CHAPTER - IV

MATERIALS AND METHODS
CHEMICALS:

Chemicals used in these studies were of analytical grade, obtained either from E.Merck (India) Ltd. or Glindia Ltd. Bovine serum albumin, sucrose, lactose, 4-aminoantipyrine, glucose oxidase (Type V), horseradish peroxidase, p-nitroaniline, L-\textalpha -alanine-p-nitroanilide, \textgamma-glutamyl-p-nitroanilide, N-acetylneuraminic acid, L-fucose, D-galactose, D-glucose, D-glucosamine, cholesterol, digitonin, \textit{Ulex europeus} agglutinin, wheat germ agglutinin, peanut agglutinin, dithiothreitol, SDS molecular weight markers, viz. carbonic anhydrase, egg albumin, 8-galactosidase, myosin and phosphorylase B were purchased from Sigma Chemical Company, U.S.A.

The radiolabelled compounds, $^{14}$C-\textit{U}-D-glucosamine hydrochloride (100 mCi/mmol), $^{14}$C-\textit{U}-D-mannose (166 mCi/mmol), [2-$^{14}$C]-acetate (54.4 mCi/mmol) and $^{125}$I as sodium iodide were purchased from Radioisotope Division, Bhabha Atomic Research Centre, Trombay, Bombay. L-[5, 6-3H]-Fucose (45 Ci/mmol) was obtained from Radiochemical Centre Amersham, U.K.

Nutritional cellulose was purchased from CSIR Centre For Biochemicals, Delhi while salt mixture (U.S.P.XIV) was from Sisco Research Laboratories Pvt. Ltd., Bombay.

EXPERIMENTAL DESIGN:

Adult male mice (2 to 3 months-old) of LACA strain obtained from the Central Animal House of the University were used. The animals were fed different diets for 21 days. The composition of various diets is given in Table 1. All the diets were isocaloric. Control animals
Table 1: Composition of various diets fed to mice.

<table>
<thead>
<tr>
<th>Dietary constituent</th>
<th>Control</th>
<th>Low protein</th>
<th>High protein</th>
<th>High fat</th>
<th>Fat-free</th>
</tr>
</thead>
<tbody>
<tr>
<td>Casein (Vitamin free)</td>
<td>18</td>
<td>8</td>
<td>30</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Starch (Potato)</td>
<td>25</td>
<td>30.5</td>
<td>19</td>
<td>7</td>
<td>36.5</td>
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<tr>
<td>Sucrose</td>
<td>25</td>
<td>30.5</td>
<td>19</td>
<td>7</td>
<td>36.5</td>
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<tr>
<td>Corn Oil</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>26</td>
<td>-</td>
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<tr>
<td>Cellulose (Nutritional)</td>
<td>14</td>
<td>13</td>
<td>14</td>
<td>34</td>
<td>1</td>
</tr>
<tr>
<td>Salt mixture (U.S.P. XIV)</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
</tbody>
</table>

100 g of each diet provides 385 calories.
were pair-fed. Animals in the normal-fed group were given a diet containing 18% protein ad libitum. There were 8-10 animals for each group and each dietary regimen was given to at least four such groups. Body weights of animals in various experimental groups were recorded every third day. A record of the daily consumption of diet by animals in each group was also kept. The time of sacrifice was always maintained between 9.00 a.m. and 10.00 a.m. to avoid rhythmic changes.

The animals were sacrificed under light ether anaesthesia. Starting from the ligament of Treitz, entire small intestine was removed and thoroughly washed with ice-cold saline. The first two-third portion of the small intestine was used for various biochemical studies.

ESTIMATION OF NUCLEIC ACIDS (DNA AND RNA):

Method of Munro and Fleck (1966) was used to extract RNA and DNA from homogenate prepared in 50 mM sodium maleate buffer, pH 6.8.

Reagents:

1) 2.2 N Perchloric acid.
2) 1.0 N Perchloric acid.
3) 0.22 N Perchloric acid.
4) 0.3 N KOH.

Procedure:

1. To 0.1 ml of the sample, 2.5 ml of 0.22 N cold perchloric acid was added and tubes were kept in ice bath for 10 min.

2. After centrifugation of tubes for 15 min at 3000 rpm, supernatant was discarded and the tubes kept inverted over folds of dry filter paper for 5-10 min.
3. 3.2 ml of 0.3 N KOH was added and tubes incubated at 37°C for 1 hr.

4. 0.8 ml of 2.2 N cold perchloric acid was added and the samples centrifuged at 1000 rpm for 5-10 min.

5. The supernatant (containing RNA) was carefully removed and the absorbance read at 260 nm (0.1 O.D. unit = 128 µg RNA/ml in original solution).

6. The residue was further washed twice with 5.0 ml of 0.1 N HClO₄.

7. It was treated with 1.0 N HClO₄, kept at 80°C for 1 hr and centrifuged.

8. The supernatant (containing DNA) was removed and its absorbance read at 260 nm (0.1 O.D. unit = 4.5 µg DNA/ml in original solution).

CELLULAR ENZYMES:

Assay of Transaminases: Glutamate-oxaloacetate transaminase (GOT, EC 2.6.1.1) and glutamate-pyruvate transaminase (GPT, EC 2.6.1.2) in intestinal homogenates, prepared in 50 mM sodium maleate buffer, pH 6.8, were estimated according to the method of Reitman and Frankel (1957). GPT produces pyruvate while the catalytic action of GOT produces oxaloacetate which decarboxylates spontaneously to pyruvate. Pyruvic acid so formed reacts with 2,4-dinitrophenylhydrazine in alkaline medium to give a brown colored, 2,4-dinitrophenylhydrazone, which absorbs maximally at 510 nm.

Reagents:

i) Standard pyruvate solution: Stock pyruvate solution (20 mM) was prepared by dissolving 220 mg sodium pyruvate in 100 ml of 0.1 M
phosphate buffer, pH 7.4. Working standard solution (1 mM) was made by diluting 1.0 ml of the stock solution to 20 ml with phosphate buffer.

ii) Phosphate buffer (0.1 M, pH 7.4): 5.65 g of dry anhydrous disodium hydrogen phosphate and 1.35 g of dry anhydrous potassium dihydrogen phosphate were dissolved in 500 ml of water to get 0.1 M phosphate buffer with pH 7.4.

iii) GOT substrate (200 mM DL-aspartic acid, 2 mM α-ketoglutarate): 2.66 g of DL-aspartic acid and 0.029 g of α-ketoglutaric acid were dissolved in 50-60 ml of phosphate buffer; pH was adjusted to 7.4 with 1 N NaOH. Final volume was made to 100 ml with phosphate buffer.

iv) GPT substrate (200 mM alanine, 2 mM α-ketoglutarate): 1.8 g of alanine and 0.029 g of α-ketoglutaric acid were dissolved in 50-60 ml of phosphate buffer; pH was adjusted to 7.4 with 1 N NaOH and final volume was made to 100 ml with phosphate buffer.

v) 2,4-Dinitrophenylhydrazine (1 mM): 19.8 mg of DNPH was dissolved in 10 ml of conc. HCl and final volume made to 100 ml with water. The solution was stored in a brown bottle at room temperature.

vi) 0.4 N NaOH.

Procedure:

1. 0.4 ml of GOT or GPT substrate was taken in different tubes.
2. The tubes were kept at 37°C for 5 min for pre-equilibration.
3. 0.1 ml of sample was added and incubated at 37°C for 60 min in case of GOT and for 30 min for GPT assay.
4. 0.5 ml of DNPH was added to each tube to stop the reaction and the samples were mixed well.

5. After 20 min, 5.0 ml of 0.4 N NaOH was added and the tubes allowed to stand at room temperature for 10 min. O.D. was measured at 510 nm.

Assay of Lactate Dehydrogenase (LDH, EC 1.1.1.27): LDH in intestinal homogenates was assayed by the method of Vassault (1983). In this method, the rate of decrease of extinction at 340 nm (caused by the oxidation of NADH by excess pyruvate in the presence of LDH) is measured. 

\[
\text{Pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{L-Lactate + NAD}^+
\]

Reagents:

i) Tris-NaCl (81.3 mM-203.3 mM, pH 7.2): 4.924 g of Tris and 5.94 g of NaCl were dissolved in 200-300 ml of water; pH was adjusted to 7.2 with 2 N HCl and final volume made to 500 ml with water.

ii) Tris-NaCl-NADH (81.3 mM-203.3 mM-0.244 M): 8.5 mg of NADH was dissolved in 50 ml of solution (i).

iii) Tris-NaCl-Pyruvate (81.3 mM-203.3 mM-9.76 mM): 10.7 mg of pyruvate was dissolved in 10 ml of solution (i).

Procedure:

1. To 2.5 ml of solution (ii) was added 0.05 ml of the enzyme sample in different tubes.

2. The tubes were incubated at 30°C for 5 min for pre-equilibration.

3. 0.5 ml of solution (iii) was added to each sample.

4. The decrease in absorbance at 340 nm vs the blank was recorded for 3-4 min at 30 sec intervals.
5. The decrease in absorbance over the linear part of the curve was measured for calculations.

**PREPARATION OF MICROVILLUS MEMBRANES (MVM):**

MVM were isolated and purified following the method of Kessler *et al.* (1978). Except otherwise mentioned, all procedures were carried out at 0° - 4°C. Thoroughly washed intestinal segments were evverted and stored at -20°C, if not used immediately. The tissue was homogenized (10%, w/v) in 50 mM mannitol-2 mM Tris-HCl (pH 7.2) and filtered through two layers of cheese cloth. Anhydrous CaCl₂ was added, with constant stirring, to the homogenate to a final concentration of 10 mM. After 15 min, the contents were centrifuged at 2000 x g for 10 min in REMI C-24 centrifuge, the pellet (P₁) was discarded and the supernatant was recentrifuged at 42000 x g for 15 min in IEC ultracentrifuge. The pellet was suspended in 20 vol of 50 mM sodium maleate, pH 6.8 and recentrifuged at 42000 x g for 15 min. The pellet (P₂) obtained was suspended in the same maleate buffer. Purity of the membranes was assessed by assaying the brush border sucrase and alkaline phosphatase (AP) activities. The final membrane preparation exhibited 10 to 15-fold enrichment of sucrase and AP activities over the crude homogenate. This membrane preparation was used for various biochemical studies.

**Dry Weight of MVM:** Small 5 ml beakers were taken and their initial weights recorded. To each beaker, 1.0 to 2.0 ml of MVM sample was added. The beakers were kept in an oven, at 100°C, for 24 hr for the membranes to dry. The beakers were weighed next day and the procedure was repeated till a constant weight of beakers was obtained.
MVM dry weight was determined by subtracting initial weight of beakers from their final weight.

**PREPARATION OF CELL FRACTIONS FROM SMALL INTESTINE:**

Epithelial cells were isolated following the modified method of Stern (1966) as described by Weiser (1973a). The intestine was rinsed thoroughly with a solution containing 0.154 M NaCl and 1 mM dithiothreitol (DTT) to remove the mucus. It was filled with solution A (see flow diagram) and incubated at 37°C for 15 min. Citrate present in solution A dissociated the cells partially. After discarding solution A, the intestine was filled with solution B containing 1.5 mM ethylene-diamine tetraacetic acid (EDTA) and phosphate-buffered saline (PBS). Tissues were incubated at 37°C for different intervals of time as shown in Fig 3. By a series of incubations and washings of intestinal loops, sequential fractions of isolated epithelial cells were obtained that appeared to define a gradient of cells from villus-tip to lower villus and crypt-base areas which was evident by analysis of brush border sucrase and AP activities in different cell fractions. The process not only separated crypt cells from villus cells but appeared also to separate cells as a villus to crypt gradient. Sequentially isolated cells were pooled into three fractions: 1-3, 4-6 and 7-9 representing, respectively, villus, mid-villus and crypt cells.

**ESTIMATION OF PROTEIN:**

Protein was assayed by the method of Lowry et al. (1951).
Fig. 3. Flow diagram for the preparation of epithelial cells.

Small Intestine

\[
\text{Rinsed thoroughly with 0.154 M NaCl, 1 mM DTT} \\
\text{Filled with solution A} \\
\text{37°C, 15 min} \\
\text{Solution discarded} \\
\text{Intestine filled with solution B} \\
\text{37°C} \\
\text{Solutions in plastic, conical centrifuge tubes} \\
\text{900 x g, 5 min} \\
\text{Cells suspended in PBS (no Ca\textsuperscript{2+}, Mg\textsuperscript{2+})} \\
\text{900 x g, 5 min} \\
\text{Final suspension of cells in 50 mM sodium maleate buffer, pH 6.8}
\]

Composition of Solution A

- KCl 1.5 mM
- NaCl 96 mM
- Sodium citrate 27 mM
- \(\text{KH}_2\text{PO}_4\) 8 mM
- \(\text{Na}_2\text{HPO}_4\) 8 mM
- pH 7.3

Composition of Solution B

- PBS (no Ca\textsuperscript{2+} or Mg\textsuperscript{2+})
- EDTA 1.5 mM
- DTT 0.5 mM

<table>
<thead>
<tr>
<th>Fraction number</th>
<th>Incubation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4</td>
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<tr>
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<td>8</td>
<td>10</td>
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<td>9</td>
<td>15</td>
</tr>
</tbody>
</table>
Reagents:
(i) Standard protein solution: 20 mg of BSA was dissolved carefully, to avoid foaming, in 100 ml of water and stored at 4°C.
(ii) Folin and Ciocalteu's reagent.
(iii) Sodium carbonate solution (2% in 0.1 N NaOH).
(iv) Copper sulfate solution (1%).
(v) Sodium potassium tartrate solution (2%).
(vi) Lowry's reagent (Reagent C) was prepared by mixing 49.0 ml of (iii), 0.5 ml of (iv) and 0.5 ml of (v) just before use.

Procedure:
1. To 0.1 ml of the appropriately diluted sample, 0.9 ml of water and 5.0 ml of alkaline reagent (vi) was added.
2. It was mixed well and allowed to stand for 10 min.
3. 0.5 ml of Folin-Ciocalteu's reagent (1:1, v/v) was added.
4. The contents were vigorously shaken and allowed to stand for 30 min at room temperature.
5. O.D. was measured at 720 nm.
6. A blank and appropriate standard samples were run simultaneously.

Assay of Alkaline Phosphatase (AP, EC 3.1.3.1): AP activity was assayed according to Bergmeyer (1963) using p-nitrophenylphosphate as a substrate to yield p-nitrophenol having $\lambda_{\text{max}}$ at 420 nm. The phosphatase activity is directly proportional to the amount of p-nitrophenol liberated per unit time.
Reagents:

(i) p-Nitrophenol standard solution (0.1 μmol/0.1 ml): 13.9 mg p-nitrophenol was dissolved in 100 ml water.

(ii) Buffered substrate (0.5 M Glycine-NaOH buffer, 5.5 X 10^{-3} M p-nitrophenylphosphate, pH 10.5): 375 mg glycine, 10 mg MgCl\textsubscript{2} and 165 mg p-nitrophenylphosphate (sodium salt) were dissolved in 42 ml of 0.1 N NaOH, pH was adjusted to 10.5 with NaOH and final volume made to 100 ml with water.

(iii) 0.1 N NaOH.

Procedure:

1. 1.0 ml of buffered substrate was taken in different tubes.
2. The tubes were incubated at 37°C for 5 min for pre-equilibration.
3. 0.1 ml of sample was added and incubated for 15 min at 37°C.
4. After incubation, 5.0 ml of 0.1 N NaOH was added to stop the reaction.
5. The p-nitrophenol liberated was measured at 420 nm.

Assay of Disaccharidases: Sucrase (EC 3.2.1.48) and lactase (EC 3.2.1.23) activities were assayed by measuring D-glucose liberated from the respective disaccharides, using a glucose-oxidase-peroxidase system. The method used was a modification of procedure described by Dahlqvist (1964).

Reagents:

1. Standard glucose solution: Stock glucose standard was prepared by dissolving 180 mg pure D-glucose in 10 ml water. Working
glucose standard was made by diluting 0.1 ml of the stock solution to 10 ml with water (0.1 ml of working glucose standard contained 0.1 μmol of glucose).

(ii) Sodium maleate buffer, 50 mM (pH 6.8): 44.4 ml of 0.2 M NaOH was added to 50 ml of 0.2 M maleic acid and volume made to 200 ml with water.

(iii) Sucrose solution (0.2 M): 684 mg of sucrose was dissolved in 10 ml of 50 mM sodium maleate buffer.

(iv) Lactose solution (0.2 M): 684 mg of lactose was dissolved in 10 ml of 50 mM sodium maleate buffer.

(v) Glucose-oxidase-peroxidase reagent:

a) Tris buffer (1 M): 60.5 g Tris was dissolved in 400 ml of water, pH adjusted to 7.2 with 1 N HCl and final volume made to 500 ml with water.

b) p-Hydroxybenzoic acid solution (0.1 M): 1.38 g of p-hydroxybenzoic acid was dissolved in 25 ml of water, pH adjusted to 7.0 with 2 N NaOH and final volume made to 100 ml with water.

c) 82 mg of 4-aminoantipyrine and 5 mg of peroxidase were dissolved in water. 1.0 ml of glucose-oxidase (Sigma Type V) was added to it.

(a), (b) and (c) were mixed and the final volume made to 1000 ml with water. In the final solution, the concentration of Tris was 0.5 M and of p-hydroxybenzoic acid 0.01 M.
Procedure:

1. To 0.1 ml of the substrate (sucrose or lactose), 0.3 ml of sodium maleate buffer (pH 6.8) was added.
2. Tubes were kept in a water bath at 37°C for 5 min.
3. 0.1 ml of sample was added and incubation was done for 30 min at 37°C.
4. 4.0 ml of glucose-oxidase-peroxidase reagent was added to each tube and allowed to stand at 37°C for 60 min. O.D. was measured at 500 nm.

Assay of Leucineaminopeptidase (LAP, EC 3.4.11.1): LAP activity was determined by the method of Goldbarg and Rutenburg (1958) with some modifications. The enzymatic hydrolysis of the substrate L-leucine-p-nitroanilide liberates p-nitroaniline which is diazotized with sodium nitrite, converted to an azo dye and pink colored complex formed absorbs maximally at 540 nm.

Reagents:

(i) Standard p-nitroaniline solution: Stock solution (1 µmol/ml) was made by dissolving 13.8 mg of p-nitroaniline in 100 ml water and it was diluted five times with water to get the working solution (0.2 µmol/ml).

(ii) Buffered substrate: 20 mg of L-leucine-p-nitroanilide was dissolved in 50 ml of water and then 50 ml of 0.2 M phosphate buffer (pH 7.0) was added.

(iii) Sodium nitrite (0.1%).

(iv) Acetic acid (10%).
(v) Ammonium sulfamate (1%).

(vi) Coloring reagent (0.05%): 50 mg N-(1-naphthyl)-ethylene diamine dihydrochloride was dissolved in 100 ml of water.

Procedure:
1. 0.5 ml of the buffered substrate was taken in different tubes.
2. The tubes were preincubated at 37°C for 5 min for equilibration.
3. 0.1 ml of sample was added and incubated for 10 min at 37°C.
4. 2.0 ml of 10% acetic acid was added to terminate the reaction.
5. 1.0 ml of sodium nitrite (0.1%) was added to each tube.
6. 1.0 ml of ammonium sulfamate (1%) was added.
7. 1.0 ml of the coloring reagent (vi) was added and O.D. was read at 540 nm.

Assay of γ-Glutamyltranspeptidase (γ-GTP, EC 2.3.2.2): The method of Naftalin et al. (1969) was used to assay the γ-GTP activity. The enzymatic transfer of γ-glutamyl group from γ-glutamyl-p-nitroanilide to glycyl-glycine liberates p-nitroaniline which is diazotized with sodium nitrite, converted to an azo dye and a pink colored complex is formed, which absorbs maximally at 540 nm.

Reagents:

(i) Standard p-nitroaniline solution: Stock (1 μmol/ml), 13.8 mg/100 ml; working standard (0.2 μmol/ml).

(ii) 0.1 M glycyl-glycine in 1M Tris (pH 7.8).

(iii) 22 mg γ-glutamyl-p-nitroanilide was dissolved in 10 ml of 0.1 M glycyl-glycine-Tris (ii) and heated up to 56°C in a water bath for
5-10 min. It was shaken vigorously to dissolve, cooled and filtered. Higher temperature and prolonged heating was avoided to prevent the substrate from being hydrolysed.

(iv) Sodium nitrite (0.1%).
(v) Acetic acid (10%).
(vi) Ammonium sulfamate (1%).
(vii) Coloring reagent (0.05%): 50 mg N-(1-naphthyl)-ethylenediamine dihydrochloride was dissolved in 100 ml distilled water.

Procedure:
1. 0.5 ml of buffered substrate was taken in different tubes.
2. The tubes were preincubated for 5 min at 37°C for equilibration.
3. 0.1 ml of sample was added and incubated for 10 min at 37°C.
4. 2.0 ml of 10% acetic acid was added to terminate the reaction.
5. 1.0 ml of sodium nitrite (0.1%) was added to each tube.
6. 1.0 ml of ammonium sulfamate (1%) was added.
7. 1.0 ml of the coloring reagent was added and the O.D. was read at 540 nm.

Assay of p-Nitrophenyl-glycosidases: Glycosidases (p-nitrophenyl-β-D glucosidase and p-nitrophenyl-β-D-galactosidase) were assayed by the method of Agrawal and Bahl (1968). p-Nitrophenyl (PNP)-α- or β-D-glycopyranoside is hydrolyzed by the appropriate glycosidase liberating p-nitrophenol and the monosaccharide. The enzyme activity is followed simply by the estimation of p-nitrophenol which forms a yellow chromogen under alkaline condition with a λ_{max} of 400 nm.
Reagents:

i) Standard p-nitrophenol solution (1 μmol/ml): 13.9 mg p-nitrophenol was dissolved in 100 ml of water.

ii) Sodium acetate buffer, pH 5.0 (0.1 M):

a) 0.4 M acetic acid: 2.3 ml acetic acid was diluted to 100 ml with water.

b) 0.4 M sodium acetate: 5.44 g of sodium acetate (trihydrate salt) was dissolved in 100 ml of water.

14.8 ml of (a) was mixed with 35.2 ml of (b) and the final volume made to 100 ml with water.

iii) 0.2 M Na₂CO₃: 10.6 g of Na₂CO₃ was dissolved in 50 ml of water.

iv) Substrates PNP-β-D-glucopyranoside and PNP-β-D-galactopyranoside (10 mM): 30.12 mg of substrate was dissolved in 10 ml of sodium acetate buffer.

Procedure:

1. 0.6 ml of substrate PNP-β-D-glucopyranoside (for β-D-glucosidase assay) or PNP-β-D-galactopyranoside (for β-D-galactosidase assay) was taken in different tubes.

2. The tubes were incubated at 37°C for 5 min for pre-equilibration.

3. Sample containing 150-250 μg protein was added to each tube and incubated at 37°C for 10 min.

4. The reaction was stopped by the addition of 2.0 ml of 0.2 M Na₂CO₃.

5. The yellow color formed as a result of liberated p-nitrophenol was measured at 400 nm.
Kinetic Studies with AP, Sucrase and LAP:

(i) **Effect of substrate concentration:** To study the effect of substrate concentration on AP, the different concentrations of substrate p-nitrophenylphosphate used were 0.889, 1.778, 2.667, 3.556 and 4.445 mM. Sucrase activity was determined at 8, 16, 24, 32 and 40 mM concentration of sucrose. Similarly, LAP activity was measured at 0.08, 0.16, 0.24, 0.32 and 0.40 mM concentration of L-leucine-p-nitroanilide. Kinetic parameters ($K_m$, $V_{max}$ and correlation coefficient) of the enzymes were calculated by feeding the raw data into programmable T160 calculator adapted for Lineweaver-Burke equation (1934).

(ii) **Effect of temperature:** The effect of temperature on various enzymes was determined by assaying the enzyme activities at 6°C, 12°C, 18°C, 24°C, 30°C, 37°C and 42°C. Arrhenius analysis was carried out using the formula

$$\ln V = -\frac{E}{RT} + C$$

where $R = 1.98$ Cal/mole, $T$ is the absolute temperature, $E$ is the energy of activation, $V$ is the specific activity of the enzyme and $C$ is the constant. The data were fed to the calculator adapted for the Arrhenius equation to calculate the value of $E_a$ and correlation coefficient.

(iii) **Heat inactivation:** Heat inactivation pattern of AP and sucrase was studied by heating the enzyme preparations at 50°C for 0, 5, 10, 15, 20 and 30 min and then assaying the enzyme activities at 37°C. LAP activity was determined after heating the enzyme at 60°C for various time intervals. The data were plotted as the enzyme specific activity ($V$) vs time (in min) and T/2 (the time in which the enzyme is half inactivated) was determined from the graph.
Expression of Enzyme Activities: All enzyme activities were calculated in enzyme units, defined as the amount of the enzyme required to hydrolyze 1 μmol of the substrate per min under standard assay conditions. Results were expressed as units/mg protein. Appropriate blank and standard samples were run simultaneously for each enzyme assay.

Estimation of Total Hexoses: Total hexoses were estimated by the method of Roe (1955) using Anthrone reaction. This is based on the formation of furfural derivatives, in conc. $\text{H}_2\text{SO}_4$, which react with anthrone to form a blue-green color.

Reagents:

(i) Standard galactose solution: Stock galactose standard was prepared by dissolving 10 mg of galactose in 1.0 ml of water. Working galactose standard was made by diluting 0.2 ml of the stock solution to 10 ml with water yielding a final concentration of 0.2 mg/ml.

(ii) Anthrone reagent: 720 ml of conc. $\text{H}_2\text{SO}_4$ was added to 280 ml of water; 500 mg of anthrone and 10 g of thiourea were added to this mixture, while it was warm. The solution was thoroughly mixed, cooled and stored in a refrigerator.

Procedure:

1. Sample containing 300-400 μg of protein was taken and the volume was made to 1.0 ml with water.

2. 5.0 ml of cold anthrone reagent was added and shaken vigorously.
3. Tubes, covered with glass beads, were heated in a boiling water bath for 15 min.

4. After cooling the tubes for 20 min, O.D. was read at 620 nm.

**Estimation of Total Hexosamines:** Total hexosamines were determined using Elson-Morgan reaction after initial acid hydrolysis of the protein, following the method of Gatt and Berman (1966). The reaction involves treatment with acetylacetone followed by the reaction of the resulting chromogen with p-dimethylaminobenzaldehyde (Ehrlich reagent) in acid solution to produce red color which absorbs at 530 nm. 2-Methylpyrrole is the major chromogen produced.

**Reagents:**

(i) Standard glucosamine solution: Stock glucosamine standard was prepared by dissolving 20 mg of D-GlcNH₂ in 2.0 ml of water. Working standard was made by diluting 0.05 ml of stock solution to 10 ml with water giving a final concentration of 50 µg/ml.

(ii) 6 N HCl.

(iii) 2 M Na₂CO₃: 10.6 g of Na₂CO₃ was dissolved in 50 ml of water.

(iv) 1.5 M Na₂CO₃: 7.95 g of Na₂CO₃ was dissolved in 50 ml of water.

(v) Ethanol (95%).

(vi) Acetylacetone (2%): 0.2 ml of acetylacetone was diluted to 10 ml with 1.5 M Na₂CO₃. Freshly prepared solution was used.

(vii) Ehrlich reagent: 333 mg of p-dimethylaminobenzaldehyde was dissolved in 5.0 ml of ethanol and 5.0 ml of conc. HCl. The reagent was prepared fresh for each estimation.
Procedure:

1. Sample containing 100-150 μg of protein was taken and the volume was made to 0.4 ml with water.

2. 0.2 ml of 6 N HCl was added and the samples, in tightly capped tubes, were kept at 100°C for 10 hr.

3. Samples were neutralized by adding 0.4 ml of 2 M Na₂CO₃ and were shaken vigorously.

4. 0.5 ml of reagent (vi) was added and the tubes were kept in a boiling water bath for 20 min.

5. After cooling the tubes, 1.0 ml of ethanol and 0.5 ml of Ehrlich reagent was added with vigorous shaking.

6. O.D. was read at 530 nm after 5 min.

Estimation of Fucose: Fucose was estimated according to the method of Dische and Shettles (1948). Methylpentoses react with -SH compounds in presence of sulfuric acid and give yellowish-brown color with an absorbance maximum at 396 nm. The hexoses also show a yellow color under these conditions but because of the symmetric shape of the absorption curve of hexoses with regard to the peak at 415 nm, the absorption at 430 nm was found to be equal to that at 396 nm. The difference in O.D. at 396 nm and 430 nm is, therefore, strictly proportional to the concentration of the methylpentoses.

Reagents:

(i) Standard fucose solution: 20 mg of fucose was dissolved in 100 ml of water.
(ii) 0.5 N $\text{H}_2\text{SO}_4$.
(iii) $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ mixture (6:1, v/v).
(iv) Cysteine-hydrochloride solution (3%): Freshly prepared solution was used.

Procedure:

1. 0.4 ml sample containing 400-800 µg protein was mixed with 0.1 ml of 0.5 N $\text{H}_2\text{SO}_4$ and kept at 100°C for 4 hr in sealed glass tubes.
2. After centrifugation at 3000 x g (epithelial cell sample) or at 10000 x g (MVM), to 0.4 ml of supernatant, water was added making final volume 1.0 ml.
3. The tubes were cooled for 10 min at 4°C.
4. 4.5 ml of ice-cold $\text{H}_2\text{SO}_4/\text{H}_2\text{O}$ (6:1, v/v) mixture was added and aliquots were thoroughly mixed.
5. The tubes were kept in a boiling water bath for 10 min and then cooled.
6. To each tube, 0.1 ml of freshly prepared 3% cysteine-hydrochloride reagent was added.
7. After 90 min, O.D. was read at 396 nm and 430 nm. Difference in the O.D. (O.D. 396 - O.D. 430) gave the reading for fucose.

Estimation of Sialic Acid: Sialic acid was estimated by the method of Skoza and Mohos (1976). Glycosidically-bound sialic acid is released and subjected to periodic acid/thiobarbituric acid reaction, leading to the formation of a prechromogen, $\beta$-formylpyruvic acid which subsequently forms a red chromogen with an absorbance maximum at 549 nm. Extraction of this chromophore with dimethyl sulfoxide (DMSO) forms a chromophore which is stable for several days.
Reagents:

(i) Standard NANA solution: 15 mg of NANA was dissolved in 100 ml of water.

(ii) 0.5 N H$_2$SO$_4$.

(iii) Sodium periodate (0.025 M): 535 mg of NaIO$_4$ was dissolved in 12.5 ml of 1.0 N H$_2$SO$_4$ and volume made to 100 ml with water.

(iv) Sodium arsenite (2% in 0.5 N HCl): 2 g of sodium arsenite was dissolved in 25 ml of 2 N HCl and volume made to 100 ml with water. Only the clear supernatant was used.

(v) Thiobarbituric acid (TBA, 0.1 M): 1.44 g of TBA was dissolved in 50-60 ml water, pH was adjusted to 9.2 with 2 N NaOH and volume made to 100 ml with water.

(vi) DMSO.

Procedure:

1. 0.4 ml sample containing 200-400 μg protein was hydrolyzed with 0.1 ml of 0.5 N H$_2$SO$_4$ at 80°C for 1 hr.

2. After centrifugation at 10000 x g, 0.4 ml of supernatant was mixed with 0.4 ml of water.

3. 0.2 ml sodium periodate reagent was added and the tubes were kept at 37°C for 30 min.

4. 0.2 ml sodium arsenite solution was added and the tubes were shaken till the yellow-brown color disappeared.

5. After adding 2.0 ml of TBA to each tube, samples were heated in a boiling water bath for 8 min.
6. The tubes were cooled and 1.0 ml DMSO was added to each. The color developed was read at 540 nm.

Appropriate blank and standard samples were run simultaneously for each estimation.

LECTIN BINDING STUDIES:

Labelling of Lectins with $^{125}$I: Various lectins viz. wheat germ agglutinin (WGA), Ulex europeus agglutinin, (UEA), and peanut agglutinin (PNA) were labelled with Na$^{125}$I using chloramine T method (Matrisian et al., 1985). 0.2 ml of the reaction mixture containing 100 µg lectin, 0.3 mCi Na$^{125}$I, 500 µg chloramine T and 0.2 M phosphate buffer, pH 7.3, was incubated at 30°C for 30 sec. The reaction was terminated by adding 0.1 ml of sodium metabisulfite (10 mg/ml). The iodinated lectins were purified in a Sephadex G-25 column (15x0.5 cm) eluted with 10 mM Tris-HCl, pH 7.2, in 5.0 ml fractions. The fractions containing the labelled protein were extensively dialyzed against 10 mM Tris-HCl buffer, pH 7.2. The specific activity of the $^{125}$I-lectins varied between $6 \times 10^4$-$1 \times 10^5$ cpm/µg protein.

$^{125}$I-Lectin Binding Assay: The binding of various lectins to MVM was determined by incubating membranes (24-28 µg protein) at 25°C for 30 min with $^{125}$I-lectin (2-3 µg) in a total volume of 0.2 ml made up with PBS, pH 7.4. The reaction was terminated by adding 4 ml of ice-cold PBS and filtering through a 0.2 µm Millipore filter (EGWP, Millipore Corp., Bedford, MA) under suction. The filter was washed twice with 3 ml of cold PBS and dried. Radioactivity was counted in EC $^{125}$I-gamma counter. Nonspecific binding to the membrane, measured in the presence
of 0.15 M α-L-fucose (UEA₁), N-acetylneuraminic acid (WGA) or lactose (PNA) was negligible for the three lectins. Binding to the filter was less than 6% of the total radioactivity added to the reaction mixture. The lectin binding to MVM was expressed as µg lectin bound/mg protein after subtracting the nonspecific binding from that of total.

INCORPORATION OF LABELED SUGARS INTO MVM:

Incorporation of [¹⁴C]-mannose (4 µCi/10 g body wt), [³H]-fucose (4 µCi/10 g body wt) or [¹⁴C]-glucosamine (2.5 µCi/10 g body wt) into the MVM was determined after injecting the labeled sugar intraperitoneally to fasted mice. The animals were sacrificed after 2 hr and MVM were isolated and purified as described earlier.

For delipidation of MVM, 3-4 mg of protein in 2.0 ml of 50 mM sodium maleate, pH 6.8, was treated with 8 vol of chloroform-methanol (2:1, v/v). The contents were vigorously shaken, allowed to stand at room temperature for 30 min and centrifuged at 10000 x g for 10 min. The organic phase was removed, the pellet resuspended in 10 ml of sodium maleate buffer and recentrifuged at 10000 x g for 10 min. The supernatant was discarded and the pellet resuspended in maleate buffer.

Suitable aliquots of intact and delipidated membrane preparation were digested in 10% KOH and radioactivity was counted in LKB Rack Beta Liquid scintillation counter after adding 8.0 ml of Bray's scintillation fluid.

Composition of Bray's scintillation fluid: 60 g Naphthalene, 4 g PPO, 0.2 g POPOP, 20 ml ethylene glycol, 20 ml glacial acetic acid and 100 ml
methanol were mixed and final volume was adjusted to one litre with 1,4-dioxan.

Separation of Microvillar Proteins by SDS-PAGE: 0.5 ml of membrane suspension containing 1 mg of protein was solubilized by adding SDS to a final concentration of 1%. The suspension was heated in a boiling water bath for 10 min. Sucrose was added to make a 25% solution and bromophenol blue was used as the tracking dye. Gel electrophoresis was performed using 5 mm x 130 mm gels. The gel solution contained 7% acrylamide, 0.177% bis-acrylamide, 0.13% SDS, 0.1 M Tris-maleate buffer, pH 7.6 and 0.4% N,N,N,N-tetramethylethylendiamine. Gel formation was initiated by ammonium persulfate. The gel solution was carefully overlaid with 0.1% SDS and the gels were allowed to stand at room temperature for at least 12 hr before use. Samples of BBM containing 100-150 µg protein were applied to each gel well. The samples were overlaid with 0.05 M Tris-maleate (pH 7.6) containing 0.1% SDS. Electrophoresis was carried out at 6 mA per gel for 5-6 hr. After electrophoresis, the gels were stained for 2-10 hr with Coomassie brilliant blue following the method of Weber and Osborn (1969). Gels were destained in a solution containing methanol, acetic acid and water (2.5:1:6.5, v/v).

Apparent molecular weight was estimated by applying 10 µg of marker proteins to gels. The proteins used included myosin (205 kDa), β-galactosidase (116 kDa), phosphorylase B (97 kDa), bovine albumin (66 kDa), egg albumin (45 kDa) and carbonic anhydrase (29 kDa). When migration of these proteins was plotted against the log molecular weight,
a linear relationship was found (Fig. 4). For molecular weight determinations, marker proteins were electrophoresed simultaneously with brush border proteins.

Radioactive Counting of Acrylamide Gel: Radioactivity in gel was counted by slicing the gel. The gel was sliced into 2 to 3 mm segments using a razor blade. Each slice was incubated in 0.2 ml of 30% \( H_2O_2 \) at 37°C for 16 hr or until the gel dissolved. It was counted for radioactivity after adding Bray's scintillation fluid.

BBM LIPID ANALYSIS:

Extraction of BBM Lipids: Membrane lipids were extracted by the method of Folch et al. (1957). 3.0 ml of membrane suspension containing 25-30 mg protein was extracted with 60 ml of chloroform-methanol (2:1, v/v) mixture for 20 min at 55°C. The contents were filtered in a graduated cylinder and the residue on the filter paper was washed twice with 5-10 ml of chloroform-methanol (2:1, v/v). To the combined filtrate, 0.7% KCl (20% of the total volume of extract) was added and mixed vigorously. The mixture was allowed to stand so as to separate the aqueous and chloroform (lipid) layers. The upper aqueous phase was removed by aspiration with a pasteur pipette and lower layer was washed thrice, each time with 5.0 ml of chloroform-methanol-KCl (0.7%), 3:48:47 (v/v). The washed lower layer was transferred to a round bottom flask and evaporated to dryness under vacuum at a temperature below 45°C. To the residue was added 5.0 ml of chloroform-methanol-water (64:32:4, v/v) mixture and evaporated to dryness. Dried lipid was dissolved in 50 ml of chloroform, filtered through Whatman No.1
Fig. 4: Standard curve for molecular weight determination.
filter paper and the filtrate again dried under vacuum at 45°C. The final residue was dissolved in 2.0 ml aliquots of chloroform thrice and transferred to a glass stoppered tube and the solvent was evaporated. The volume was made up to 5.0 ml with chloroform and the lipid extract was preserved at -20°C. Stream of nitrogen gas was passed through the round bottom flask at various steps if any water droplets were seen adhering to the sides of the flask.

Estimation of Total Lipids: Small 5 ml beakers, with their initial weights recorded, were placed in a desiccator containing anhydrous calcium chloride. To each beaker, 1.0 ml of lipid extract was added. Vacuum was created inside desiccator by suction and beakers were allowed to remain there overnight for the lipid extract to dry. Beakers were weighed next day and the procedure was repeated till a constant weight of beakers was obtained. Total lipids were estimated by subtracting the initial weight of the beakers from their final weight.

Estimation of Phospholipid Phosphorus: Phospholipid phosphorus was estimated by the method of Ames (1966). Total phospholipids were obtained by estimating inorganic phosphate and multiplying it by a factor of 25 (taking average molecular weight of phospholipids as 775).

a) Ashing procedure for total phosphate: The sample of organic phosphate and a drop of magnesium nitrate solution in a small test tube are taken to dryness by shaking the tube in flame or in a sand bath. This procedure ashes organic phosphate in a few seconds and is coupled with the very sensitive inorganic phosphate method.
Reagents:
(i) Magnesium nitrate (10% in 95% ethanol).
(ii) 0.5 N HCl.

Procedure:
1. To 0.1 ml of lipid extract in different tubes was added 0.15 ml of Mg(NO₃)₂.
2. The material was heated to dryness and ashed by keeping the tube in the sand bath until the brown fumes disappeared and a white fused mass was left.
3. The tubes were allowed to cool and 1.5 ml of 0.5 N HCl was added to each tube.
4. Tubes, capped with marbles, were heated in a boiling water bath for 15 min to hydrolyze any pyrophosphate formed in the ashing to phosphate.
5. Appropriate standard was run through the ashing procedure as Mg(NO₃)₂ is known to cause a small lowering of the extinction.

b. Estimation of inorganic phosphate:

Reagents:
i) Ascorbic acid (10%).
ii) Ammonium molybdate tetrahydrate (0.42% in 1 N H₂SO₄): To 0.42 g ammonium molybdate was added 2.86 ml conc. H₂SO₄ and final volume made to 100 ml with water.
iii) Coloring reagent: One part of (i) was mixed with six parts of (ii) to get the coloring reagent.
Procedure:

1) 3.5 ml of coloring reagent (iii) was added to the tubes containing inorganic phosphorus in 1.5 ml of 0.5 N HCl (1.5 ml water in case of blank).

2) The tubes were incubated at 45°C for 20 min.

3) O.D. was read at 820 nm.

Estimation of Total Cholesterol: Total membrane cholesterol in the lipid extract was estimated colorimetrically using glacial acetic acid-FeCl₃ reagent as described by Zlatkis et al. (1953).

Reagents:

(i) Standard cholesterol solution (1 mg/ml): 100 mg of cholesterol was dissolved in 100 ml of glacial acetic acid.

(ii) Ferric chloride reagent: Stock solution (10%) was prepared by dissolving 5 g of anhydrous FeCl₃ in 50 ml of glacial acetic acid. Working FeCl₃ solution (0.1%) was prepared fresh, just before use. For this, to 25-30 ml of conc. H₂SO₄ was added 1.0 ml of stock solution with continuous stirring. The final volume was made to 100 ml with conc. H₂SO₄.

(iii) Glacial acetic acid.

Procedure:

1. 0.1 ml of lipid extract was taken in different tubes and evaporated to dryness.

2. 3.0 ml of glacial acetic acid was added to each tube.
3. 2.0 ml of working FeCl₃ reagent was added and mixed well.
4. The tubes were kept in dark for 30 min and O.D. measured at 540 nm.

**Estimation of Free Cholesterol:** Free cholesterol was estimated by the method of Courchaine et al. (1959).

**Reagents:**

1) Acetone-95% ethanol (1:1, v/v).
2) Digitonin solution (1%): 1.0 g of digitonin was dissolved in 50 ml of 95% ethanol and diluted to 100 ml with water. The solution was stored in the dark.
3) Acetone.

**Procedure:**

1. 0.1 ml of lipid extract was taken in different tubes and evaporated to dryness.
2. 1.0 ml of acetone-ethanol and 1.0 ml of digitonin were added to each tube and the samples were mixed well.
3. After 10 min, the tubes were centrifuged at 3000 rpm for 5 min. The residue was of free cholesterol.
4. The residue in different tubes was dissolved in 4.0 ml of acetone and the tubes centrifuged at 3000 rpm for 5 min.
5. After centrifugation, the residue was allowed to drain for 5 min and then dissolved in 3.0 ml of glacial acetic acid.
6. To each tube, 2.0 ml of working FeCl₃ reagent was added and mixed well.
7. O.D. was read at 540 nm after keeping the tubes in dark for 30 min.

Estimation of Triglycerides: Triglycerides were estimated by the method of Van Handel and Zilversmit (1957).

Reagents:

i) Standard triglyceride solution: Stock solution (500 mg/100 ml) was prepared by dissolving 500 mg of trioleate in 100 ml of chloroform. Working standard solution (50 μg/ml) was made by diluting the stock standard solution 1:100 with chloroform.

ii) 0.1 N alcoholic KOH: 280 mg of potassium hydroxide was dissolved in 50 ml of absolute alcohol.

iii) Sodium periodate (0.05 M): 107 mg of sodium periodate was dissolved in 10 ml of water.

iv) Sodium sulfite (20%): 2.0 g of sodium sulfite was dissolved in 10 ml of water.

Reagents (iii) and (iv) were prepared fresh, just before use.

v) 0.4 N H$_2$SO$_4$.

vi) Chromotropic acid (0.2%): 500 mg of chromotropic acid was dissolved in 10 ml of water and 250 ml of 66% H$_2$SO$_4$ was added to it. The solution was mixed well and preserved in a brown glass bottle in cold.

Procedure:

1) 0.1 ml of lipid extract was taken in different test tubes and evaporated to dryness.
2) 0.5 ml of alcoholic KOH solution was added to each tube.
3) The samples were refluxed at 70°C for 20 min and subsequently kept in a boiling water bath till the smell of alcohol disappeared.
4) 0.2 ml of 0.4 N H$_2$SO$_4$ was added to each tube.
5) 0.1 ml of sodium periodate solution was added.
6) After 10 min, 0.2 ml of sodium sulfite solution was added to different tubes.
7) After adding 8.0 ml of chromotropic acid to each tube, the samples were mixed well and kept in a boiling water bath for 30 min.
8) The tubes were cooled under tap water and the O.D. was read at 570 nm.
   Appropriate blank and standard samples were run simultaneously for each estimation.

**Separation of Phospholipid Fractions Using Thin Layer Chromatography (TLC):**

a) Preparation of TLC plates:

**Materials:**
Silica gel G, glass plates 0.4 x 20 x 20 cm, Desaga applicator (adjustable), drying rack to hold ten 20 x 20 cm plates and mounting board to take five 20 x 20 cm plates.

**Procedure:**

The plates, washed thoroughly and dried at room temperature, were rewashed with chloroform or acetone. Silica gel G slurry was prepared by mixing quickly 30 g of silica gel G in 60 ml of water. Thus, the gel used per plate was 10-12 g. The slurry was immediately
added to reservoir chamber of applicator (adjusted to 0.25 mm thickness) and it was pulled at a steady rate across the series of plates on the mounting board, from left to right. The plates were allowed to dry at room temperature and placed in the drying rack. They were put for a pre-run in the solvent mixture to be used for developing and then activated at 110°C for at least one hr before applying the sample.

b) Separation of phospholipids: 100-150 ml of solvent mixture (CHCl₃ : CH₃OH : 7 N NH₃, 65:25:4, v/v) was added to the TLC jar. A strip of filter paper, soaked with the solvent mixture, was kept along the inner wall of the jar to allow its proper saturation. 7-10 µg of lipid phosphorus was applied per spot and the plate was chromato­graphed immediately after application of the sample. The solvent was allowed to ascend to within 1-2 cm of the top of the plate. The plate was removed and the solvent allowed to evaporate. It was exposed to iodine vapours by placing it in iodine chamber. Phospholipid fractions were found to appear as yellowish brown spots.

c) Quantitation of phospholipids from chromatoplates: The spots on the developed plates were marked with a sharp needle and their Rf values noted. They were scraped off directly into the test tubes. Phosphorus content in each spot was estimated after digestion with 10% Mg(NO₃)₂ prepared in 95% ethanol.

Radioactive Incorporation Studies: In order to ascertain the dynamics of membrane lipids, the incorporation of [2-¹⁴C]-acetate into the intestinal BBM lipids was studied. Overnight-fasted animals were injected intraperitoneally with [2-¹⁴C]-acetate (specific activity 54.4
mCi/mmol) at the dose of 10-15 μCi/10 g body wt. Animals were sacrificed 2 hr after the injection of [2-¹⁴C]-acetate. Intestines were removed, cleaned thoroughly with ice-cold saline and everted. Tissues from 6-8 animals were pooled, BBM isolated and purified as described above. The membrane lipids were extracted according to Folch et al. (1957). Phospholipid fractions were separated by TLC. The spots corresponding to different lipid fractions were directly scraped into scintillation vials containing 8.0 ml of scintillation fluid and counted for radioactivity.

**STATISTICAL ANALYSIS:**

Statistical analysis of the data was done using Student's 't' test and values having p < 0.05 were considered to be statistically significant.