene and mounted in DPX.

**CYTOCHEMICAL TECHNIQUES**

Different cytochemical techniques were employed for the localization of carbohydrates, proteins, nucleic acids and lipids.

**Periodic-acid Schiff (PAS)**: Test for polysaccharides (Hotchkiss, 1948; Casselman, 1954 and Pearse, 1968): Zenker fixed paraffin sections were oxidised in 0.5% aqueous periodic acid for 5 min, rinsed in distilled water, dried and treated with Schiff's reagent for 30 min The pink colour was obtained after washing the sections in water due to
conversion of 1:2 glycol groups into dialdehyde by periodic acid which combine with the Schiff's reagent to form a pink dye. The sections were dehydrated rapidly in different grades of alcohol, cleared in xylene and mounted in DPX.

Acetylation and KOH reversal techniques of McManus and Cason (1950) were employed for control. Acetylation with acetic anhydride and pyridine (16:24; v/v) mixture for 20 h, blocked the reaction of 1:2 glycol groups with periodic acid.

The positive PAS reaction could also be reverted after treatment with 0.1 N KOH for half an hour. A negative reaction after KOH reversal, therefore, confirmed the presence of 1:2 glycol groups.

Himes and Moriber's technique for simultaneous detection of DNA, polysaccharides and proteins (Himes and Moriber, 1956). Zenker-fixed paraffin sections were used for the localization of polysaccharides, DNA and proteins. The sections were hydrolysed in 1 N HCl at 60°C for exactly 12 min and stained in Azure-A which stains the DNA. The slides were washed and oxidised with 0.5% aqueous periodic acid for 5 min and immersed in Schiff's reagent for 30 min after rinsing in distilled water. This stains the carbohydrates. The slides were then washed well in running tap water and treated with naphthol yellow to demonstrate the proteins. These were then dehydrated directly in tertiary butyl
alcohol, cleared in xylene and mounted in DPX. Sites containing DNA stained blue while polysacchrides were pink and proteins yellow.

**Sudan black B (SBB) method for lipids** (Baker, 1944, 1949, 1956; Pearse, 1968): For the detection of lipids in general, the gelatin sections of the tissue fixed in formaldehyde-calcium were stained in the saturated sol of Sudan black B in 70% alcohol and then differentiated in 70% alcohol, downgraded up to water and mounted in glycerine jelly. Lipids were stained blue.

**Nile blue sulphate (NBS) technique** for neutral and acidic lipids (Cain 1947). Formaldehyde calcium (post-chromed) gelatin sections were stained in 1% aqueous sol of Nile blue sulphate at 60°C, differentiated in 0.1% acetic acid and mounted in glycerine jelly. This technique is based on the principle that neutral lipids dissolve the oxazone and combined with the free base to out only the oxazone and free base (both red in colour) from the aqueous sol of Nile blue whereas acidic lipids dissolve the oxazone and combined with the free base to form blue lipid soluble compounds (Cain, 1947). The acid lipids stain blue while the neutral lipids stain pink.

**Mercuric bromophenol blue (Hg-BPB) method for proteins** (Mazia et al., 1953; Pearse, 1968). For the detection of proteins in general, the Zenker - fixed paraffin sections were downgraded, washed and brought to
They were then stained in mercuric bromophenol blue sol (1% mercuric chloride and 0.05% bromophenol blue in 2% aqueous acetic acid) for 10 min, differentiated in 0.5% acetic acid for 2 min, dehydrated in tertiary butyl alcohol, cleared in xylene and mounted in DPX. The sites containing proteins were stained in various shades of blue.

Feulgen reaction for DNA (Feulgen and Rossenback, 1924; Pearse, 1968). For the detection of nucleic acids, Zenker-fixed paraffin sections were hydrolysed in 1N HCl at 60 °C for an optimum time of 5 min, followed by rinsing in distilled water. Then the sections were stained in Schiff's reagent (de Tomasi, 1936) for 30 min. Mild hydrolysis released aldehyde groups from the deoxypentose sugar of DNA which then reacted with Schiff's leucobasic fuchsin to form a pink dye.

Carbol-fuchsin: Smear slides of spermatozoa were dried and fixed in methanol : acetic acid (3:1) for 10 min followed by staining in carbol fuchsin for 35 min. The slides were then differentiated in butanol for 5 min and mounted in euperol. All the slides were examined under high power of Light microscope to see the morphology of the spermatozoa.

STAINING FOR TEM

Filtered the stains before use. Pipetted out a small amount of uranyl acetate sol in a clear watch glass. Placed the grid carrying the section down on to the stain.
Placed a wooden or card-board cover as the staining was effective when carried out in dark. Stained for 10 to 15 min. Took each grid and washed in 2 lots of 50% ethanol and 2 lots of double distilled water with continuous agitation. Dried carefully on a filter paper (taking care that the sections carrying the surface was not coming in contact with the filter paper.

Placed a few ml of lead citrate in watch glass and placed the grids on to the stain for 5 to 10 min. Washed each grid briefly in 0.1N sodium hydroxide and then in 2 lots of double distilled water. Dried the grids and stored in the grid boxes.

So now the sections were ready for observation in the transmission electron microscope.

MORPHOMETRIC STUDY OF TESTIS SECTIONS

Quantitative histological study of spermatogenic events was performed from randomly selected sections of the testis. Classification of Leblond and Clermont (1952) was followed for identification of cellular associations present. Deviation in the normal cellular population reflected the cell types affected and the degree of damage. For morphometric analysis, intratubular cells were counted in tubules.

METHODS OF BIOCHEMICAL ANALYSIS

Simultaneous extraction of lipids, phospholipids, proteins and nucleic acids: The method of Schneider (1945)
was employed for the extraction of lipids, proteins and nucleic acids. A 10% tissue homogenate was prepared in chilled 10% trichloroacetic acid (TCA) in a motor driven teflon-glass homogenizer at full speed for 1 min and centrifuged for 10 min at 2,500 xg. Supernatant was discarded and precipitates were recentrifuged after suspending in 5 ml of 10% TCA. Supernatant was discarded again and the precipitates suspended in a mixture of distilled water and 95% ethyl alcohol (1:4, v/v). After centrifugation the supernatant was collected and precipitates suspended in 5 ml of ethyl alcohol and centrifuged again. This was repeated once again. The residue from last centrifugation was extracted by suspending it in 10 ml of ethyl alcohol:ether mixture (3:1, V/V), at 60 °C for 4 min and the supernatant was collected after centrifugation. The entire procedure was repeated. All the four supernatants were combined and used for estimation of total lipids and phospholipids.

The residue left over from the above extraction was suspended in a mixture of distilled water (1.2 ml) and 10% TCA (1.3 ml) and heated in a water bath at 90-95 °C for 15 min. Supernatant was collected after centrifugation and precipitates resuspended in 2.5 ml of 5% TCA and centrifuged. Supernatant was again collected and the combined supernatant was used for the estimation of DNA and
RNA. The precipitates were dissolved in 10 ml of 1N NaOH by heating in a water bath at 90°C. Proteins were estimated in this fraction.

**Estimation of total lipids**: Total lipids were estimated by the method of Pandey et al. (1963).

Reagents:
1) Acid dichromate sol (2 g potassium dichromate in 100 ml Conc H₂SO₄).
2) Standard: 500 mg % Olive oil in absolute alcohol.

Procedure: 5 ml of lipid extract was taken in a test tube and evaporated to complete dryness and 3 ml of freshly prepared acid dichromate sol was added to it and heated for 15 min in boiling water bath. A green colour was developed. After cooling at room temp, 8 ml of distilled water was added and absorbance of the colour was read at 590 nm. Blank and standard tubes were run simultaneously.

**Phospholipids**: Phospholipid estimation was done according to the method of Bartlett (1959), as modified by Marinetti (1962), in which lipid phosphorous was taken as a measure of total phospholipids present.

Reagents:
1) 70% Perchloric acid.
2) 2.5% acid ammonium molybdate sol
3) Amino naphthol sulphonylic acid (ANSA)
4) Standard KH₂PO₄ - 34.2 mg/100 ml of distilled water.
Procedure: Five ml of lipid extract was taken in scrupulously clean tube and evaporated to dryness. The residue was digested with 1 ml of perchloric acid in a hot sand bath until the colour changed from white to black, yellow and then colourless. To the cooled digest, was added 7.0 ml of distilled water, 0.5 ml of 2.5% ammonium molybdate sol and 0.2 ml of ANSA reagent. Blue colour was developed by heating in a boiling water bath for exactly 7 min and intensity of the colour was read at 600 nm. Blank and standard tubes were also run at the same time. The lipid phosphorous thus obtained was multiplied by a factor of 25 to get the amount of phospholipids present in the aliquot.

Thin layer chromatography (TLC): The various lipid fractions of tissue were separated by thin layer chromatography and then subjected to quantitative chromogenic analysis.

Extraction of tissue lipids: Extraction of lipids was carried out by the method of Folch et al. (1957) as follows:

1. The tissue was weighed, cut into small pieces and ground in a pestle and mortar with aqua regia-washed sand and 20 vol of chloroform: methanol mixture (2:1, v/v).

2. The extract was filtered into a graduated cylinder and residue again extracted with chloroform: methanol mixture.
3. To the filtrate was added 0.7% KCL (20% of vol of extract) and after shaking vigorously, the mixture was allowed to stand for a few hours to let the aqueous and lipid layers separate.

4. The upper aqueous layer was removed carefully and lower layer washed two more times with 5 ml of washing mixture (chloroform:methanol:water, 3:48:47, v/v, Folch upper phase).

5. The lower lipid layer was then put in a round bottom flask and evaporated to dryness in vacuum under reduced pressure and at a temp around 40°C in a rotary evaporator (Buchi type).

6. The residue was washed with 5 ml of chloroform:methanol (2:1, v/v), dried and then washed with chloroform:methanol (2:1, v/v) containing 4% water (CHCl₃:CH₂OH:H₂O, 64:32:4, v/v) and evaporated to dryness. The process was repeated once again.

7. Lastly, only chloroform was added and evaporated to dryness. The dried lipids were dissolved in 10 ml chloroform, filtered through whatman No 1 filter paper, evaporated to dryness, dissolved in a known vol of chloroform:methanol (2:1, v/v) and stored at -20°C.

8. The upper layer from step 4 is taken in separate tubes, salt removed and redissolved in chloroform:methanol mixture (2:1, v/v). Sialic acid was
estimated in this fraction. Triglycerides (TG), Free Fatty acids (FFA), Cholesterol (Chol), total lipids and phospholipids were estimated in the lipids extract obtained in step 8.

**Estimation of total lipids**: Total lipids were estimated according to the method of Fringes and Dunn (1970).

Principle: A bluish-green colour was developed with the help of phospho-vanillin in the presence of concentrated sulphuric acid and the absorbance read at 540 nm.

**Reagents:**

a) 0.6% vanillin sol in distilled water (600mg/100 ml of water).

b) Phosphovanillin reagent: 80 ml orthophosphoric acid and 20 ml of vanillin sol (0.6%) were mixed. The sol was prepared fresh.

c) Standard: 500 mg % Olive oil in absolute alcohol.

**Procedure**: Two ml of concentrated sulphuric acid was added to 0.1 ml of lipid extract. The contents were shaken and the tubes kept in a boiling water bath for 15 min. After cooling at room temp 5 ml of phosphovanillin reagent was added to 0.2 ml of the sol. Sets of standards and blank were also run simultaneously. The optical density was measured at 540 nm.
Total cholesterol: Cholesterol was estimated by the method of Zlatkis et al (1953).

Principle: Cholesterol in the presence of Conc sulphuric acid and glacial acetic acid formed a violet coloured complex with ferric chloride which could be measured at 540 nm.

Reagents:
a) Acetone - alcohol mixture 2 equal vol of acetone and alcohol.
   b) Glacial acetic acid.
   c) FeCl₃ sol: 10g FeCl₃.6 H₂O/100 ml of acetic acid glacial.
   d) Conc sulphuric acid
   e) Colouring reagent (working FeCl₃ sol)

Procedure: To 0.5 ml of the lipid extract was added 9.5 ml of acetone : alcohol mixture (1:1, v/v) and kept in water bath at 60-70°C for 10 min. The vol was made upto 10 ml with 1:1 acetone alcohol. After centrifugation for 5 min at 2000xg 2 ml of the supernatant was taken and dried completely in a water bath at 60°C. Three ml of glacial acetic acid (GAA) and 2 ml of working FeCl₃ sol was added to each tube. After shaking vigorously, the tubes were kept for 30 min in dark.
A brown colour was developed the intensity of which was measured at 540 nm. Blank and standard tubes were run simultaneously using doubly crystallized cholesterol standards.

**Phospholipids**: Phospholipids were estimated by determining the lipid-bound phosphorous (Bartlett, 1959) as modified by Marinetti (1962).

**Principle**: When acid hydrolysate of lipid was treated with molybdate, it formed phosphomolybdic acid, which in turn was reduced by the addition of ANSA to produce a blue colour. The intensity of the colour was measured at 830 nm.

**Reagents**

a) Perchloric acid (PCA)

b) 2.5% ammonium molybdate sol (2.5 g/100 ml water)

c) Amino-naphthol-sulphonic acid (ANSA) reagent

d) Standard: \( \text{KH}_2\text{PO}_4 \) -34.2 mg/100ml distilled water.

**Procedure**: To 0.1 ml of lipid extract, was added 0.9 ml of PCA and digested in a hot sand bath till the sol became colourless. To the tubes were added 7.0 ml distilled water. 0.5 ml of 2.5% ammonium molybdate reagent and 0.2 ml of ANSA in that order. Colour was developed by keeping in a boiling water bath for exactly 7 min and the intensity of the colour was read at 600 nm. Blank and standard tubes were run under similar conditions.
Triglycerides: Tg were estimated by the method of Sidney and Bernand (1977).

Principle: Tg from the total lipids were extracted with isopropanol and hydrolyzed by refluxing with alcoholic KOH to yield glycerol and free fatty acids (FFA). Glycerol reacts with sodium metaperiodate giving rise to formaldehyde which in the presence of acetyl-acetone and ammonium ions produced yellow colour which had absorption maxima at 425 nm.

Reagents: 
- a) Heptane
- b) Isopropanol
- c) Sod-m-Periodate sol (0.6%) 600 mg/100 ml water + 50 ml of glacial acetic acid
- d) Ammonium acetate (15.4 g/100 ml water)
- e) 6.25 M Potassium hydroxide sol (35 g KOH in 100 ml water)
- f) Acetyl-acetone reagent (1.5 ml of acetyl acetone diluted to 200 ml with ammonium acetate)
- g) Standard: Triolein, 500 mg/50 ml of isopropanol.

Procedure: To 0.1 ml of lipid extract, 2.0 ml of heptane and 3.5 ml of isopropanol were added and mixed for 30 sec. One ml of Conc H₂SO₄ was added and mixed thoroughly in a vortex mixer for 30 sec. The tubes were kept till the two layer separated. From each tube, 0.8 ml of upper phase was
taken and 4 ml of isopropanol and 2 drops of KOH (6.25 M) were added. After incubation for 10 min at 60°C, 0.4 ml of sodium-m-periodate and 2 ml of acetyl-acetone were added and kept for incubation for 10 min at 60°C. This was then cooled for 3 min and the intensity of the colour was read at 425 nm. Simultaneously, blank and standard were also run.

**Free-Fatty acids**: FFA were estimated by the method of Novak (1965).

**Principle**: Cobalt nitrate reagent was used to form soaps with FFA and complex of cobalt with α-nitroso-β betanaphthol was measured at 540 nm.

**Reagents**: a) Dole's reagent [40 parts of isopropanol + 10 parts of heptane plus 1 part of 1 N \( \text{H}_2\text{SO}_4 \) (0.1 ml conc \( \text{H}_2\text{SO}_4 \) in 3.5 ml of distilled water)].

b) Chloroform heptane sol (5:1, v/v)

c) Cobalt-nitrate reagent
d) α-nitroso-β-naphthol reagent
e) 0.028 g of Palmitic acid/50 ml Dole’s reagent

**Procedure**: To 0.2 ml of the lipid extract, 1 ml of Dole’s reagent, 1.2 ml heptane and 2 ml of water were added. After shaking for 1 min to 1.2 ml of its upper phase 2.0 ml \( \text{CHCl}_3 \) :heptane (5:1, v/v) and 2 ml cobalt nitrate reagent were added. After vortexing for 3 min, each tube was centrifuged
at 2500 g. To 2.4 ml of upper phase from each tube 3, ml colouring reagent was added and read at 540 nm after 30 min. Blank and standards were also run simultaneously.

**Preparation of thin layer plates:** Glass plates (20x20 cm) were washed, dried, rinsed with acetone and dried again. These were then coated with 0.25 mm thick layer of silica gel G, as in the method of Mangold (1961). A slurry was made with 30 g of silica gel dissolved in 60 ml of distilled water. The silica gel layered plates were air dried and activated for 1 h at 110 °C before applying the lipid samples.

**Separation of phospholipids:** A suitable aliquot of lipid extract was spotted on the activated plate for one dimensional TLC. The plates were run in a rectangular glass jar (10"x10"x5"). Prior to running the plates, the jar was saturated with solvent vapours by lining it with filter paper soaked in the solvent mixture. The solvent system for separation of phospholipids consisted of:

- chloroform:methanol:14N ammonia (v/v)
  - 65 : 25 : 4

**Iodine exposure** (Stahl, 1969): After the above solvent systems had run almost the entire length of the plate, the plate was taken out of the jar, dried and exposed to iodine vapours. I vapour demarcated the lipids as yellow-brown spots.
Quantitation of phospholipid fractions from chromatoplates: For quantitative analysis, the spots of the various phospholipid fractions were separately scrapped off the plate into test tubes for phosphorous estimation. After adding 1.0 ml of 60% perchloric acid (PCA), the tubes were kept in a hot sand bath and the lipids were digested till the sol changed colour from yellow to colourless. After cooling the tubes at room temp, 7.0 ml of distilled water, 0.5 ml of 2.5% ammonium molybdate sol and 0.2 ml of ANSA reagent were added to them in that order and heated in a boiling water bath for exactly 7 min. Silica gel did not interfere in the development of colour. The tubes were centrifuged at 2000 g for 10 min to settle down the silica gel particles before measuring the absorbance at 600 nm. Amount of phospholipid in each spot was calculated taking into consideration the total amount of lipid extract applied on the plate.

Separation of neutral lipids: Known amount of lipid extract (30-50 µl vol) was spotted in freshly activated plates and various neutral lipid fractions were separated by running in a manner similar to that given for phospholipids. The solvent mixture consisted of:

petroleum ether:diethyl ether:acetic acid (v/v)

90 : 10 : 1
Exposure to iodine vapours revealed the spots of the various neutral lipid classes. These were identified by comparison with simultaneously run authentic standards.

Quantitation of neutral lipid component classes:

i) Cholesterol and cholesterol esters: The spots corresponding to cholesterol and cholesterol esters were scrapped off the plates into centrifuge tubes and 2.0 ml of chloroform:methanol (2:1, v/v) was added to each tube to extract the lipid. After centrifugation, the supernatant was collected into test tubes by decanting and the procedure was repeated two more times. Estimation of cholesterol and cholesterol esters was carried in the dried extract by the method of Zlatkis et al. (1953).

Reagents: a) Glacial acetic acid  
b) FeCl₃ sol (10 g FeCl₃·6H₂O/100 ml of glacial acetic acid)  
c) Conc H₂SO₄  
d) FeCl₃ Colouring reagent  
e) Standard cholesterol (100 mg/100 ml acetic acid)

The supernatant from the above step was evaporated to dryness and then was added 3.0 ml of glacial acetic acid.
and shaken. Then the tubes were kept in the dark for 30 min after adding 2 ml of colouring reagent. Optical density was read at 540 nm. Blank and standard tubes were run at the same time.

ii) Free-fatty acids: Free fatty acids were estimated by the method of Lowery and Tinsley (1976).

Reagents: a) Benzene
   b) Cupric acetate - pyridine reagent
   c) Standard - 1 mg palmitic acid/5 ml chloroform

The lipid spots were scrapped and to it was added 5 ml of benzene to extract the lipid. The mixture was warmed slightly and to it was added 1.0 ml of cupric acetate pyridine reagent. The contents were mixed for 2 min and then centrifuged for 5 min. The absorbance of the upper layer was recorded at 715 nm. Standard and blank tubes were run simultaneously.

iii) Triglycerides

Triglyceride estimation was carried out by the method of Sidney (1973).

Reagents: a) Heptane
   b) Conc $\text{H}_2\text{SO}_4$
   c) KOH (6.25 M)
   d) Isopropanol
e) Sodium metaperiodate (0.600 g/ml water and 50 ml of glacial acetic acid)
f) Acetyl-acetone reagent (1.5 ml of acetyl acetone was diluted to 200 ml with ammonium acetate
g) Standard - 1 mg glycerol trioleate/ml of chloroform

Procedure: To the spots scrapped from the plates was added 2.0 ml of heptane and mixed well. Then after adding 1 ml of conc H₂SO₄ and mixing thoroughly, the tubes were centrifuged. To 0.8 ml of the upper phase, was added 4 ml of isopropanol and 2 drops of 6.25 M KOH and the mixture was incubated for 10 min at 60°C. Then 0.4 ml of sodium metaperiodate and 2.0 ml of acetyl acetone were added. Again the mixture was incubated at 60°C for 10 min. This was cooled for about 5 min at room temp and OD was read at 425 nm. Standard curve was prepared from the various concentrations of glycerol trioleate.

Cholesterol from the tissue homogenates

Cholesterol in the tissue homogenates was estimated by the procedure of Zarrow et al. (1964).

Reagents: a) Glacial acetic acid
b) Ferric chloride colouring reagent (0.1 ml of 10% FeCl₃ in phosphoric acid+9.9 ml Conc H₂SO₄
c) Standard-Cholesterol (1mg/ml acetic acid)
Procedure: To one ml of 1% tissue homogenate prepared in acetic acid was added 2 ml of glacial acetic acid and 2.5 ml of colouring reagent. The tubes were shaken well and allowed to stand for 15-20 min. Optical density was read at 560 nm. Blank and standard tubes were run at the same time.

Estimation of nucleic acid: Both deoxyribose nucleic acid (DNA) and ribose nucleic acid (RNA) were estimated according to the procedure of Schneider (1945).

Estimation of DNA

Principal: Deoxyribose nucleic acid, after the treatment with diphenylamine under acidic condition forms a blue coloured compound which has an absorption maxima at 600 nm. The reaction is not specific for DNA but is given by 2-deoxypentoses under acidic conditions. The straight chain form of 2-deoxypentose (sugar of DNA) is converted to a highly reactive levulinic aldehyde which in turn forms a blue complex with diphenylamine.

Reagents: a) Dische diphenylamine reagent

b) Standard: 10mg DNA/100 ml distilled water

Procedure: 2.0 ml of extract was mixed with 4.0 ml of freshly prepared diphenylamine reagent (DPA). This sol was then heated in a water bath at 90°C for 10 min. Blank and standard tubes were run under similar conditions. Tubes were cooled and optical density was read at 600 nm.
**Estimation of RNA**

Principal: For estimation of RNA, orcinol reagent was used, the furfural formed from ribose reacts with orcinol to produce a green colour which is read at 660 nm.

Reagents: a) Orcinol reagent  
   b) Standard - 10mg RNA/100 ml distilled water.

Procedure: To 2.0 ml of the extract was added, 2.0 ml of Orcinol reagent (freshly prepared) and heated in a boiling water bath for 20 min. Blank and standard tubes were run at the same time. The optical density was read at 660 nm.

**Proteins:** Estimation of proteins was carried out according to the modified biuret technique of Gornall *et al.* (1949).

Reagents: a) Biuret reagent  
   b) Standard - Bovine serum albumin (BSA) (10μg/ml of distilled water)

Procedure: To 1 ml of protein extract was added 4 ml of biuret reagent. The sol was kept at room temperature for 30 min before reading the intensity of the colour at 540 nm. Blank and standard tubes were run simultaneously.

**Glycogen:** Extraction of glycogen was performed by the method of Heatley (1935). Tissue glycogen was released by heating in the presence of a strong alkali and this was precipitated by addition of ethanol. Addition of a coprecipitant, sodium sulphate, helped to give a quantitative yield of glycogen.

Reagent: a) 30% KOH sol
b) Saturated sodium sulphate sol

c) Ethanol (95%, v/v)

Procedure: In 2 ml of 30% KOH, 200 mg of tissue was digested by heating in a boiling water bath for 20-30 min. The digest was cooled in ice and then 0.2 ml of saturated sodium sulphate sol was added and mixed well. Glycogen was precipitated by adding 5 ml of ethanol. The contents were heated to boil and then cooled. After centrifugation, the supernatant was discarded and the alcohol removed by heating in water bath for a few min. The process was repeated by adding ethanol for complete extraction. The precipitates were dissolved in 2 ml of distilled water and then proceeded for estimation of glycogen by anthrone method.

Estimation of glycogen: The estimation of glycogen content in the extract was done according to the method of Seifter et al. (1950).

Reagents: a) Anthrone reagent- 2 g anthrone/1 litre of Conc \( \text{H}_2\text{SO}_4 \)

b) Standard - glycogen, 10mg/100 ml of distilled water

Procedure: 4.0 ml of freshly prepared anthrone reagent was added to 1.0 ml of glycogen extract while the tubes were in ice. Then the tubes were kept in boiling water bath for 10 min with a glass marble on the top of the tube to prevent
water loss by evaporation. After cooling, the optical density of the blue-green colour was read at 620 nm. Blank and standard tubes were run simultaneously.

**Phosphatases** Acid phosphatase, EC 3.1.3.2 and alkaline phosphatase, EC 3.1.3.1): The estimations of acid and alkaline phosphatase activity were done according to the method of Bodansky (1933) and Shinowara et al. (1941) as modified by Natelson (1963) respectively. The activity of these enzymes was recorded as the amount of inorganic phosphate liberated in a specific time under optimum conditions of pH and temp and expressed as mg inorganic phosphate liberated/g of tissue/h at 37°C.

The inorganic phosphate released was estimated by the method of Fiske and Subbarow (1925). This phosphate reacted with molybdic acid to form phosphomolybdic acid, which on treating with amino-naphthol sulphonic acid (ANSA), gave a blue colour. The colour could be compared with that of the standard phosphate sols processed similarly.

Reagents: a) Buffered acid phosphatase substrate (pH 5.2) b) Buffered alkaline phosphatase substrate (pH 8.5) c) 10% Trichloroacetic acid (TCA) d) 2.5% acid ammonium molybdate sol e) ANSA reagent 6) Standard $\text{KH}_2\text{PO}_4-34.2$ mg/100 ml of distilled $\text{H}_2\text{O}$
Procedure: To 0.5 ml of 10% aqueous tissue homogenate, was added 1.0 ml of either acid or alkaline phosphatase buffered substrate and incubated at 37°C for 1 h. After incubation 1.0 ml of 10% TCA was added to stop the reaction. The tubes were shaken, allowed to stand for 5 min and then centrifuged at 2500 \( \times \) g for 10 min. Control tubes were proceeded simultaneously in which 1 ml of 10% TCA was added prior to incubation to inactivate the enzyme.

To 1 ml of the above supernatant from test and control tubes, was added 7.0 ml of distilled water, 0.5 ml of acid ammonium molybdate sol and 0.2 ml of ANSA reagent. The tubes were kept in a boiling water bath for exactly 7 min. They were then cooled and absorbance read at 600 nm. Blank and standards were proceeded simultaneously.

Hexokinase (Glucose-ATP phosphotransferase EC 2.7.1.1) : The method of Crane and Sols (1955) was followed for the estimation of this enzyme.

Reagents:

- a) ATP-magnesium salt sol (pH 7.0)
- b) Histidine-tris-EDTA buffer (0.1M)
- c) 0.1M glucose sol (17.1 mg glucose/5 ml D.W.)
- d) 5% TCA
- e) Standard: \( \text{KH}_2\text{PO}_4 \) (34.2mg/100 ml distilled H\(_2\)O)

Procedure: 0.2 ml each of ATP-Mg sol, histidine-tris-EDTA buffer, 0.1 M glucose and 0.3 ml of distilled water were warmed together at 30°C. 0.1 ml of supernatant from 1%
homogenate was added, mixed thoroughly and incubated for 30 min at 37°C. Reaction was stopped with 1.0 ml of 5% TCA. In control tubes, TCA was added prior to incubation. Nucleotides were quantitatively absorbed on Norit charcoal whereas glucose-6-phosphate was not absorbed from TCA sol. After centrifugation, 1 ml of the supernatant was taken and to it, was added 2 ml of distilled water, 1 ml of acid ammonium molybdate and 0.5 ml of ANSA reagent. The intensity of this colour was read at 595 nm. One unit of enzyme activity was defined as that amount which liberated 1 g of stable phosphorus in 30 min at 37°C.

Amylase (α-1, 4 glucan. 4. glucanohydrolase, EC 3.2.1.1) : The method of Sumner (1921) was employed to estimate the activity of this enzyme.

Reagent: a) Starch substrate sol (2 g of soluble starch and 40 mg NaCl in 100 ml of 0.05 M phosphate buffer, pH 7.0)

b) Phosphate buffer (0.05 M, pH 7.0)

c) 3, 5 dinitrosalicylic acid reagent (DNSA)

d) Standard-maltose (100 mg/100 ml distilled water)

Procedure: To 0.2 ml of 10% aqueous tissue homogenate, were added 2 ml of substrate, 1 ml of phosphate buffer and 0.2 ml of water and the mixture was incubated for 30 min at 37°C. The reaction was terminated with 3 ml of DNSA reagent. In the control tubes DNSA was added prior to incubation. Tubes
were immersed in a boiling water bath for 10 min and then cooled. Each tube was diluted to 20 ml with distilled water and the intensity of the colour was read at 540 nm. Blank and standards were run simultaneously.

**Fructose 1, 6-diphosphatase (FDP) (D-fructose-1,6-diphosphate-1-phosphohydrolase, EC 3.1.3.11):** Enzyme activity was estimated by the method of McGilvery (1955) in which phosphorous liberated from FDP was determined.

**Reagents:**

a) **Substrate** - 0.05M fructose-1,6-diphosphate (FDP), 53 mg of sodium salt of FDP in 2.5 ml distilled water.

b) 0.05 M MgSO$_4$ (1.234 g MgSO$_4$ in 100 ml distilled water)

c) 0.005 M MnCl$_2$ (99.99 mg MnCl$_2$ in 100 ml distilled water)

d) 10% TCA

e) Ammonium molybdate

f) ANSA reagent

g) Boric acid - NaOH buffer (0.05M, pH 9.5).

h) **Standard** - KH$_2$PO$_4$ (34.2 mg/100 ml distilled water)

**Procedure:** To 0.4 ml of 10% aqueous tissue homogenate were added 0.2 ml of substrate, 0.1 ml of MgSO$_4$, 0.1 ml of MnCl$_2$, 0.4 ml of boric acid - NaOH buffer and 0.3 ml of distilled water. The incubation of the mixture was done for 1 h at 37°C. After which, 1 ml of 10% TCA was added to terminate the
reaction while in control tubes TCA was added before incubation. After centrifugation, 1 ml of supernatant was taken and to it were added 2 ml of distilled water, 1 ml of acid ammonium molybdate and 0.5 ml of ANSA. The intensity of the colour was read at 595 nm. One unit of enzyme activity was defined as that amount which liberated 1 μ mole of phosphorus with linear kinetics in 1 h time.

**Glycogen phosphorylase** (α-1, 4-glucon : orthophosphate glucosyl transferase, EC 2.4.1.1) : The method of Niemeyer *et al.* (1961) was employed for the determination of phosphorylase activity in the presence of AMP.

Reagent: 
a) Substrate - 0.025M sol of glucose-1-phosphate (monosodium salt - 9.42 mg in 1 ml distilled water)
b) Citrate buffer (0.067 M, pH 6.0)
c) 0.0025 M adenosine monophosphate sol (1.023 mg AMP in 1 ml of distilled water).
d) 1% glycogen (1 g of glycogen in 100 ml of water)
e) 0.075 M sodium fluoride (3.15 mg NaF in 1 ml distilled water)
f) 10% Trichloroacetic acid (TCA)
g) 2.5% ammonium molybdate
h) ANSA (Amino naphthol sulphonic acid) reagent
h) Standard - KH$_2$PO$_4$ (34.2 mg/100 ml of distilled water)
Procedure: To 0.2 ml of 10% aqueous tissue homogenate was added 0.1 ml of substrate, 0.1 ml of 0.0025M AMP, 0.5 ml of citrate buffer pH 6.0, 0.1 ml of sodium fluoride, 0.1 ml of glycogen and 0.1 ml of distilled water and the mixture was incubated at 37°C for 1 h after incubation, the reaction was terminated with 1 ml of chilled 10% TCA, which in case of control tubes was added prior to incubation. Tubes were centrifuged and to 1 ml of supernatant was added, 2 ml of distilled water, 1 ml of ammonium molybdate and 0.5 ml of ANSA. Colour intensity was read at 595 nm. A unit of enzyme was expressed as that amount which liberated 1 µg of orthophosphorus from glucose-1-phosphate by 100 mg tissue per h at 37°C.

Glucose-6-phosphatase (D-glucose-6-phosphate phosphohydrolase, EC 3.1.3.9): The activity of this enzyme was estimated according to the procedure of Swanson (1955).

Reagents:

a) Substrate—Monosodium salt of glucose-6-phosphate (0.1M-28.4 mg in 1 ml distilled water)

b) Citrate buffer (0.1 M, pH 6.2)

c) 10% trichloroacetic acid (TCA)

d) 2.5% ammonium molybdate

e) ANSA reagent

f) Standard - KH₂PO₄ (34.2 mg/100 ml distilled water)
Procedure: The reaction mixture consisting of 0.2 ml of 10% aqueous tissue homogenate, 0.1 ml of glucose-6-phosphate substrate (0.1M monosodium salt), 0.3 ml of citrate buffer (0.1 M, pH 6.2) and 0.2 ml of distilled water was incubated for 1 h at 37°C. After incubation, 1 ml of cold 10% TCA was added to stop the reaction. In the control tubes, the TCA was added prior to incubation. The tubes were centrifuged and to 1.0 ml of supernatant was added, 2 ml of distilled water, 1 ml of ammonium molybdate and 0.5 ml of ANSA. The intensity of the colour was read at 595 nm. The unit of enzyme activity was defined as that amount which liberated 1 μg of phosphorous in 1 h at 37°C under standard assay conditions.

Glucose-6-phosphate isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9): The method of Slein (1954) was followed for estimation of the enzyme activity.

Reagents: a) Substrate-monosodium salt of glu-6-PO (0.1M-28.4 mg in 1 ml distilled water)
b) Tris-HCl buffer (0.1M, pH 9.0)
c) 30% HCl (21.33 ml of water was added to 100 ml of conc HCL)
d) 0.1% alcoholic resorcinol
e) Standard - Fructose (10 mg/100 ml distilled water)

Procedure: A 10% aqueous tissue homogenate (0.4 ml) along with 0.1 ml of substrate, 0.4 ml of tris buffer and 0.2 ml
of distilled water was incubated for half an hour at 37°C. Reaction was stopped after incubation by addition of 3.5 ml of 30% HCl, which in case of control tubes was added prior to incubation. Then 1 ml of 0.1% alcoholic resorcinol was added to all the tubes, which were heated for 10 min at 80°C. After cooling at room temp and centrifuging, colour intensity of the supernatant was read at 520 nm. The unit of the enzyme activity was defined as that amount which caused a change of units in O.D. due to fructose-6-phosphate in 30 min isomerization at 37°C.

**Adenosine triphosphatase (Mg++ ATPase, EC 3.6.1.3):**

The enzyme, ATP phosphohydrolase was estimated by the method of Kielley (1955).

**Reagents**

a) Tris buffer (0.2 M, pH 7.6)

b) MgCl₂ sol (5 mM-101.65 mg MgCl₂ in 100 ml distilled water)

c) ATP sol (5 mM sodium salt - 29.35 Mg ATP in 10 ml distilled water)

d) 10% TCA

e) 2.5% ammonium molybdate

f) ANSA

g) Standard: KH₂PO₄ 34.2 mg/100 ml distilled water

**Procedure:** To 0.2 ml of 10% tissue homogenate (in 0.25 M sucrose sol) was added 0.2 ml of tris buffer, 0.2 ml of ATP sol and the mixture was incubated at 37°C for 30 min. The
reaction was stopped by adding 2 ml of 10% TCA, which had been added to control tubes before incubation. Tubes were centrifuged and to 1 ml of the supernatant were added 2 ml of distilled water, 1 ml of acid ammonium molybdate and 0.5 ml of ANSA. The intensity of the colour was read at 595 nm. One unit of enzyme activity was defined as that amount which liberated 1 µg of phosphorous in 30 min at 37°C.

Lactate dehydrogenase (Lactose:NAD oxido-reductase (LDH), EC 1.1.1.27) : Method of King (1959) was employed for the estimation of enzyme activity.

Reagents:

- a) Glycine buffer (0.1M) pH 8.5
- b) Buffered substrate
- c) NAD sol (10 mg in 2 ml distilled H₂O)
- d) Dinitrophenyl hydrazine reagent (DNPH) 20 mg dissolved in hot INHCL to a final vol of 100 ml
- e) 0.4 N NaOH (1.6 g NaOH distilled water to a vol of 100 ml)
- f) Standard-11 mg sodium pyruvate/100 ml of buffered substrate.

Procedure: To 0.2 ml of the tissue homogenate (in 0.25M sucrose) was added, 1 ml of buffered substrate and incubated at 37°C for 15 min. Then 0.2 ml NAD was added. Tubes were shaken and incubated again and exactly after 15 min was added, 1 ml of DNPH reagent. Tubes were incubated again for 15 min and then 10 ml of 0.4 N NaOH was added and the
The intensity of the colour was read at 440 nm within 1-5 min. One unit of the enzyme was defined as change in O.D. per min by 100 mg tissue.

**Succinate dehydrogenase (SDH) (EC 1.3.99.1):**

Activity of the enzyme was determined by the method of Kun and Abood (1949).

**Reagents:**

- a) Phosphate buffer (0.1M, pH 7.4)
- b) 0.2 M sodium succinate (5.4 g in 100 ml H₂O)
- c) Triphenyl tetrazolium chloride (TTC) (100 mg in 100 ml distilled water)
- d) Acetone
- e) Standard- sodium dithionite (1 μg/100 ml distilled water)

**Procedure:** Reaction mixture consisting of 1 ml of 10% tissue homogenate (in 0.25 M sucrose), 0.5 ml of phosphate buffer, (pH 7.4), 0.5 ml of sodium succinate and 0.5 ml of TTC was incubated at 37°C for 1 h. Then to it was added 3.5 ml of acetone and the tubes were centrifuged. Optical density of the supernatant was read at 420 nm. Enzyme activity was defined as change in O.D. in a specific time.

**Citric Acid**

This was estimated in the seminal vesicles as described by Oser (1976).

**Reagents:**

- a) 18N H₂SO₄ (50 ml H₂SO₄ made up to 100 ml with distilled water)
- b) Potassium bromide - bromine sol
- c) 5% potassium permaganate
d) Hydrogen peroxide

e) Thiourea sol (2 g of sodium tetraborate was dissolved in 4% thiourea sol)

f) n-heptane

g) Standard-Citric acid (0.1 mg/100 ml distilled water)

Procedure: A 10% tissue homogenate was made and 1 ml of it was centrifuged. To the supernatant was added 0.04 ml of 18 N $\text{H}_2\text{SO}_4$ and after mixing thoroughly the sol was evaporated to 0.4 ml in a paraffin oil bath at 100-120°C. After cooling, 0.04 ml of potassium bromide-bromine sol was added and kept for 10 min. Then after adding 0.1 ml of 5% potassium permanganate, the tubes were cooled to about 10 °C and 2 drops of 6% hydrogen peroxide were added to decolourise the permanganate. The tubes were shaken thoroughly after adding 1.3 ml of n-heptane to extract the pentabromoacetone and then centrifuged. To 1.0 ml of the organic supernatant was added 3.5 ml of thiourea sol and the tubes were shaken well for about 5 min. After centrifuging for 5 min, the colour intensity of the pale yellow aqueous phase was read at 445 nm. Blank and standards were run alongwith it.

**Ascorbic Acid**: The method of Natelson (1963) was used for estimation of ascorbic acid in the seminal vesicle.

Reagents: a) 10% TCA

b) DNPH reagent
c) 65% $\text{H}_2\text{SO}_4$ (to 100 ml of Conc $\text{H}_2\text{SO}_4$ was added 63.32 ml of water)

d) Standard - Ascorbic acid (0.1 mg/100 ml distilled water)

Procedure: To 0.4 ml of 10% tissue homogenate in distilled water, was added 1.6 ml of 10% TCA. The contents were mixed and allowed to stand in ice for 5 min. After centrifugation, 0.5 ml of supernatant was taken and to it was added 0.2 ml of DNPH reagent. The tubes were mixed, stoppered and incubated for 3 hs at 37 °C after which they were chilled in an ice bath. The tubes were then allowed to stand for 30 min after adding 0.8 ml of cold 65% $\text{H}_2\text{SO}_4$. Optical density was read at 520 nm. Blank and standards were run simultaneously.

Sialic acid: The estimation of sialic acid was carried out in epididymis according to the method of Warren (1959).

Reagents: a) 0.1N $\text{H}_2\text{SO}_4$

b) Sodium periodate sol - 0.2M
c) Sodium arsenite sol
d) Thiobarbituric acid (600 g of it was dissolved in 0.5 M sodium sulphate sol
e) Cyclohexanone

Procedure: To 0.2 ml of 10% tissue homogenate in distilled water was added 0.5 ml of 0.1N $\text{H}_2\text{SO}_4$ and heated for one h at 80 °C. To 0.2 ml of this extract was added 0.1 ml of sodium periodate sol. After shaking thoroughly, the tubes were allowed to stand for 20 min. 0.1 ml of arsenite sol was
added and the tubes were shaken till yellow-brown colour disappeared. To this was added 3 ml of thiobarbituric acid and again the contents were shaken vigorously and then heated in a boiling water bath for 15 min. After cooling for 5 min, 4.3 ml of cyclohexanone was added and centrifuged. Optical density of upper reddish cyclohexanone phase was read at 590 nm. Blank was also run.

**Glucose-6-phosphate-dehydrogenase (G6PDH) (EC 1.1.1.49)**

Its activity was determined by the method of Julian and Reithal (1961).

**Principle:** Glucose-6-phosphate was used as substrate which when reacted with NADP in the presence of G6PDH formed gluconate-6-P, NADPH and H⁺. The change in optical density at 340 nm per unit time gave the measure of the G6PDH activity.

**Reagents:**

- a) Triethanolamine buffer (0.1 M)
- b) MgCl₂ sol - 2g/100 ml water
- c) G-6-PO₄ (35 µM) - 10 mg/ml water
- d) NADP sol - 10 mg/ml water

**Procedure:** 10% homogenate of testis and 5% homogenate of epididymis were prepared in triethanol amine buffer (0.1 M, pH 7.6). Homogenates were centrifuged at 3000 g to settle the cell debris. For the estimation of enzyme, 2.59 ml of triethanolamine buffer (0.1M, pH 7.6), 0.2 ml of 0.1M MgCl₂ sol, 0.1 ml of 35 mM glucose-6-phosphate sol and 0.1 ml of 0.11 mM NADP sol were taken. Reaction was started by
addition of the sample. Sample taken for testis was 0.01 ml of supernatant of a 10% homogenate and for epididymis, 0.01 ml of supernatant of a 5% homogenate. The OD was read after 1, 2, 3, 4, 5 min at 340 nm. Mean was calculated from the measured $\Delta E/\text{min}$. $\Delta E$ stands for the differences in absorbance per min.

**Malate dehydrogenase (MDH) (EC 1.1.1.37):** MDH activity was estimated by the method of Davidson and Cortner (1967).

**Principle:** Oxaloacetate was used as the substrate for the estimation of MDH activity. This in the presence of coenzyme NADH was converted into L-malate with the oxidation of NADH into NAD. The change in optical density at 340 nm per unit time was taken as a measure of the malate dehydrogenase activity.

**Reagents:**

a) Phosphate buffer (0.1 M, pH 7.5)

b) Oxaloacetate sol 15 mM (2 mg/ml of phosphate buffer)

c) NADH sol 12 mM (10 mg/ml of phosphate buffer)

**Procedure:** 10% homogenate of testis and 5% homogenate of epididymis were prepared in cold phosphate buffer (0.1M, pH 7.5). Homogenates were centrifuged at 4 °C at 3000 x g to settle the cell debris. For the estimation of the enzyme, 2.83 ml of phosphate buffer (0.1 M, pH 7.5), 0.1 ml of oxaloacetate sol (0.5 mM) and 0.05 ml of NADH (0.2 mM) were taken. Reaction was started by addition of the sample. Sample taken for testis was 0.01 ml supernatant of a 10%
hopmogenate and 0.01 ml of supernatant of a 5% epididymis homogenate. The O.D. was read after 1,2,3,4,5 min at 340 nm. Mean was calculated from the measured ΔE/min, as described for G6PDH.

Hydroxymethyl glutaryl-CoA reductase (HMG-CoA) reductase (EC 1.1.1.34): The activity of this enzyme was estimated by the method of Rao and Ramarkrishnan (1975).

Principle: Acetyl-CoA and acetoacetyl-CoA reacts to form hydroxymethyl glutaryl-CoA (HMG-CoA) which by the enzyme HMG-CoA reductase was converted into mevalonic acid. HMG-CoA and mevalonic concentration in the tissue homogenate, were estimated in terms of absorbancy and the ratio between the two was taken as an index of activity of enzyme which catalyzes the conversion of HMG-CoA to mevalonate. The ratio was increased when the activity decreased and the ratio decreased when the activity was increased.

HMG-CoA was determined by reaction with Hydroxylamine at pH 5.5 and subsequent colorimetric measurement of the resulting hydroxamic acid by formation of complex with ferric salt. Mevalonate interferes at acid or neutral pH. Alkaline hydroxylamine was used to estimate specifically HMG-CoA. Mevalonate was estimated by reaction with the same reagent but at pH 2.1. At this pH, lactone form of mevalonate readily reacted with hydroxylamine to form hydroxymate.
Reagents:

a) Saline arsenate (1 g saline arsenate/1000 ml of saline.

b) Diluted PCA - 5 ml of PCA/100 ml of water

c) Hydroxylamine hydrochloride (HAHCL) reagent

d) Hydroxylamine reagent for HMG-CoA

Procedure:

Fresh testis and epididymis homogenates were prepared in arsenate-saline sol (1 g/1 l saline). Equal vol of 10% homogenate and dilute PCA (50 ml/l H₂O) was mixed. After waiting for 5 min it was centrifuged at 2000xg for 30 min. To 1 ml of the supernatant, 0.5 ml of fresh hydroxylamine reagent (equal vol of 2 mol/l) hydroxylamine hydrochloride and water for mevalonate and equal vol of hydroxylamine hydrochloride and NaOH (4.5 M/1) for hydroxymethyl glutaryl-CoA). These reagents were mixed freshly before use. This was mixed properly and after waiting for 5 min, 1.5 ml of ferric chloride reagent (5.2 g TCA and 10 g ferric chloride in 50 ml of 0.65 M HCl and diluted to 100 ml with the later) was mixed. After 10 min, optical density was read at 540 nm.

**Triglyceride lipase (TGL)(EC 3.1.1.3)**: was estimated by the method of Kunze et al (1974).

Principle: The release of FFA from Tg has been regulated by the enzyme, Tg lipase. Ediol (a commercially available emulsion of 50% coconut oil) was used as substrate. The enzyme acted on it to release FFA which was then extracted
with Dole's reagent. FFA in the presence of Co(NO₃) formed soaps which gave colour reaction with α-nitroso-β-naphthol which was measured at 540 nm.

Reagents:

a) Ediol for Tg lipase
b) KRB buffer (pH 7.4)
c) 5% FFA free BSA sol - 5 g of FFA free BSA/100 ml of KRB buffer
d) Sucrose sol 0.25M (0.855 mg/100 ml of water)
e) Dole's reagent - 40 parts of isopropanol + 10 parts of heptane + 1 part of 1N H₂SO₄.
f) α-Nitroso-β-Naphthol colouring reagent

Procedure: After sacrificing the animals, testis and epididymis were immediately placed in Krebs-Ringer-phosphate (KRB) buffer (pH 7.4) which contained 5% fatty acid free albumin. pH of albumin sol was adjusted to 7.4 by addition of NaOH. Homogenization and centrifugation were carried out at 4 °C. Extract of fat pad were prepared by homogenizing the tissue in 3 ml of 0.25 M sucrose per g of tissue. Homogenate was centrifuged at 12,000 xg for 10 min. A fat cake accumulating at the top of the tube containing epididymis tissue was discarded. Remaining supernatant was saved for assay of lipolytic activity. Lipolytic activity was measured in the extract, containing 0.1 ml of diluted ediol, 0.5 ml of 20% fatty acid free albumin, pH 6.8, 0.2 ml of 0.06 M phosphate buffer, pH 6.8 and sufficient distilled water to make a final vol of 2.0 ml. This was incubated at 37 °C in
shaking water bath. The incubation was stopped by adding the mixture of 0.5 ml Dole's reagent, 1.5 ml heptane and 1 ml H₂O. This was shaken vigorously. 0.5 ml of the extract were added 2 ml Chloroform : heptane (5:1), 0.5 ml heptane and 2 ml cobalt reagent. This was mixed in a vortex mixer for 3 min and centrifuged for 15 min at 2500 x g. 2.4 ml of the upper phase was taken and 3 ml of α-Nitroso-β-Naphthol colouring reagent was added. The color developed was read after 30 min at 540 nm.

Post-heparin lipolytic activity (PHLA) (EC 3.1.1.34): PHLA was estimated by the method of Boberg and Carlson (1964).

Principle: Normal blood does not contain appreciable quantities of this enzyme. However, following injection of heparin lipoprotein lipase is released. Therefore, treatment with heparin is a necessary step. Apparently a lipase released into the blood hydrolyzes the neutral fat in chylomicron fraction. Substrate of this enzyme activity is intralipid which consists of Tg emulsions. Apoprotein CII is the activator for enzyme PHLA which comes from the serum added in the initial reaction mixture. Lipase from sample acts on the substrate and release of glycerol and FFA takes place. FFA is extracted and quantitated with Dole's reagent.

Reagents: a) Heparin - 500 L.U./ml of normal sline

b) Saline 0.145M - 8.562 g/100 ml water

c) 10% BSA - 100 μg/100 ml of KRB buffer (pH 8.5)
d) Tris-HCl buffer 1.35M

Procedure: Heparin in the dose of 100 IU/kg body weight was injected intravenously through ear vein. A zero min sample was taken before injecting heparin. Then blood was drawn after 10 min in heparinized tubes. The reaction mixture contained 20 ml of 10% W/V bovine serum albumin in Krebs-Ringer buffer (KRB) (pH 8.5), 4.3 ml of Tris-HCl (1.35 M, pH 8.5), 14 ml of 10% intralipid (1:1 intralipid and normal serum) and 7 ml of heparin sol (500 IU/ml). The reaction mixture was incubated at 37°C for 1 h in a shaking water bath. To 1.5 ml of the above substrate, 0.9 ml of 0.145 M NaCl and 0.1 ml of heparinized plasma was added. After shaking the contents thoroughly, it was incubated at 37°C for 1 h. One ml of the above mixture was taken and FFA was extracted with 5 ml of Dole's mixture to which 2 ml of H₂O and 3 ml of heparin were added. 5 ml of the extract, 0.5 ml heptane, 2 ml chloroform : heptane (5:1) and 2 ml cobalt reagent were added. This was vortex mixed for 3 min and then centrifuged for 15 min at 2500xg in cold. To 2.4 ml of upper phase, 3 ml of colouring reagent (α-nitroso-β-naphthol) was added. This was shaken very well and the colour was read at 540 nm after 30 min.
Fractional turnover rate of lipid Intravenous fat tolerance test (IVFTT), an index of rate of removal of circulating fat (triglycerides) was performed according to Salman and Hans (1973).

Intralipid (synthetic chylomicron suspension) in the dose of 2 ml per animal was injected through the tail vein and blood was collected at 0 min and at 10 min interval thereafter up to 40 min from the retro-orbital plexus. Serum was separated at 4°C and the amount of fat in it was estimated by measuring the turbidity in a spectrophotometer (Kontron Uvikon 860) at 280 nm using the absorbance mode following the procedure of Pownall et al. (1978). The fractional turnover rate (k) and half life (t1/2) of circulating fat was calculated from the slope of the line, following the first order rate equation, \( \log A_\infty - A_o / \log A_\infty - A_T \), where \( A_T \), \( A_\infty \) and \( A_o \) represented the absorbance at time \( T \), infinity and o, respectively. From the values of \( k \), the t1/2 was calculated by the equation \( t1/2 = (\ln 2/k) \) where \( \ln^2 = 0.693.t1/2 \) is defined as the time required for a 50% decrease in initial turbidity.

Amino transferases (Transaminases) These were estimated by the method of Reitman and Frankel (1957).

1. Aspartate transaminases (L-aspartate : 2 oxo-glutrate aminotransferase formerly known as glutamate oxaloacetate transaminase (GOT) (EC 2.6.1.1)).
2. **Alanine transaminase** (L-alanine : 2-Oxoglutarate amino transferase, formerly known as glutamate pyruvate transaminase (GPT) (EC 2.6.1.2)

**Reagents:**

a) Aspartate transaminase substrate  
b) Alanine transaminase substrate  
c) 2,4-dinitrophenyl hydrazone sol (DNPH)  
d) 0.4 N NaOH  
e) Standard-Oxaloacetic acid 2 mM (26.8 mg/100 ml buffer)

**Procedure:** To 0.1 ml of serum was added 0.5 ml of aspartate or alanine transaminase substrate and incubated at 57°C for one h in case of GOT and 30 min in case of GPT. In control tubes DNPH was added prior to incubation. Then to it was added 0.5 ml of DNPH sol. After 20 min, was added 5.0 ml of 0.4N NaOH. Absorbance of the colour was read at 510 nm after 10 min.

**Bilirubin** The Van den Bergh test (Van den Bergh and Snapper, 1913) as described by Natelson (1963) was used to determine the serum bilirubin level.

**Principle:** The technique is based upon the reaction of bilirubin with diazotized sulfanilic acid to be converted to purple azobilirubin. This purple colour can be used as a quantitative measure for serum bilirubin.

**Reagents:**

a) Sulfanilic acid sol  
b) Diazotizing reagent  
c) Methanol
d) Standard - bilirubin (5 mg/100 ml water)
Procedure: 0.1 ml of serum was added to 1.0 ml of distilled water and 1.5 ml of methanol. After shaking thoroughly, to it was added 0.3 ml of diazo reagent. Blank (using 0.1 ml of distilled water instead of serum) and standard tubes were run at the same time. Optical density was read after 30 min at 540 nm.

Total protein, albumin and albumin/globulin ratio:
Biuret reagent was used to estimate serum total protein and albumin, according to the procedure described by Natelson (1963). The globulin was quantitated by subtracting the albumin from the total protein content of serum.
Percentage globulin = percentage total protein - percentage albumin

Total protein
Reagents a) Bloor's reagent i.e. ethanol : diethyl ether (3:1, v/v).
b) Biuret reagent (half strength)
c) Standard - bovine serum albumin
Procedure: To 0.1 ml of serum was added 4 ml of Bloor's reagent to remove total lipids and bilirubin which otherwise would interfere in the protein estimation. The supernatant was discarded after centrifugation and to the residue was added 6 ml of Biuret reagent (half strength). Colour was allowed to develop for 20 min before reading the optical density at 540 nm. Half strength Biuret served as blank.
Albumin

Reagents: a) 25% sodium sulphite sol
   b) Ether
   c) Biuret reagent (full strength)
   d) Standard - bovine serum albumin

Procedure: To 0.1 ml of serum was added 2.9 ml of 25% sodium sulphite and 2 ml of ether to precipitate the globulins. After shaking thoroughly, the tubes were centrifuged, the ether layer aspirated and the protein precipitated in the bottom was removed and suspended in water. To 1.5 ml of aqueous aliquot was added 1.5 ml of Biuret reagent (full strength) and the absorbance of the colour was read at 540 nm.

Serum protein concentrations were calculated as follows:

\[
\text{% protein in serum} = \frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times \text{% protein in standard}
\]

The albumin/globulin (A/G) ratio was determined from the protein and albumin concentrations in serum.

\[
\text{A/G ratio} = \frac{\text{% of albumin}}{\text{% of total protein} - \text{% of albumin}}
\]

Glucose: Determination of serum glucose level was carried out as described by Natelson (1963)
Principle: When protein free blood serum was heated with alkaline copper sol, cuprous oxide was formed which when reacted with phosphomolybdic acid sol gave a blue colour to the sol which can be compared with that of standard.

Reagents
a) Tungstic acid
b) Alkaline copper reagent
c) Phosphomolybdic acid
d) Standard - 0.1% Glucose sol

0.02 ml of serum was diluted with 0.2 ml of distilled water and to this diluted serum was added 0.5 ml of tungstic acid. The contents were mixed thoroughly and then centrifuged. To 0.5 ml of supernatant was added 0.5 ml of alkaline copper reagent and heated for 10 min in a boiling water bath. After cooling, 1 ml of phosphomolybdic acid was added and mixed well. Colour was allowed to develop for 10 min and then its intensity was read at 420 nm. Blank and standard tubes were run simultaneously. Serum glucose was calculated as

\[
\frac{\text{Absorbance of unknown}}{\text{Absorbance of standard}} \times 100 = \text{mg glucose/100 ml}
\]

Phosphatases: Serum acid and alkaline phosphatases were estimated by the same procedure described for tissue phosphatases, using 0.05 ml of serum instead of tissue homogenates.

Total lipids, Phospholipids, Triglycerides, Cholesterol, FFA:

Total lipids, phospholipids, triglycerides, cholesterol...
terol and FFA were estimated by employing the same method used for the estimation of tissue lipids as described earlier. Instead of lipid extract of a tissue, a suitable vol of serum was used for the estimation of the lipids.

**High density lipoprotein-cholesterol (HDL-C)** was separated by the method of Lopes-Virella et al. (1977).

**Principle:** At neutral pH in the presence of magnesium (Mg++) ions, phosphotungstate precipitates low density lipoprotein (LDL), very low density lipoprotein (VLDL) and chylomicrons. From the supernatant, HDL-C was estimated by the method of Zlatkis et al. (1953).

**Reagents:**

a) Phosphotungstatic acid (phosphotungstic acid 45 g/l containing 160 ml of 1 M NaOH)

b) 1M NaOH sol
c) MgCl₂ sol
d) FeCl₃ reagent

**Procedure:** To 0.5 ml of serum, 0.05 ml of sod. phosphotungstate and 0.025 ml of MgCl₂ was added and centrifuged at 3000 x g for 25 min. 0.5 ml of the supernatant was taken. After drying the tubes, HDL-C was estimated with the FeCl₃ reagent.

**HDL and LDL-cholesterol** were separated by the method of Dana and Michael (1973).
Principle: In the presence of sodium dodecyl sulphate (SDS) (10 percent in 0.15 M saline), VLDL forms a pellet at the bottom of the tube. From the supernatant HDL-C and LDL-C were estimated by the method of Zlatkis (1953).

Reagents: a) Sodium dodecyl sulphate sol
d) EDTA

Procedure: Instead of serum, 2 ml of EDTA-plasma was taken in polypropylene tubes and to it 0.15 ml of 10% SDS (in 0.15 M saline at pH 9.0) was added and mixed properly. This was incubated for 2 h at 35 °C. After incubation, it was centrifuged for 10 min at 4 °C at 3000Xg. VLDL was separated in the form of a pellet while the clear supernatant contributed to HDL. The pellet was removed and estimated for HDL + LDL-C by the method of Zlatkis (1953) in the supernatant.

VLDL Cholesterol can be calculated by subtracting HDL-C and LDL-C from total cholesterol.
LDL-cholesterol can be calculated by subtracting HDL-C from HDL-C + LDL-C.

MEASUREMENT OF INTESTINAL UPTAKE

Starting from the ligament of Trietz, 20-25 cm portion of the intestine was used. Tissues were flushed with ice cold saline, thoroughly cleaned and everted using a thin stainless steel rod. All uptake studies were completed within 2 hours after sacrificing the animals. Duodenum
portion was used to study the calcium uptake while uptake of sugar and amino acids were measured in jejunum portion using tissue accumulation method of Crane and Mandelstam (1960).

Everted rings (0.3 to 0.5 cm) were cut and incubated in 25 ml Erlenmeyer flask containing 5 ml of oxygenated (95% O₂ and 5% CO₂) Kreb's Ringer bicarbonate buffer, pH 7.4 containing 5 mM D-glucose or L-Leucine or L-alanine with trace amounts (5µCi/100 ml KRBbuffer) of ¹⁴C radiolabelled substrate respectively. Incubations were carried out in a metabolic shaker at 37°C for 5 min with shaking rate of 150-160 oscillations/min. This shaking rate was used to overcome the effects of unstirred water layers on the kinetics of nutrient uptake as suggested by Lherminier and Alvarado (1981). Intestinal uptake of calcium was determined by incubating the tissues in 5 ml of oxygenated Tris-HCl buffer (140 mM NaCl, 6 mM KCl and 4 mM Tris buffer, pH 6.9) containing 1 mM calcium along with trace amounts of radiolabelled Ca-CaCl₂. Incubations were carried out for 30 min.

At the end of incubation period, the tissues were removed, gently blotted, weighed and the radioactivity taken up was determined after digesting the tissues in 20% (v/v) KOH as described by Robinson and Alvarado (1971). 5 ml of the scintillation fluid (containing 60 g naphthalene, 4 g PPO, 0.2 g POPOP, 20 ml ethylene glycol, 20 ml glacial acetic acid, 100 ml of absolute methanol and the final vol
raised to one litre with 1,4-dioxan) was added, the radioactivity was measured in a Beckman 8-liquid scintillation counter (with 90% efficiency). The tissue uptake was calculated and expressed as unit/g tissue, where one unit represents the μ moles of the substrate taken up per 5 min at 37°C.

Enzyme assays: Jejunal portion (20 cm) of the gut was removed, flushed with ice cold saline and everted. Enzymes were assayed in small intestine tissue homogenates prepared by homogenizing the intestines in 10 mM sodium maleate buffer, pH 6.8 and centrifuged at 1000 x g for 10 min. The supernatant was removed and used for enzyme determinations.

Sucrase, lactase, maltase, alkaline phosphatase and leucine amino peptidase were assayed both in the above supernatant and in the partially purified brush border membrane fragments.

Preparation of intestinal brush border membranes:
Intestinal brush border membranes were isolated and purified following the method of Schmitz et al. (1973).

A known weight of the small intestine (approximately 4-6 g) pooled from 3 animals was minced and homogenized in 1 mM Tris-50 mM mannitol buffer, pH 7.4 for 1.5 min in an electrically driven homogenizer at 4°C. 5% (w/v) homogenate was prepared and passed through two layers of cheese cloth. To the above filterate, anhydrous CaCl₂...
(enzyme grades quality) was added with constant stirring to a final concentration of 10 mM. After leaving in the cold for 10-15 min, the filtrate was centrifuged at 2000xg for 10 min in a Sorvall RC-5B centrifuge at 0-4 °C.

Pellet obtained from the previous step was discarded while the supernatant was recentrifuged at 43,000xg for 20 min. Supernatant obtained was discarded, pellet was suspended in 20 vols of 50 mM sodium maleate, pH 6.5 and re-centrifuged at 42,000xg for 20 min. The supernatant was again discarded and the precipitates were suspended in 50 mM sodium maleate buffer containing 0.02% sodium azide. The final membrane preparation obtained was similar to the P₂ fraction of Schmitz et al. (1973) and was used as such for various biochemical studies. The membrane was essentially free from other subcellular contaminants such as mitochondria, microsomes, basolateral membrane and DNA. There was a 5-9 fold membrane purification as determined by marker enzyme assays.

**Alkaline phosphatase assay in BBM**

Alkaline phosphatase activity was assayed according to the method of Bergmeyer (1963).

Principle: The activity of alkaline phosphatase was determined by using p-nitrophenyl phosphate which gets hydrolyzed by the action of the phosphatases in alkaline pH to p-nitrophenol. The yellow colour of p-nitrophenol was measured at 410 nm.
Reagents: a) Buffered substrate: 0.5 M glycine buffer containing $5.5 \times 10^{-3}$ M p-nitrophenyl phosphate, pH 10.5, 375 mg glycine, 10 mg MgCl$_2$ and 165 mg p-nitrophenyl phosphate (sodium salt) were dissolved in 42 ml of 0.1N NaOH and diluted to 100 ml with distilled water. pH was set to 10.5.

b) p-nitrophenol standard: 13.9 mg p-nitrophenol was dissolved in 100 ml distilled water (1 ml = 1μmole p-nitrophenol).

Procedure: One ml of the buffered substrate, p-nitrophenyl phosphate (sodium salt) was taken in different test tubes. Tubes were incubated for 5 min to attain a temp of 37 °C. 1 ml of suitably diluted enzyme was added and incubation was done for 15 min at 37 °C. After incubation, enzyme reaction was terminated by adding 5 ml of 0.1N NaOH to each tube, the optical density of p-nitrophenol liberated was read at 420 nm using Bausch and Lomb colorimeter. Blank and standard (p-nitrophenol concentration: 0.1-0.5μmole) were run simultaneously.

Leucine aminopeptidase assay (EC 3.4.1.1):

Leucine aminopeptidase activity was determined following the method of Goldberg and Ruttenberg (1958).
Principle: The enzymatic hydrolysis of the substrate L-leucyl-β-naphthylamide, liberates β-naphthylamine which is diazotised with sodium nitrite and converted to a blue coloured azo dye, which is read colorimetrically at 580 nm.

Reagents:

a) Buffered substrate: Dissolved 20 mg of L-leucyl-β-naphthylamide in 100 ml of 0.2M phosphate buffer, pH 7.0.

b) Sodium nitrite (0.1%): Dissolved 100 mg of NaN₃ in 100 ml of distilled water.

c) Ammonium sulfamate (0.5%): Dissolved 500 mg of ammonium sulfamate in 100 ml of distilled water.

d) Colouring reagent: 50 mg of N-l/naphthylene ethylene diamine dihydrochloride was dissolved in 100 ml of alcohol.

e) β-naphthylamine standard: Stock 10 mg/100 ml in 2N HCl. Working standard (100 g/ml): 1 ml of stock standard was diluted to 10 ml with 2N HCl.

Procedure: 0.5 ml of the buffered substrate was taken in different tubes. To each tube 0.1 ml of the enzyme was added, mixed well and incubated in a water bath for 30 min. at 37 °C. 0.5 ml of 2N HCl was then added to stop enzyme reaction. To each tube 1 ml of 0.1% NaNO₂ added, after waiting for exactly 3 min at room temp 0.5 ml of 0.5% ammonium sulfamate...
was added. After 3 min 2 ml of coloring reagent was added. Tubes were allowed to stand for 30 min and optical density was read at 580 nm in Bausch and Lomb colorimeter.

7. Blank and Standards (8-naphthylamine concentration 10-60μg) were also run simultaneously.

**Glucose-6-phosphatase assay** (EC 3.1.3.9):

The activity of glucose-6-phosphatase was determined by the method of Swanson (1955).

Reagents
- a) **Buffered substrate**: Dissolved 28.4 mg of monosodium salt of glucose-6-phosphate in 1 ml of 0.1 M citrate buffer, pH 6.5.
- b) **10% TCA**: Dissolved 10 g trichloroacetic acid in 100 ml distilled water.
- c) 2.5% ammonium molybdate
- d) ANSA reagent
- e) Standard - KH₂PO₄ (34.2 mg/100 ml distilled water).

Procedure: 0.1 ml of buffered substrate was taken in different test tubes. To this 0.1 ml of the supernatant was added, the tubes were incubated for 15 min at 37°C. After incubation, 2 ml of 10% TCA was added in each tube to stop the enzyme reaction. After incubation, tubes were centrifuged and 1 ml of the supernatant was taken for inorganic phosphorus assay by the method as described earlier.
Lactate dehydrogenase (LDH) assay (EC 1.1.1.27):

Lactate dehydrogenase was assayed according to the method of Wooton (1964). The pyruvate formed reacts with DNPH with the formation of pyruvate-2-4-dinitrophenyl hydrazine.

Reagents:

a) Sorenson glycine buffer (0.1M): 7.505g of glycine and 5.85g NaCl were dissolved in one litre of distilled water.

b) NAD - 5mg/ml

c) Buffered substrate: 5.8 ml of 60% sodium lactate (pH 10.0) was mixed in 125 ml of 0.1M glycine buffer and 75 ml of 0.1N NaOH.

d) 0.4N NaOH - 16g NaOH/litre.

e) Colouring reagent: 200 mg DNPH is dissolved in 1 N HCl. Then it was cooled and vol was made to one litre with 1N HCl.

f) Standard: 11 mg sodium pyruvate/100 ml of buffered substrate.

Procedure: One ml of buffered substrate was pipetted in different tubes. Blank and standard pyruvate were run simultaneously, 0.1 ml of the supernatant obtained from homogenate was added to each tube. Then 0.2 ml of freshly prepared NAD sol (5 mg/ml) was added, mixed well and incubated for 15 min at 37°C. 1 ml of DNPH reagent was added.
to each tube, mixed well and incubated for another 15 min at 37°C. 10 ml of 0.4 N NaOH was added to all the tubes, mixed and optical density was read at 440 nm.

Assay of disaccharidases

The activities of sucrase (EC 3.2.1.48), maltase (EC 3.2.1.20) and lactase (EC 1.10.3.2) were determined by measuring the D-glucose liberated from the respective sugars using glucose-oxidase peroxidase system of Dahlqvist (1964).

Principle: Glucose + $O_2^+$ $H_2O$ $\rightarrow$ GOD Gluconate + $H_2O_2$

$H_2O_2$ + colouring reagent $\rightarrow$ POD Coloured complex + $H_2O$

Where GOD is glucose oxidase; POD is peroxidase and colouring reagent is GOD reagent.

Reagents: a) Sodium maleate buffer, pH 6.0 (50 mM)

b) Sucrose sol (0.2 M): Dissolved 684 mg in 10 ml sod maleate buffer (50 mM).

c) Maltose sol (0.2 M): Dissolved 684 mg in 10 ml sod maleate buffer (50 mM).

d) Lactose sol (0.2M): Dissolved 684 mg in 10 ml sod maleate buffer (50 mM).

e) GOD Reagent i) Tris- Dissolved 12.1 g tris in 50 ml of distilled water and pH was adjusted to 7.2 with 1N HCl; Vol was made to 1 litre with distilled water. ii) p-OH-benzoic acid-0.276g/20 ml, of water pH is adjusted to 7.0 with 2N NaOH. iii) 4-amino
antipyrene-16.4 mg. iv) Peroxidase - 1 mg.
v) Glucose oxidase - 0.2 ml (2 mg)
and the total vol was made to 200 ml.
f) Standard Glucose: Stock glucose standard was prepared by dissolving 180 mg of analytical grade glucose in 10 ml distilled water. Working glucose standard was prepared by diluting 0.1 ml of the stock glucose sol to 10 ml with distilled water, this was equivalent to 1 mM and 0.1 ml of it contained 0.1 µmol of glucose.

Procedure: 0.1 ml of the substrates, sucrose or lactose or maltose was taken in different tubes. To this 0.3 ml of sodium maleate buffer (50 mM) was added, and thoroughly mixed. 0.1 ml of suitably diluted enzyme was added and tubes were incubated for 30 min at 37°C. After incubation, 4 ml of GOD reagent was added and kept for 30 min. at 37°C and optical density was measured at 500 nm. and standard glucose samples (0.1-0.5 µmole) were run simultaneously.

EXTRACTION OF BRUSH BORDER MICROVILLUS MEMBRANE LIPIDS

Membrane lipids were extracted by the method of Folch et al. (1957) as given below.

1. 1.5 ml of the brush border preparation (10-15 mg protein) was pipetted in to conical flask containing 50-60 ml of chloroform:ethanol (2:1) mixture and thoroughly agitated. The mixture was left for 15 min at 55°C.
2. The contents were filtered in a graduated cylinder and the residue left on the filter paper was washed three times with 10 ml of chloroform:methanol, 2:1 (v/v).

3. To the combined filtrates 0.9% KCl (20% of the total vol of extract) was added and vigorously mixed. The mixture was allowed to stand in cold so as to separate the aqueous and chloroform (lipid) layers.

4. Upper aqueous phase was removed by aspiration with a pasteur pipette and kept for the estimation of ganglioside-bound sialic acid, while the lower layer was washed three times with 5 ml mixture of chloroform:methanol:potassium chloride (0.9%), 4:48:47 (v/v).

5. The washed lower layer was transferred to a round bottomed flask and evaporated to dryness under vacuum at a temp below 45°C.

6. To the residue 5 ml of chloroform:methanol, 2:1 (v/v) containing 4% water (chloroform:methanol:water, 64:32:4:, v/v) was added and evaporated to dryness. The process was repeated two times.

7. Dried lipid was washed with chloroform and filtered through Whatman No 1 Filter paper and the filtrate was dried.

8. The vol was made to 3 ml with chloroform and lipid extract was preserved at -20°C.
Stream of nitrogen gas was passed through the round bottomed flask at steps 5, 6 and 7 if any water droplets were seen adhering to sides of the flask.

Estimation of total lipids, phospholipids and cholesterol was done according to the methods already described for tissue lipid extracts.

Ganglioside-bound sialic acid in the BBM was estimated by the method of Warren (1959) as described earlier.

Protein estimation in BBM and Intestinal tissue homogenates was done according to the modified Lowry method, the SDS-Lowry method of Lees and Paxman (1972), using bovine serum albumin (BSA) as standard.

Reagents:
- a) 5% sodium dodecyl sulphate (SDS) in 0.5N NaOH
- b) Copper tartarate sol: i) 50 ml of each of 2% Na tartrate and 1% CuSO₄ ii) 2.5 ml of 1 N NaOH.
- c) Folin Phenol reagent 1N-Dilute the 2N reagent 1:1 with distilled water
- d) 2% Na₂CO₃
- e) Standard: BSA - 2 mg/ml H₂O

Procedure: Added 0.5 ml of 5% SDS in 0.5 N NaOH to samples, standards and Blanks and dissolved thoroughly. Added 2.5 ml copper carbonate sol (mixed 50 ml of 2% Na₂CO₃ and 1 ml of copper tartarate sol). Added 0.25 ml Folin reagent and mixed immediately. After 45 min read at 750 nm.
STATISTICAL ANALYSIS

Student's 't' test to see whether the difference between the mean value of two groups (mean value with S.D. of control and experimental groups) is statistically significant or not, the student's 't' test was applied. The calculated values of 't' were compared with the theoretical value taken from the standard tables and the corresponding 'p' values were determined. The value of 't' was calculated in the following manner:

\[
S = \sqrt{\frac{(n_1-1)SD_1^2 + (n_2-1)SD_2^2}{n_1 + n_2 - 2}}
\]

\[
t = \frac{X_1 - X_2}{S} \sqrt{\frac{n_1n_2}{n_1 + n_2}}
\]

where

- \( n_1 \) = total no. of readings of control group
- \( n_2 \) = total number of readings of experimental or treated groups
- \( SD_1 \) = standard deviation of control group
- \( SD_2 \) = standard deviation of treated group
- \( X_1 \) = mean of control animals
- \( X_2 \) = mean of treated animals
- \( S \) = pooled standard deviation
- \( n_1 + n_2 - 2 \) = degrees of freedom