CHAPTER-I

ISOLATION AND CHARACTERIZATION OF INTESTINAL EPITHELIAL CELLS
4.1 Isolation and Characterization of Intestinal Epithelial Cells

4.1.1 Introduction:

In the past few years several methods have employed for the isolation of epithelial cells from the small intestine of rat and guinea-pig (Section 1.16.2). In the present study the intestinal cells were isolated from small intestine of the rat and that of guinea-pig by the method of Weiser (1973a) because of its distinct advantages (Section 1.16.2). Isolation of the cells from two species was performed to establish the suitability of these cells for various chemico-biological interactions in vitro, and as well as to understand the sensitivity and selectivity of the cells corresponding to villus-crypt surface of the small intestine, to the xenobiotics interaction in vivo and in vitro. The cells isolated are to be ensured that they are structurally and functionally intact before they are used as a model system for the proposed studies presented in this report. For this matter, a number of parameters were applied to establish the viability and structural integrity of the cells isolated.

4.1.2 Results

4.1.2.1 Isolation of epithelial cells corresponding to the villus tip-to-crypt surface of small intestine of rat:

The cells were isolated in a total of nine fractions (Section 3.4) and the efficiency of the methodology
in obtaining cells in gradient fractions from villus tip-to-the crypt region of small intestine of rat was established by monitoring cell specific biochemical markers in the sequentially eluted cell fractions. The enzymatic activities of \( \gamma \)-glutamyl-transpeptidase, sucrase and alkaline phosphatase were highest in the differentiated cells of the villus region and their activities declined progressively towards the crypt region where these activities were at the level of detectability (Fig.1). On the contrary, the incorporation of \( ^3\text{H} \)-thymidine was the highest in the proliferating crypt cells which declined steadily towards the villus tip as the cells differentiate and migrate towards the villus tip. These data are consistent with the observations of others (Fortin Magana et al., 1970; Weiser, 1973a; Pinkus et al., 1977; Porter et al., 1982) thus confirming that the procedure of isolation of cells correspond to the villus tip-to-crypt distance.

### 4.1.2.2 Isolation of epithelial cells from rat and guinea-pig small intestine:

Modification of the method of Weiser (1973a) was employed to isolate total enterocytes from guinea-pig and rat small intestine (Hegazy et al., 1983). The time limit of incubation with EDTA and dithiothreitol was reduced and the number of incubations were minimized. This resulted in a homogeneous pool of isolated enterocytes of the intestinal mucosa with high viability. The cell yield from a 60 cm segment of rats weighing 225-250 gm was 174±15.2 mg cell protein, where as in case of guinea-pigs (425-450 gm, b.wt.) the total cellular
Fig. 1 Gradient elution of rat small intestinal epithelial cells from villus tip to the crypt. The isolation of cells (section 3.4), determination of marker enzymes (section 3.12) and incorporation of $^3$H-thymidine into the cells (section 3.11) were as described in Materials and Methods. The data are the mean from at least four separate experiments. The coefficient of variation was less than 10%.
yield of 267±28 mg cell protein was achieved.

4.1.2.3 **Morphological aspects of isolated intestinal cells:**

Microscopic examination of the sequentially isolated intestinal cells revealed that clumping prevailed in cells isolated from villus tip region but the size and number of clumps decreased as one moves down to crypt region, where individual cells were evenly distributed and aggregates very rarely seen. Villus tip cells were recognisable under light microscopy by their oblong shape and conspicuous brush border (Fig.2a) and constituted the principal cell type isolated in the first three fractions; mid-villus (fr.4-6) and lower-villus or crypt cells (fr.7-9) were mostly spherical (Fig.2b). The electron microscopy of the villus cells revealed long microvilli (Fig.3a) with well developed endoplasmic reticulum and mitochondria (Fig.3b).

The total enterocytes isolated from guinea pig revealed a homogeneous preparation of intestinal epithelial cells, with few clumps of not more than 4-10 cells. Cells with elongated shape and prominent long brush border and several small oval shaped cells were visible with a relatively small brush border (Fig.4) and in some cases only slight notchy ridge was seen at one end of the cell. The enterocytes isolated from rat small intestine had a similar cellular morphology as those isolated from guinea pig except that the number of clumps in case of enterocytes isolated from rats were more than in cell preparation
Fig. 2 Phase contrast photomicrograph of freshly isolated intestinal cells. The cells were incubated in Eagle's MEM with Earle's salts at 37°C for 15 min, and then fixed by addition of formaldehyde drop by drop so that the final concentration was 10% (magnification x 100).

(a) villus tip cells; (b) crypt cells.
Fig. 3  Transmission electron microscopy of villus tip cells isolated from the rat small intestine.

(b) The ultrastructure of villus tip cells showing:

- Desmosomes (D);
- Nucleus (N);
- Mitochondria (M);
- Microvilli (V).

Endoplasmic reticulum, intercellular spaces, vesicles and the tight junctions at the luminal border of villus-tip cells can also be seen. (Magnification, X 1400)

(a) Ultrastructure of the brush border area of the same cell at magnification (X 4800).
Fig. 4 Phase contrast photomicrograph of the pooled intestinal cells: The enterocytes isolated from guinea pig small intestine were processed as described in Fig. 2. The photomicrograph shows a monodispersed preparation of both the villus and crypt cells. (Magnification X 100).
from guinea-pig small intestine.

4.1.2.4 Viability of the cells:

The enterocytes isolated from guinea-pig small intestine revealed 90±5% viability and that of the rat was 88±5% (Section 3.6). The viability of cells amongst isolated cell fractions of the rat small intestine varied from fraction to fraction; the lowest viability of approximately 85±5% was observed in villus tip cells (fr.1) which increased with each subsequent elution of cells isolated in gradient fractions so that the mid-villus cells (fr.4-6) had a viability of 88±3% and crypt cells (fr.7-9) of 96±2%.

4.1.2.5 Respiration rate of isolated enterocytes:

The rat of O₂ utilization in the intestinal epithelial cells isolated from the small intestine of rat was lower than that of guinea-pig. The guinea-pig cells had an O₂ consumption rate of 42±2.4 u atoms/min/gram protein against 31.6±2.2 u atoms/min/gram protein of rat intestinal cells when the cellular respiration was measured in the presence of 10 mM glucose (Table 1). Intestinal cells from both the species registered a linear increase in O₂ consumption at least for 60 min. The basal respiration rate was not affected either by 5 mM BOHB or 3 mM succinate or Ca²⁺ (Table 1). Both glucose (10 mM) and glutamine (5 mM) were better substrates than BOHB in stimulating the rate of O₂ consumption (Table 2). The per cent increase in O₂ consumption on addition of the substrates were similar in rat and guinea-pig enterocytes. Addition of rotenone
Table 1: Effect of various substrates and inhibitors on the rate of oxygen consumption of rat and guinea-pig enterocytes

<table>
<thead>
<tr>
<th>Experimental regimen</th>
<th>Oxygen consumed (u atoms/min/gm prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>rat</td>
</tr>
<tr>
<td>None</td>
<td>16.6 ± 1.2</td>
</tr>
<tr>
<td>BOHB (5 mM)</td>
<td>19.4 ± 0.8</td>
</tr>
<tr>
<td>Succinate (3 mM)</td>
<td>16.8 ± 1.3</td>
</tr>
<tr>
<td>Glucose (10 mM)</td>
<td>31.6 ± 2.2</td>
</tr>
<tr>
<td>Glucose + Methanol (10 ul)</td>
<td>30.0 ± 2.1</td>
</tr>
<tr>
<td>Glucose + Anti-A (6 uM)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Glucose + Anti-A + Suc (3 mM)</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>Glucose + Rot (10 uM)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Glucose + Rot + Suc (3 mM)</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>Glucose + CaCl₂ (1.2 mM)</td>
<td>31.8 ± 1.8</td>
</tr>
</tbody>
</table>

50 ul aliquots of the cell suspension (3 mg cell protein) were added to 3 ml Hanks balanced salt solution and the O₂ utilized was measured polarographically at 30°C (Section 3.10.1). Rotenone and antimycin-A were introduced in 10 ul of methanol. The controls received 10 ul of methanol. Results expressed are mean±SD from 4 separate experiments.
Table 2: Time Course of O\textsubscript{2} Consumption by enterocytes of the small intestine of rat and guinea pig

<table>
<thead>
<tr>
<th>Experimental Regimen</th>
<th>Rate of O\textsubscript{2} uptake (u atoms O\textsubscript{2} consumed/min/gram prot.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 min</td>
</tr>
<tr>
<td>Rat intestinal cells:</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>16.4 ± 2.2</td>
</tr>
<tr>
<td>Cells+GLUC</td>
<td>30.2 ± 1.8</td>
</tr>
<tr>
<td>Cells+GLUT</td>
<td>26.5 ± 2.2</td>
</tr>
<tr>
<td>Cells+GLUC+GLUT</td>
<td>34.8 ± 1.9</td>
</tr>
<tr>
<td>Cells+BOHB</td>
<td>18.8 ± 1.0</td>
</tr>
<tr>
<td>Guinea pig intestinal cells:</td>
<td></td>
</tr>
<tr>
<td>Cells</td>
<td>21.2 ± 1.6</td>
</tr>
<tr>
<td>Cells+GLUC</td>
<td>42.0 ± 2.6</td>
</tr>
<tr>
<td>Cells+GLUT</td>
<td>30.1 ± 0.6</td>
</tr>
<tr>
<td>Cells+GLUC+GLUT</td>
<td>44.8 ± 2.7</td>
</tr>
<tr>
<td>Cells+BOHB</td>
<td>24.2 ± 1.2</td>
</tr>
</tbody>
</table>

16-20 mg of intestinal cell preparations were incubated in 4 ml of O\textsubscript{2}-saturated HBSS devoid of glucose and glutamine. Glucose (10 mM), glutamine (5 mM) and BOHB (5 mM) as shown were added and the cells were incubated at 30°C in a shaking water bath. During incubation the flasks were continuously gassed with oxygen. Aliquots (500 ul) were removed at the indicated time intervals and added to 2.5 ml of O\textsubscript{2}-saturated HBSS and the O\textsubscript{2} uptake was monitored polarographically. Data expressed are the mean±SD from 3 different experiments.
and antimycin-A, the potent inhibitor of the respiratory chain and uncoupler of oxidative phosphorylation, brought about complete inhibition of the cellular respiration (Fig.5).

4.1.2.6 Respiration rate in isolated cell fractions corresponding to the villus-crypt surface of small intestine:

The rate of respiration in sequentially isolated cells corresponding to the villus-tip to-crypt of the rat intestinal epithelium is shown in Fig.6. The endogenous rate of respiration was highest in cell preparations of the villus region (fr.1-6) whereas in the highly undifferentiated crypt cells (fr.7-9) it was 2 to 5-fold lower than that found in cell fractions of the upper villus. The average mean rate of respiration of the sequentially isolated cells when pooled together was comparable to the rate observed in total cells (Table 2). Both L-glutamine (5 mM) and glucose (10 mM) stimulated significantly the rate of $O_2$ consumption in all cell fractions. The stimulation was more pronounced in the upper villus cells with readily available source of energy such as glutamine and glucose while it was relatively less significant in the cells of the crypt region (fr.7-9). Respiration rate of cells from fraction 1-4 was stimulated by 100-114% and that of fraction 5-6 was increased by 70% and that of fraction 7-9 by 42-29%. The total isolated enterocytes had a basal rate of 16-18 u atoms $O_2$ consumed/min/gram protein which increased to 29 u atoms $O_2$ consumed/min/gram protein in the presence of 10 mM glucose (Fig.6).
Fig. 5  Polarographic tracings of the oxygen consumption rate by guinea pig small intestinal cells. The tracing shows the oxygen consumption of a single preparation directly after isolation. Concentration of the substrates and inhibitors used: Glucose 10 mM, L-glutamine (GLUT) 5 mM, BOHB 5 mM, Succinate (SUC) 3 mM, Rotenone (ROT) 10 uM, Antimycin-A (Anti-A) 6 uM.

Fig. 6  Rate of respiration in sequentially isolated intestinal cells: Effect of L-glutamine and glucose. O₂ uptake was measured in 3 ml HBBS containing cells (3 mg prot.) HBSS (without glucose) (●) or 5 mM L-glutamine (*) or 10 mM Glucose (▲). Data are from polarographic tracings representative of at least three separate experiments. Each value is the mean±S.D. of three experiments.
Inhibition characteristics of the cellular respiration of intestinal cells with rotenone and antimycin-A in the presence of various substrates was found similar to that of guinea-pig intestinal cells shown in the polarographic tracings of Fig.5. Rotenone completely inhibited the rate of respiration.

4.1.2.7 *In vitro* incorporation of tritiated thymidine and 1-\(^{14}\)C-DL-leucine in isolated guinea-pig enterocytes:

The *in vitro* rate of \(^3\)H-thymidine incorporation in the isolated enterocytes measured as acid insoluble count showed a time-dependent linear incorporation rate for 60 min (Fig.7). Enterocytes when incubated with labelled 1-\(^{14}\)C-DL-leucine incorporated amino acid into acid insoluble fraction curvilinearly at least for 60 min when saturation plateau was attained (Fig.8). An inhibition in the rate of incorporation of 1-\(^{14}\)C-DL-leucine was observed on addition of uncouplers and inhibitors of respiratory chain. Ouabain, rotenone and cyanide produced a similar and significant inhibition of about 75% which indicated that leucine uptake is dependent both on cation transport and energy level of the metabolically active cells. Sodium azide and dinitrophenol brought about 57% and 65% percent inhibition in the rate of 1-\(^{14}\)C-DL-leucine incorporation. Endosulfan a known inhibitor of the respiratory chain (Dubey *et al.*, 1984) at 60 \(\mu\)M concentration inhibited the leucine uptake by 32% (Table 3).
Fig. 7  **In vitro** incorporation of tritiated thymidine into acid-insoluble fraction. Standard incubation conditions and procedures for measurement of radioactivity are described in section 3.11. Results shown are mean±S.D. from at least 3 different experiments with variation of less than 15%.

Fig. 8  Protein synthesis in isolated intestinal epithelial cell suspension of the guinea pig. Standard incubation conditions and measurements are described in section 3.11.
Table 3: Effect of metabolic inhibitors on the uptake of DL-leucine in the intestinal epithelial cells of the guinea pig

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% inhibition</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cells + Saline</td>
<td>Nil</td>
<td>-</td>
</tr>
<tr>
<td>Cells + 0.5% DMSO</td>
<td>5 ± 1.2</td>
<td>-</td>
</tr>
<tr>
<td>Cells + 2.5 mM K-Cyanide</td>
<td>70 ± 6.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cells + 0.5 mM Ouabain</td>
<td>76 ± 12.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cells + 0.4 mM DNP</td>
<td>65 ± 8.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cells + 2 mM NaN₃</td>
<td>57 ± 6.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cells + 0.25 mM Rotenone</td>
<td>75 ± 9.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Cells + 60 uM Endosulfan</td>
<td>32 ± 4.8</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Intestinal cells (6 mg) were preincubated for 3 min at 37°C in 2 ml of Hanks basal salt solution supplemented with 10 mM Hepes and 0.05% bovine serum albumin. Inhibitors at indicated concentrations were added to the incubation medium in 10 ul of saline or DMSO and incubated for 2 min, after which 2 uci of C¹⁴-DL-leucine was added. After 15 min of incubation 200 ul aliquots were drawn and added to 10% chilled TCA. Radioactivity was measured in the acid precipitable protein (Section 3.11). The control value was 258±28 cpm/mg protein. Results expressed are mean±SD from 3 different experiments.
4.1.3 Discussion

The sequential elution of epithelial cells as villus tip-to-crypt gradient (Weiser, 1973a) has been applied in the present study. The profile of cells isolated in nine sequential fractions corresponded to the villus tip-to-crypt gradient in situ. This was established by measuring the distribution of the activities of γ-glutamyltranspeptidase, sucrase, alkaline phosphatase and incorporation of the $^3$H-thymidine in gradient profile of cells isolated in nine serial fractions 1-9. These findings are consistent with the observations of other (Weiser, 1973a; Pinkus et al., 1977; Raul et al., 1977; Porter et al., 1982). The methodology provides simple and interesting system for studying biochemical changes during proliferation and differentiation stages of cells. The isolated cells either total or in gradient fractions appear to be metabolically active and viable for in vitro studies. This is evidenced from our results on rate of cellular respiration and from the trypan blue exclusion frequency of the cells. The rate of oxygen utilized by isolated intestinal cells has been taken as a measure of functional integrity and metabolic capability of the cells (Iemhoff et al., 1970; Evans et al., 1971; Reiser and Christiansen, 1971; Windmueller and Spaeth, 1974; Towler et al., 1978; Watford et al., 1979; Hegazy et al., 1983). As such the intestinal cells might, therefore, allow to predict the harmful nature of xenobiotics due to the interference of the latter with cellular respiration. Further these studies also demonstrate that cells from different regions of the
epithelium along the villus crypt surface respond differently
to \( O_2 \) consumption and its stimulation by nutrients. This appears
to depend on the functional characteristics and energy
requirements of the cells which undergo continuous differentiation
from crypt to villus tip so as to take up the absorptive
functions. Consequently the high rate of oxygen uptake by cells
of the villus tip and upper half of the mid villus apparently
is a reflection of their metabolic role in the absorption and
assimilation of nutrients. This coincides with the activities
of brush border enzymes found in the villus-crypt surface of the
intestinal mucosa (Weiser, 1973a; Pinkus et al., 1977; Raul et al.,
1977; Porter et al., 1982). The major advantage of sequential
elution of cells by Weiser's method is that one can qualitatively
and quantitatively localize and characterize the various biochemical
functions along the villus-crypt surface.

The viability of the guinea-pig cells was
found better than cells isolated from rat's small intestine. The
cell suspension obtained did not contain clumps and cell were
more or less single cell suspension. This is perhaps due to low
content of mucus secreted by these cells (Dawson and Bridges,
1979b; Pinkus et al., 1983). Enterocytes isolated from rats had
a tendency to clump. The production of more mucus in rat
intestinal mucosa than in guinea pigs is well known (Evans et al.,
1971). The shortening of total incubation time from 50 min to
16 min with EDTA buffered solution helps in avoiding the
viability loss brought about by exposure to EDTA (Hoffman and
Kuksis, 1979) and yielding a cell preparation with better viability
The guinea-pig intestinal cells are therefore more suitable in studying cellular transport and in \textit{in vivo} regulation studies. Both the guinea-pig and rat intestinal cells are found to maintain a linear rate of oxygen consumption at least for 60 min as observed by others (Towler \textit{et al.}, 1978; Watford \textit{et al.}, 1979). However, the guinea-pig enterocytes showed better viability than rat cells when incubated for more than 90 min. Similar observations were made by others (Dawson and Bridges, 1979a; Pinkus \textit{et al.}, 1983).

The cells from both the species were found to respond normally to their preferred substrates glucose and L-glutamine (Windmueller and Spaeth, 1974; 1975) in stimulating the rate of respiration. Further cellular respiration was found to be sensitive to the inhibitors and uncouplers of oxidative phosphorylation, e.g. antimycin-A, rotenone, DNP, cyanide, NaN\textsubscript{3}, etc. Addition of succinate to rotenone treated and untreated cells did not alter the rate of \textit{O}\textsubscript{2} utilization which indicated that the plasma membrane of the cells have not been damaged during the isolation procedure, and the cells possess well coupled bioenergetics. Respiratory coefficient and cellular respiration in the presence of sufficient amount of substrates are utilized as useful criteria to assess the quality of cells isolated (Baur \textit{et al.}, 1975; Singh and Schwarz, 1981; Hegazy \textit{et al.}, 1983). Observations with some preparations of sequentially isolated cells reveal that only cells isolated in fr.1 (villus tip cells) respond marginally to succinate.
stimulation, which could possibly be due to the loss in mem-
brane integrity of the cells isolated in this fraction, as
these cells are almost in the shedding stage (Crane, 1979).

**In vitro** incorporation of uridine and leucine into the cellular macromolecules was consistent with
the findings of (Hartmann et al., 1982a; Hegazy et al., 1983;
Schwenk et al., 1984). Incorporation of leucine and uridine
indicated that the enterocytes continue to synthesize protein
and RNA by the cooperative action of the cell membrane,
mitochondria, nucleus and cytoplasm, which is also evident by
the significant decrease brought about in the incorporation of
leucine into the macromolecules by inhibitors of energy
metabolism and ouabain. This indicated that the cell
preparations are metabolically active (Reiser and Christiansen,
1971). The **in vivo** and **in vitro** studies with tritiated thymidine
have revealed that highly dividing crypt cells incorporate
\(^{3}H\)Tdr maximally because, the cells are, known to have high
mitotic index (Weiser, 1973a; Raul et al., 1977; Lawson et al.,
1982; Hartmann et al., 1982a). The incorporation of radioactive
thymidine was poor in mature cells of the villus providing
adequate evidences that cells isolated in different fractions
correspond to the villus tip, mid-villus and crypt regions.

In conclusion the suspension of intestinal epithelial cells isolated from the small intestine of guinea-pig
and rat appear suitable for various biochemical and
pharmacological studies. Different populations of cells can be
easily isolated from the villus-crypt surface of intestinal epithelium. This offers the possibility to study and monitor the sensitivity and selectivity of various cell types to xenobiotics interaction and to predict their vulnerability to the cyto-cum-genotoxic action of xenobiotics.
CHAPTER II

LOCALIZATION AND CHARACTERIZATION OF MONOOXYGENASES -
ALONG THE VILLUS-CRYPT SURFACE OF THE RAT SMALL
INTESTINE
4.2 Localization and characterization of Monooxygenases along the Villus-Crypt Surface of the Rat Small Intestine

4.2.1 Introduction

The small intestine is the major portal of entry for a large number of chemicals and drugs into the body system. The cells responsible for their absorption and transport are the epithelial cells lining the villus-crypt surface of the intestinal lumen. The cells in the crypt actively divide, differentiate and migrate upward to the villus-tip so that the cells in the upper third of the villus are fully mature and differentiated. Various studies have indicated that the drug metabolizing activity of the small intestine could be modulated to actively metabolize xenobiotics modifying their fate and bioavailability (Vaino and Hietanen, 1980), although the basal drug metabolizing activity is found considerably lower than in the liver (Chhabra, 1979). A number of reports showed the high sensitivity of intestinal monooxygenases to induction by polycyclic aromatic hydrocarbons and to compounds present in cigarette smoke or certain foods (Pantuck et al., 1976; Stohs et al., 1976a; Schiller and Lucier, 1978; Borm et al., 1982). The activity of the monooxygenases is higher in the proximal part of the small intestine (Wattenberg et al., 1962; Chhabra, 1979) declining towards the caudal end. The activity has been found to be mainly localised in the villus cells and to be almost at the
levels of detection in the crypts (Hoensch et al., 1975, 1976; Porter et al., 1982) where cell proliferation is actively occurring. The fact that various cell types along the villus-crypt surface represent different stages of differentiation and proliferation there is a possibility that these cell types and their monooxygenases might be expressing differential sensitivity towards xenobiotics or their modulators. This would affect the steady state level of drugs and environmental carcinogens in situ and may simultaneously influence the functions of these cells. Crypt cells which rapidly proliferate might, therefore, be the preferred target for cytotoxic and genotoxic action of chemicals. Thus it would be more important to know the capacity of the cells along the villus-crypt surface for activation and inactivation of chemicals. This involves the isolation of the epithelial cells corresponding to the villus-tip to crypt surface of the small intestine, so that the drug biotransforming competence of these cells could be quantitated, localized and characterized.

Wattenberg et al. (1962) observed the highest arylhydrocarbon hydroxylase activity histochemically in the mid-villus while others (Porter et al., 1982) established biochemically the preferential localization of this enzyme activity in isolated cells functions. These studies, however, don't examine the competence of the various cell types of villus-crypt surface to oxidise other model drug substrates, i.e. the presence and activity of other cytochrome P-450 isozymes. Cytochromes P-450 are known to exhibit a wide variety of substrate specificity, differential induction pattern and tissue distribution (Lu and West, 1980).
Based on various lines of evidence the cytochrome P-450 are broadly classified into two major groups: 1) The cytochrome P-450 forms which predominantly are present in liver and inducible by compounds typified by phenobarbital (PB) and express preferential specificity towards substrates, e.g. aldrin, ethylmorphine, nitrosamine or aflatoxin B₁ (Wolff et al., 1979; Lake and Paine, 1982; Loquet and Wiebel, 1982; Wiebel et al., 1984a, 1984b). 2) the cytochrome P-448 forms which are ubiquitous in nature and inducible by polycyclic aromatic hydrocarbons (Lake and Paine, 1982; Wiebel et al., 1984a,b). Benzo(a)pyrene is metabolized by cytochrome P-448 forms while 7-ethoxycoumarin is metabolized by both types of monooxygenases (Lake and Paine, 1982).

An attempt, therefore, has been made in the present study to quantitate and characterize the major monooxygenase activities directed towards the substrates, benzo(a)pyrene, 7-ethoxycoumarin and ethylmorphine in cells isolated in sequential fractions (Weiser, 1973a) corresponding to the villus tip-to-crypt gradient in the small intestinal epithelium of the rat.

4.2.2 Results

4.2.2.1 Isolation of epithelial cells from villus tip-to-crypt gradient:

The cells isolated in gradient fraction corresponded to the villus tip-to-crypt distance (Chapter 1, Fig.1).

4.2.2.2 The effect of monooxygenase inducers on the activities of AH and 7-ECDE along the villus-crypt surface of intestinal epithelium:

The biochemical localization and distribution
of AHH activity is shown in Fig.1. The enzyme activity was predominant in the mid-villus region and at level of detection in the crypt region. However, a single oral dose of 3MC induced AHH from 5-7 fold in the villus cells and 19-26 fold in the crypt cells; the extent of AHH induction tended to increase towards the proliferating zone. It was interesting to observe a significant induction of AHH activity by PB in almost all cell fractions, though relatively much weaker than 3MC. The enzyme activity was induced 3-6 fold in crypt cells and 50-100% in cell fractions from the villus over their corresponding untreated controls. The extent of induction by 3MC was at least 4-5 fold higher than obtained by PB.

The biochemical localization and the distribution of 7ECDE along the surface of the villus-tip to the deep crypt followed a similar pattern as that of AHH (Fig.2). The enzyme activity was the highest in the mid-villus region and decreased steadily to the crypt cells where it was one-fifth of the upper villus. 3MC induced 7ECDE in an increasing order in all the cell fraction beginning from villus-tip to crypt. The extent of increase was from villus tip (2-fold) to the crypt cells (7-fold) compared to the cells from control rats. The extent of induction was lower than that of AHH. PB also induced 7ECDE significantly from 50-70% in villus cells and 2 to 3 fold in crypt cells. The capacity of PB to induce 7ECDE activity was lower than that of 3MC by a factor of almost one in all the fractions.
Fig. 1 Effect of 3MC and PB on the biochemical localization and distribution of AHH in rat small intestinal epithelial cells from villus tip-to-crypt gradient. Determination of AHH activity and treatment of animals with inducers are described in Materials and Methods (section 3.14.2). AHH activity: (*——*) Basal, (△——△) PB treated, (○——―○) 3MC treated. The data give the mean±SD from four animals.

Fig. 2 The influence of 3MC and PB on the biochemical localization of 7-ECDE in rat small intestinal epithelial cells from villus tip-to-crypt gradient. The method for the assay of enzyme activity is given in Materials and Methods (section 3.14.1). Other conditions were same as in Fig. 2. (□——□) Basal, (△——△) PB treated, (○————○) 3MC treated. The data give mean±SD from four animals.
FIG. 1

![Graph showing AH activity (DNA/µg) vs. isolated cell fractions.]](image1)

FIG. 2

![Graph showing 7-EC-O-deethylase activity (p moles/min/mg prot.) vs. isolated cell fractions.]](image2)
4.2.2.3 Expression of ethylmorphine-N-demethylase and its modulation:

EMD is a preferred substrate for PB-inducible cytochrome P-450. Previous attempts by other (Chhabra, 1979) to demonstrate EMD activity in the intestinal mucosa from untreated rats have been unsuccessful. It was interesting to note that EMD activity was below the level of detectability in cells isolated from untreated rats (Fig. 3). However, treatment with PB markedly increased the enzyme activity in all cell fractions. The maximum activity of 80 pmoles per min per mg protein was observed in cell fractions from the mid-villus region which was double the activity of crypt cells. Thus, the differentiated cells expressed the highest demethylase activity. Coadministration of dexamethasone or theophylline with PB did not significantly modulate the enzyme activity.

4.2.2.4 Time course of AHH and 7ECDE induction:

Two types of effects were discernible with two different populations of cells from rats pretreated with a single oral dose of 3MC (Fig. 4, 5). In cell fractions of the villus region the maximal AHH activity of 4-8 fold was observed at hr 24 which declined to 2-3 fold at 72 hr of a single oral exposure to 3MC. The time related in vivo stability of the induced AHH level appeared similar in all fractions 1-7 of the villus. In the crypt cells (fractions 8-9) the AHH activity was 19-26 fold at hr 24 and this did not differ much at 48 hrs, thereafter it declined to about one-third of its maximal induced level at hr 72 (Fig. 4). The time course of 7ECDE induction
Fig. 3  Expression and modulation of EMD in rat intestinal epithelial cells. The data are the mean from four animals. The coefficient of variation was less than 10%. Other conditions are same as described in Materials and Methods (section 3.14.3). EMD activity: (*——*) Basal, (o——-o) PB treated, (e——-e) PB+Dexamethasone.

Fig. 4  Time course of AHH induction by 3MC along the villus-crypt surface of small intestine. Rats were killed at the indicated time period after a single oral dose of 3MC. Data are the mean from two separate experiments of two rats each. F denotes sequence of fraction number. Other conditions were same as in Materials and Methods (section 3.14.2).
FIG. 3

ETHYLORPHINE-N-DEMETHYLASE
(μ moles/min/mg prot.)

ISOLATED CELL FRACTIONS

FIG. 4

AHF-INDUCTION (% 100 INCREASE)

3MC-TREATMENT (hrs)
followed a similar pattern as that of AHH (Fig. 5). The crypt cells appear to respond more strongly to inducers of the monoxygenases and that the maintenance of induced level followed a similar trend in all the cell types. The studies demonstrate that crypt cells express relatively greater sensitivity of monooxygenase induction by inducers. Because the magnitude of enzyme induction still remains predominantly higher in crypt cells when in mature villus cells the enzyme activity has levelled to the control value at 72 hrs of 3MC treatment.

4.2.2.5 Kinetic constants of AHH in cells from villus tip, mid villus and crypt regions of intestinal villi:

Fractions were pooled to represent upper-villus (fr.1-3), mid-villus (fr.4-7) and crypt (fr.8-9) regions. The apparent Km and Vmax values of AHH from control and 3MC-treated rats in these fractions obtained are shown in Table 1. The apparent Km values for AHH in all fractions from untreated cells obtained was 5.5 uM. The Vmax was highest in the mid-villus region followed by upper villus and crypt. 3MC-treatment enhanced the Km and Vmax values disproportionately. The Km value was increased by 3-fold in cells corresponding to villus region and marginally in the crypt cells. However, the Vmax increased by about 6-fold in the villus region and about 12-fold in the crypt cells. PB had no significant effect on Km from controls while Vmax increased by 3-fold in crypt cells and 50-70% in the villus cells.
Fig. 5  Time course of 7-ECDE induction by 3MC along the villus-crypt surface of small intestine. Conditions were same as described in Fig. 5.

Fig. 6  Inhibitory effect of 7,8-BF on the basal and 3MC induced AHH activity of the rat small intestinal cells. AHH activity was measured in pooled fractions: Villus tip (e-----e), fr.1-3, mid-villus (*)-----(*), fr.4-7, crypt (Δ-----Δ). fr.8-9 rats were given 3MC p.o. and after 24 hrs the cells were isolated. AHH activity was measured in vitro with or without 7,8-BF. 7,8-BF was added in 10 ul of DMSO before the reaction was started with BP. Controls received the vehicle only. Other conditions were same as described in Materials and Methods (section 3.14.1). a; basal, b; 3MC-treated.
Table 1: Kinetic constants of aryl hydrocarbon hydroxylase in epithelial cells isolated corresponding to villus-tip, mid-villus and crypt regions of the small intestine of the rat.

<table>
<thead>
<tr>
<th>Villus-crypt cells</th>
<th>Control</th>
<th>3-MC treated</th>
<th>BP-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\text{K}_m^+$</td>
<td>$\text{V}_{\text{max}}^*$</td>
<td>$\text{K}_m^+$</td>
</tr>
<tr>
<td>Villus-tip</td>
<td>5.5</td>
<td>14.6</td>
<td>15.4</td>
</tr>
<tr>
<td>Mid villus</td>
<td>5.5</td>
<td>21.2</td>
<td>15.4</td>
</tr>
<tr>
<td>Crypt</td>
<td>5.5</td>
<td>4.8</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* $\text{V}_{\text{max}}$ expressed as pmoles/mg protein/min

† $\text{K}_m$ values in $\mu$M

The 9 cell fractions isolated (Weiser, 1973a) were finally pooled into 3 groups. Fractions 1-3 pooled as villus tip cells, fraction 4-7 as lower and mid-villus and fractions 8-9 as crypt cells. The $\text{V}_{\text{max}}$ and $\text{K}_m$ values were obtained using Lineweaver-Burk plots. The data are the mean values from two different experiments which varied by less than 10%. Rats were treated with 3MC or PB as described in Materials and Methods (Section 3.2).
4.2.2.6 Effect of 7,8-BF on AHH activity:

7,8-BF is a potent inhibitor of 3MC-inducible hepatic monooxygenase form (Wiebel, 1980) and differentiates this from the PB-inducible monooxygenases. 7,8-BF caused a dose-dependent inhibition of both constitutive and 3MC-induced AHH of intestinal cells of upper and mid villus and crypt. The magnitude of the inhibition was similar in pooled fractions representing three zones of villus-crypt surface (Fig. 6). Both the constitutive as well as induced monooxygenase activities from intestinal cells were inhibited to the same extent by 7,8-BF indicating that the AHH form in all the cells belong to the 3MC-inducible cytochrome P-448 dependent monooxygenases.

4.2.3 Discussion

The methodology adopted offered a simple system of choice for quantitation, characterization and distribution of the monooxygenases along the villus-crypt surface of the intestinal epithelium. The three substrates used, i.e. benzo(a)pyrene, 7-ethoxycoumarin and ethyl morphine (Lake and Painé, 1982) reflect the general metabolic competence of the cells in biotransforming xenobiotics along the villus-crypt surface. For the present discussion, the broad classification of monooxygenases into cytochrome P-450 and cytochrome P-448 forms (Lu and West, 1980; Wiebel and Singh, 1980; Wiebel et al., 1984a, 1984b) has been used, though multiple forms of cytochrome P-450 have been isolated to homogeneity (Guengerich et al., 1982; Hermann and Johnson, 1982; Kaminsky et al., 1984).
These studies provide evidence that the metabolic competence of crypt cells in the oxidation of BP hitherto considered insignificant (Wattenberg et al., 1962; Porter et al., 1982), can be raised significantly by polycyclic aromatic hydrocarbon type of inducers such as 3MC. Interestingly, the enhanced level of AHH in crypt cells was nearly 3-fold higher than in the mid-villus region of the control cells where the AHH is largely localised. It is also evidenced from these studies that the intestinal cells respond more strongly to inducers than the liver (Pantuck et al., 1976; Grafstrom et al., 1979) over time-dependent induction of AHH. The induction would thereby reduce the difference between the intestinal and liver monooxygenase activity at time when the intestinal cells show maximum induction after a single oral pretreatment with 3MC as also observed by others (Stohs et al., 1977). It is therefore, quite possible that the true cytochrome P-448 dependent AHH induction in liver might be as high as in gut.

Among the isolated cell fractions, the crypt cells expressed relatively greater sensitivity of AHH induction to 3MC after a single oral exposure to inducers than cells corresponding to fractions from the upper or mid-villus region. It is not known, at present, the reasons for high degree of responsiveness of AHH induction to inducers in the crypt cells and as well as in the villus cells and maintenance of induced level for longer period in crypt cells. It is possible that crypt cells may have higher concentration of AHH receptors and 3MC or its metabolites may be increasing the enzyme synthesis by
binding with highly replicating DNA of crypts by mechanism such as activation of genome (Bresnick et al., 1984).

Although the absolute activities of cytochrome P-448 monooxygenases, AHH and 7ECDE, are not found uniformly distributed along the villus-crypt surface, the higher apparent Km for 3MC induced hydroxylases of the cell fractions from mid-villus and upper-villus than that of the crypts may not necessarily mean that the exposure of the intestinal epithelium to 3MC results in different forms or population of P-448 isozymes along the villus-crypt surface, because the enzyme is a part of multicomponent system which has not been purified for the present study of Michaelis constants. Inasmuch as the velocity of enzyme reaction depends upon the concentration of free substrate available at the active site, the high Km of AHH in villus cells from 3MC treated rats may presumably be due to the higher unspecific binding of B(a)P in these cells. The intestinal wall is known to strongly accumulate carcinogen such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (Schiller and Lucier, 1978), or 3MC (Rees et al., 1969; Stohs et al., 1977) and a slow rate of B(a)P hydroxylation (Stohs et al., 1977) and of 7-ethoxy-coumarin deethylation (Borm et al., 1982) are reported in villus cells of rat treated daily with a single oral dose of 3MC for two consecutive days. This may partly be related to enhanced Km of AHH in villus cells from 3MC treated rats.

The results have apparently indicated that 7ECDE and AHH activities are closely related in the intestinal
epithelium based upon their induction and distribution pattern. As shown by others (Hermann and Johnson, 1982) BP is metabolised by cytochrome P-450 forms-6, EC by forms-4 and -6 and that both P-450 isozymes are inducible by polycyclic aromatic hydrocarbons. This would suggest that monooxygenase forms predominant in the rat intestinal epithelium are of the types inducible by polycyclic aromatic hydrocarbons. This is also evidenced by 7,8BF inhibition of intestinal monooxygenases activity in this study demonstrating the predominance of cytochrome P-448 in the intestinal mucosa of both 3MC and untreated rats.

The EMD in small intestine of the rat was below the level of detection unlike that in the intestinal epithelium of other rodents (Chhabra, 1979). Ethylmorphine is preferentially metabolized by PB-inducible cytochrome P-450. Some workers considered PB as a poor inducer of intestinal drug metabolism while others noted induction of N- and O-dealkylation (Shirkey et al., 1979). Sharf and Ullrich (1974) demonstrated higher in vitro inducibility of ECDE with parallel increase in NADPH-cyt:* reductase by PB in the small intestine of the mice. In fact, the extrahepatic induction of monooxygenases by PB seems to be unusual which normally is confined to adult hepatic tissue. However, from the studies of aforementioned workers and that of Wiebel et al. (1984a,b) in differentiated and dedifferentiated cell lines derived from Reuber H 35 rat hepatoma it may be viewed that PB can modulate the monooxygenase activities in tissues other than the adult liver. Although EMD is expressed significantly in the entire intestinal epithelium of the
PB-treated rat, the activity is not expressed uniformly in the villus-crypt surface; the cells of the mid-villus being stronger by a factor of two than that of crypt or villus-tip. On the other hand PB caused a relative higher increase of AMH and 7ECDE in crypt cells than in the cells of the villus region. Similarly the induction of cytochrome P-448 dependent monooxygenases by PB has been observed in fetal rats at term (Kremers et al., 1981). It remains to be determined that if PB induced cytochrome P-448 isozyymes in the crypt cells are similar to 3MC-induced P-448 isozyymes.

The present results indicate that the intestinal epithelium of rat possesses at least two forms of monooxygenases which are specifically induced by different inducers. The induction of monooxygenases would, thus mean either an increase in activation or inactivation pathways resulting in formation of their reactive metabolites or detoxified products. It is therefore quite possible that the mitotically active crypt cells, which possess higher DNA/protein than the villus cells (Webster and Harrison, 1969), might become susceptible to the toxic action of xenobiotics. The rapid induction of monooxygenases and maintenance of induced level for longer time might mean to provide protection to this compartment. But the steady state level of reactive metabolites in vivo is also known to depend on other enzymes, such as conjugases. In the following chapter (4.3) it is described that the villus-crypt surface of the small intestine is also rich in conjugases and their cofactors, UDPGA and GSH. Because of these properties the intestinal cells might protect
themselves against the noxious effect of xenobiotics. The studies also indicate that exposure of intestinal epithelial cells to xenobiotics and the higher sensitivity and inducibility of their monooxygenases would render the cells capable of inactivating a considerable amount of the potentially toxic compounds and thereby reducing the harmful action in other 'target' tissues. This would reduce the systemic burden of xenobiotics during intestinal first-pass and affect their bioavailability in vivo. However, prolonged exposure to potentially toxic compounds and certain intestinal diseased conditions might affect the delicate balance of activation and inactivation and consequently alter the steady-state kinetics of the cells in the proliferation compartment. These and further studies may help in assessing and predicting the vulnerability of small intestinal epithelium to genotoxic and cytotoxic effects of xenobiotics.
CHAPTER-III

LOCALIZATION AND CHARACTERIZATION OF CONJUGATION
REACTIONS ALONG THE VILLUS-CRYPT SURFACE OF THE
SMALL INTESTINE OF THE RAT
4.3 Localization and Characterization of Conjugation Reactions along the Villus-Crypt Surface of the Small Intestine of the Rat

4.3.1 Introduction

Intestine is one of the major routes for the entry of xenobiotics into the body. It is continuously exposed to orally ingested drugs, carcinogens and other xenobiotics. The xenobiotic metabolism in the animal body involves two distinct types of reactions, phase I (Section 1.4) and phase II (Section 1.5). UDP-glucuronyltransferases (GT) and glutathione-s-transferases (GST) are the major enzymes responsible for phase II metabolism of xenobiotics (Williams, 1972; Burke et al., 1977; Jerina and Bend 1977; Dutton, 1980; Mulder, 1982; Jefcoate, 1983) which, in general, are responsible for terminating the biological activity of the foreign compounds or their metabolites by conjugating them with UDP-glucuronic acid and/or glutathione. Determination and characterization of these activities and quantitation of their cofactors in the intestinal cells, in parallel with monooxygenase functions determined in the previous chapter, would provide us with a deeper understanding of the drug biotransforming competence of the cells.

In the present study, therefore, the sequentially isolated epithelial cells were utilized to characterize, quantitatively and qualitatively, the distribution of conjugases and their co-factors along the villus-crypt surface of the small
intestinal epithelium of the rat. Various workers have determined the conjugation reactions in the intestine using microsomal preparation (Lucier et al., 1977), scraped off mucosa (Josting et al., 1976; Bock et al., 1982) or total enterocytes (Grafstrom et al., 1979; Koster and Noordhoek, 1982; Schwenk and Locher, 1985). Their observations, however, do not take into account the prevalence of various forms of GT or GST and their cofactors in the various cell types along the villus-crypt surface so as to estimate their differential sensitivity to various xenobiotics. The determination of endogenous content of UDPGA and GSH is equally essential because these cofactors appear to regulate the endogenous activities of these enzymes (Singh and Schwarz, 1981; Jefcoate, 1983). Such systematic and in-depth studies would, therefore, provide insight into the drug metabolizing potential of the small intestine, its potential role in 'first-pass' loss of drugs and its vulnerability to harmful effects of xenobiotics.

4.3.2 Results

4.3.2.1 Glucuronidation Potentials of the Epithelial Cells along the Villus-Crypt Surface of Small Intestine:

The observations in Fig.1 reveal that glucuronic acid conjugation is highly expressed in the intestinal epithelial cells of rats towards a variety of substrates (aglycons). 3-OH-BP and 4-OH-biphenyl are taken as prototype substrates towards the transferases GT₁ and GT₂ (Section 1.5.1). High GT₁ activity towards 3-hydroxybenzo(a)pyrene as substrate
was observed in the cells isolated in villus tip-to-crypt gradient fractions from the small intestinal epithelium of rat. Villus tip and mid-villus cells expressed highest GT₁ activity which declined progressively towards the crypts. The crypt cells expressed 4-times less activity than that observed in villus cells, although activity present in the crypt cells was significantly above the level of detection. 3MC induced GT₁ activity towards 3OH-BP, whereas the enzyme activity remained unaffected on PB treatment (Fig.1). In contrast to the localization of the quantity of basal GT₁ activity along the villus-crypt surface of the untreated intestinal epithelium, a progressive increase in conjugase activity was noticed in cells of 3MC-treated animals. A maximum 6-fold induction was expressed in crypt cells whereas the enzyme activity was induced by only 2-fold in the villus cells.

The glucuronidation potential of the epithelial cells towards 4-OH-biphenyl (GT₂) (Fig.2) was found to be almost 4-7 times lower than the conjugation potential towards 3-OH-BP indicating clearly that GT₁ activity is much more dominating than GT₂ activity in the intestine. Cells from the mid-villus and villus tip regions were found to express maximum GT₂ activity which gradually decreases towards the crypt region. GT₂ activity was significantly induced by PB which was unaffected by exposure of rats to 3MC. A maximum induction of 4-fold was observed in the crypt cells, while in the villi tip cells a maximum induction of only 2-fold was achieved. The pattern of PB-induced GT₂ activity followed a similar trend as
Fig. 1  Effect of 3MC and PB on the biochemical localization and distribution of GT activity towards 3-OH-BP in rat small intestinal epithelial cells from villus tip-to-crypt gradient. Determination of GT activity and treatment of animals with inducers are described in Materials and Methods (section 3.2 and 3.14.4). GT₁ activity:
(o——-o) Basal, (e——-e) 3MC treated, (*——*) PB treated. The data give the mean±SD from set of experiments.

Fig. 2  The influence of 3MC and PB on the biochemical localization of GT activity towards 4-OH-biphenyl in rat small intestinal epithelial cells from villus tip-to-crypt gradient. The method for assay is given in section 3.14.5. Other conditions are same as in Fig. 1. GT₂ activity:
(o——-o) Basal, (e——-e) 3MC, (*——*) PB. The data give the mean±SD from four sets of experiments.
Expression and modulation of UDPGA content in rat intestinal epithelial cells, isolated in a gradient fashion from villus tip-to-crypt gradient. The assay procedure is described in section 3.16. Other conditions are same as in Fig. 1. UDPGA content; (o—o) Basal, (e—e) 3MC, (*—*) PB.

Expression and modulation of GST activity in rat intestinal epithelial cells. The data are the mean±SD from four separate experiments. Other conditions are same as described in Materials and Methods (section 2.5.1.18). GST activity; (o—o) Basal, (e—e) 3MC, (*—*) PB.
3MC-induced GT$_1$ activity along the villus-crypt surface of the intestinal epithelium. A combined treatment of PB together with dexamethasone enhanced the GT$_2$ activity marginally, and similarly as that of PB alone (Fig. 2).

4.3.2.2 **Effect of inducers on the UDPGA content of the rat Intestinal Epithelial Cells along the Villus-Crypt Surface:**

The small intestinal cells of rat were found to contain significantly high amount of UDPGA (Fig. 3). The UDPGA content was found to be in the range of 0.3 to 0.8 n moles per mg of cellular protein in the intestinal cells of villus to crypt surface; the highest content was found to be in the villus-tip cells and this decreased in gradation towards the crypt cells where the amount of UDPGA was about less than half the amount present in the villus tip cells. The UDPGA content increased in the cells isolated from 3MC treated rats; a maximum 3-fold induction in the cells of mid-villus and villus tip region while a 2-fold increase in crypt cells was evident. PB treatment did not significantly alter the UDPGA level of the epithelial cells (Fig. 3).

4.3.2.3 **Localization of the glutathione-s-transferases along the Intestinal Epithelium from Villus tip-to-Crypt surface:**

The glutathione-s-transferase activity determined towards the substrate CDNB in the rat intestinal epithelium along the villus-crypt surface is shown in Fig. 4. A significant amount of GST activity was found in the crypt cells, which increased progressively as the epithelial cells
mature into villus cells. The highest activity was in fr. 3, the cells below the villus-tip. Both PB and 3MC modulated the GST activity. PB treatment brought about a maximal induction of about 3 to 4-fold in the cells of crypt region and about 2-fold induction in the mid-villus and villus-tip cells. Exposure to 3MC induced the GST activity much more strongly than PB with a maximal induction of 5-fold in the crypt cells. The induction of GST activity is therefore sensitive to both types of inducers, PB and 3MC and this induction was evident in the entire intestinal epithelium (Fig. 4). The expressions of GST activity towards other substrates such as DCNB and epoxy-prop were below the level of detection.

4.3.2.4 Glutathione Status of Cells, along the Villus-tip-to-Crypt Region:

The GSH content was quantitated in the sequentially isolated intestinal epithelial cells (Fig. 5). The mature and well differentiated cells of the upper and mid-villus (fr. 2-6), which perform the major absorption of nutrients and xenobiotics, had the lowest GSH level. The level increased by about 6-fold towards the highly proliferating crypt cells (fr. 6-9). GSH content increased steeply in epithelial cells from villus tip-to-crypt region, although the villi tip cells contained significantly high GSH content. Treatment of animals with 3MC or PB did not modulate the in vivo level of GSH content in the intestinal epithelium (Fig. 5). Considering an average of 4.2±1.1 ul per mg protein as the intracellular volume of the intestinal cells (Hegazy et al., 1983), the endogenous
Fig. 5 The influence of 3MC and PB on the expression and modulation of GSH content in rat intestinal epithelial cells, isolated in a gradient fashion from villus tip-to-crypt gradient. The assay procedure is described in section 3.15. Other conditions are same as in Fig. 1. GSH content: (○——○) Basal, (●——●) 3MC, (*——*) PB. The data give the mean±SD from four different experiments.
concentration would thus range from about 0.8 mM in villus cells to 3 mM in crypt cells.

4.3.3 Discussion

The present studies show that both the GT and GST follow a similar distribution pattern as observed for monooxygenases (chapter 4.2) along the villus crypt surface of the small intestine which indicated that mature villus cells are well equipped with xenobiotics biotransformation system. The GT activity of the intestinal cells differed for the two groups of substrates; GT$_1$ activity towards 3-OH-BP (a planar phenol) was 6-fold higher than the GT$_2$ activity towards 4-hydroxybiphenyl (a non-planar phenol) in almost all the cell fractions eluted. The ratio of the GT$_1$/GT$_2$ activity was found to be almost consistent in the villus-crypt surface of the entire mucosa of small intestine.

3-MC and PB pretreatment showed a marked and selective induction of the GT activity towards the 3-OH-benzo(a)pyrene and 4-OH-biphenyl respectively. GT is known to be induced differentially by the two prototypes of inducing agents, 3-MC and PB (Sanchez and Teply, 1974; Bock et al., 1976) and the functional heterogeneity of GT towards two groups of substrates has been found in intestinal microsomes of rat (Bock et al., 1980) and mouse (Bock et al., 1982). Present results have explored the GT activities in cell types of different degrees of differentiation and have shown that undifferentiated cells of the crypt region are more sensitive
to 3-MC and PB types of inducers. The crypt cells appear to adapt rapidly to environmental exposure by enabling them to eliminate quickly the reactive metabolites through glucuronidation. The cells acquire a conjugation potential equivalent to that of mid-villus and villus-tip cells and, therefore, might function successfully in terminating the biological activity of the foreign compounds or its metabolites. This would offer protection to the cells against harmful drugs and chemicals.

An interesting feature of the study was the induction of GT₁ activity by PB in the crypt cells; because GT₁ activity is specifically inducible by 3MC and not by PB (Bock et al., 1983) as observed in the differentiated villus cells. This could be due to proliferation of endoplasmic reticulum of the highly dividing crypt cells by PB as reported for liver (Remmer and Merker, 1963). This is in contrast to the effect of 3MC on GT₂ activity towards 4-OH-biphenyl which was not induced by 3MC in any of the cell fraction. Further, it may be observed that the induction of GT₁ by 3MC was similar to that of AHH activity (chapter 4.2.2.2) in the whole villus-crypt surface of the small intestine and that the maximal induction of AHH and GT₁ by 3MC was localized in the crypt cells. This suggests a regulatory link between 3MC-inducible cytochrome P-450-dependent monooxygenases and GT₁ within the same cells. It is possible the genes responsible for the synthesis of these enzymes may be located on the same chromosome or closely linked which would be advantageous for an efficient and safe elimination of a variety of environmental pollutants from the intestine. In mice
the induction of the phase I and phase II reactions appear to be genetically linked (Owens, 1977). A similar link might also be suggested between the 3MC-inducible GT and UDPGA synthesis as is evidenced from our results.

Significant amount of glutathione-transferase (GST) activity towards CDNB as substrate is present in the cytosol of the intestinal cells which is largely localized in the upper-and- mid-villus cells. The cellular GST activity was markedly induced by 3MC and PB with maximal induction localized in the crypt cells as found for the corresponding induction of GT and monooxygenases. As observed GST and γ-glutamyltranspeptidase (chapter 4.1) activities are highest in the upper-and mid-villus cells and correspondingly the GSH content is found much lower in these cells as compared to the crypt cells. This is because of high turnover of GSH in these cells associated with the transport of amino acids, smaller peptides and xenobiotics biotransformation. These contrasting concentrations in enzyme activities and GSH content in the villus and crypt cells are associated with structural and biochemical functions brought about during the migration of cells from undifferentiated crypt cells to the mature villus tip cells thus equipping the later for their functional activities.

GST has been shown to catalyze the conjugation of GSH with a variety of compounds that bear a sufficiently electrophilic carbon and have a hydrophobic topography (Jakoby et al., 1976; Jakoby, 1978) e.g. epoxides formed from the
oxidation of polycyclic aromatic hydrocarbons by MFO (Fjellstedt et al., 1973; Nemato et al., 1975; Nemato and Gelboin, 1975; Hayakawa and Udenfriend, 1978) and numerous halo- and nitro-benzene derivatives (Chasseaud, 1976), etc. In small intestine GST may be the principal route of epoxide biodegradation since the levels of epoxide hydrase, a microsomal detoxifying enzyme, are extremely low in this organ (Stroming and Bresnick, 1973; Clifton and Kaplowitz, 1977). High GSH and GST activity and higher sensitivity of GST to inducers along the villus-crypt surface would suggest that enzyme may be playing an important protective role against many intestinal xenobiotics which are metabolized through the microsomal MFO system. Higher sensitivity of colonic epithelium to chemicals induction of neoplastic growth could be due to low GST activity (Weisburger, 1974).

From the above discussion it may be concluded that the phase II reactions that occur parallel to the oxidative reactions or subsequent to them are much dominating in the intestinal mucosa than reported in hepatic and other extrahepatic tissues (Hietanen, 1977; Schwenk and Locher, 1985). The activities become much pronounced with inducers especially, in the highly dividing crypt cells. The crypt cells because of their high mitotic index are much prone to the geno-cum-cytotoxic action of chemicals. Higher prevalence of conjugates thus offers protection to the small intestinal mucosa by decreasing the concentration of reactive intermediates furnished by MFO in the small intestine, and
also suggested similarly by others (Mitchell et al., 1975; Owens, 1978), and hence their availability to systemic circulation. However, there are instances where the sulfhydryl (GSH) contents were found to activate the xenobiotics such as N-methyl-N-nitrosoguanidine, 1,2-dichloroethane or 1,2-dibromo-methane (Rannug et al., 1978; Wiestler et al., 1983; Kobori, 1984). Under such circumstances the higher GSH content of the crypt cells might be a determinantal factor in contributing towards the development of intestinal malignancy due to such procarcinogens. It is, thus, the delicate balance of activation and inactivation systems determining the harmful effect of xenobiotics on the intestine. The xenobiotics conjugating capability of the small intestine appear to be much benefiting to the tissue. The present studies indicate that the intestinal cells along the villus-crypt surface are equipped with systems to inactivate the xenobiotics so as to protect the intestine from large exposure of xenobiotics and as well as to reduce their load on systemic circulation.
CHAPTER-IV

PIPERINE A NOVEL POTENT INHIBITOR OF DRUG METABOLISM AND ENHANCER OF DRUG BIOAVAILABILITY: USE OF INTESTINAL CELLS AND HEPATIC TISSUE TO ELUCIDATE ITS MECHANISM OF ACTION
4.4  
Piperine a Novel Potent Inhibitor of Drug Metabolism and Enhancer of Drug Bioavailability: Use of intestinal cells and hepatic tissue to elucidate its mechanism of action.

4.4.1  
Introduction

It is evident from the review of literature (section 1.10) that environmental factors and nature of the food greatly modulate the drug metabolizing enzymes of the intestine and other tissues which might contribute to the steady-state level of drugs and chemicals in the body. It is also known that liver is the major organ contributing to the metabolism and clearance of chemicals and drugs in the body. Thus the bioavailability of xenobiotics in the body is primarily due to the drug metabolizing capacity of the liver and that of the small intestine, the major portal of entry of compounds. One of the major promising ways to increase the bioavailability of drugs in the body is, therefore, to modulate (inhibit) the activity of enzymes that participate in the metabolic activation and inactivation pathways. It is, therefore, important to explore such compounds, especially among the commonly used natural products, which might inhibit the drug metabolism and allow the drug to be retained for longer period in the body. This would offer adequate benefits to the pharmaceutical industry and the chemotherapist in reducing the cost and side effects of the drugs, especially those which are very expensive and beyond the reach of poor man. This may also
help in reducing the risks of human exposure to procarcinogens which require metabolic activation for their in vivo biological activity.

Piperine, a major active compound of black pepper (\textit{Piper nigrum} Linn) and long pepper (\textit{Piper longum} Linn) commonly used in our spices is reported to enhance the bioavailability of test drugs in experimental animals (Atal et al., 1981). It was, therefore, speculated that piperine might be enhancing the drug bioavailability by inhibiting the drug metabolizing enzymes in vivo.

This notion was conceived while working with drug metabolizing potentials of the rat small intestine where the inhibition of monooxygenase activity of its epithelial cells indicated that piperine might inhibit these enzymes. These studies were conducted both in hepatic tissue and intestinal cells because the glucuronidation potential of intestinal cells is found to be very high (chapter 4.3) while some of the monooxygenase reaction such as ethyl-morphine-N-demethylation are not profoundly expressed in these cells (chapter 4.2.2.3). The monooxygenases inhibition by piperine was therefore explored in hepatic tissues and that of glucuronidation in the intestinal cells. As such some of the monooxygenase activities of the intestinal cells appeared similar to that of the hepatic tissue based on their specificity to inducers and inhibitors (section 1.4.3). The following studies have also established the suitability of intestinal
cells as a simple system for their use in understanding the bioavailability of drugs and modulation of xenobiotics interaction.

4.4.2 Results

4.4.2.1 Inhibition of Monooxygenases:

(a) Structure of Piperine:

Piperine was extracted from black pepper and purified in our laboratory (Atal et al., 1981). The purity of the product was determined by t.l.c. and m.p. The structure of piperine is shown in Fig.1.

(b) In vivo effect of piperine:

In vivo effect of a single high dose of piperine on AHH and GT activities of liver and intestinal cells from 3MC pre-treated rats caused 40% inhibition of hepatic AHH and GT, and 55-60% inhibition of both the enzymes in the intestinal cells (Table 1). This indicated that inhibition potential is identical in both tissues.

(c) In vitro effect of piperine on hepatic drug metabolizing enzymes:

In PMS from control rats piperine significantly inhibited all the reactions studied, with more than 50% inhibition at 50 μM (Fig.2). Similarly when we examined the effect of piperine on the activities in PMS from 3MC and PB-treated rats (Fig.3) we found significant inhibition of both the test hydroxylases and UDP-glucuronyltransferase.
Fig. 1 Structure of piperine

Fig. 2 Dose-dependent in vitro inhibition of hepatic drug metabolizing enzymes by piperine. Enzyme activities were measured in PMS from control rats as described in section 3.14.1, 3.14.2, 3.14.3, and 3.14.4. Piperine at the indicated concentrations was added in 10μl of ethanol before initiating the reaction with the respective substrate. The control tubes received identical quantity of ethanol. Data are the mean from three separate experiments each determined in duplicate. SD for each point was less than 10%. The control enzyme activities taken as 100% are: AHH (o—o), 0.275±0.024 nmoles 3-hydroxybenzo(a)pyrene/min/mg prot; 7-ECDE (△—△), 0.193±0.02 nmole umbelliferone/min/mg prot; EMD (*—*), 6.85±0.55 nmoles HCHO/min/mg prot; GT (□—□), 0.76±0.09 n mole BP-3-glucuronide/min/mg prot.

Fig. 3 In vitro effect of piperine on hepatic drug metabolizing enzymes of inducers pretreated rats. Expression of data and conditions for enzyme assays are same as described in Fig. 2. Data are the mean from two separate experiments each determined in duplicate. The coefficient of variation between two experiments was less than 10%. The control induced enzyme activities expressed as moles product formed/min/mg prot. are taken as 100%. MC induced: AHH (o—o), 1.89; 7-ECDE (△—△), 0.910; GT (□—□), 1.69; PB induced; 7-ECDE (△—△), 0.329.
FIG. 1

STRUCTURE OF PIPERINE

FIG. 2

FIG. 3
Table 1: In vivo effect of piperine on AHH and GT activities of liver and small intestinal epithelial cells from 3-MC-treated rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Liver AHH</th>
<th>Liver GT</th>
<th>Intestinal cells AHH</th>
<th>Intestinal cells GT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.9 ± 0.3</td>
<td>1.77 ± 0.2</td>
<td>0.128 ± 0.03</td>
<td>2.76 ± 0.4</td>
</tr>
<tr>
<td>3-MC + Piperine</td>
<td>1.14 ± 0.1</td>
<td>1.03 ± 0.1</td>
<td>0.057 ± 0.01</td>
<td>1.08 ± 0.2</td>
</tr>
<tr>
<td>(-40%)</td>
<td>(-42%)</td>
<td>(-55%)</td>
<td>(-61%)</td>
<td></td>
</tr>
</tbody>
</table>

Piperine, 125 mg/kg, in coconut oil was given by gavage in rats pretreated with 3-MC for 24 hrs. Animals were sacrificed 2 hrs. after piperine administration. Data are the mean ± SD of four animals. Values in parenthesis indicate % inhibition of 3MC-treated group as 100%. Specific enzyme activities were expressed as given in Fig. 2.
The studies would indicate that piperine is not a specific inhibitor of a particular drug metabolizing enzyme.

(d) **Comparative influence of 7,8-benzoflavone and piperine on AHH inhibition in vitro:**

We observed piperine was equipotent to 7,8-BF in inhibiting the AHH activity of PMS from 3MC-treated rats (Fig 4).

(e) **Kinetics of inhibition of AHH and EMD in the presence of piperine:**

The apparent Km for AHH of control rat liver microsomes with benzo(a)pyrene as substrate was found to be 30 μM and the corresponding Vmax value was 0.42 nmol of 3-OH-benzo(a)pyrene formed/min/mg protein. A Dixon plot of kinetic data indicated that the inhibition was non-competitive with a Ki of 30 μM which was similar to the Km of control rats (Dixon and Web, 1979) (Fig.5). In microsomes from 3MC-pretreated rats, the apparent Km was 11 μM and the Vmax value was 1.33. The Vmax decreased similarly to that of the control microsomes in the presence of piperine and again the nature of inhibition found was non-competitive. The value of half maximal velocity Ki of enzyme inactivation was 30 μM similar to the apparent Km of AHH from control rats.

In view of the profound insolubility of benzo(a)pyrene, it was considered important to determine also the kinetics of inhibition with a soluble substrate ethylmorphine. Like AHH piperine caused non-competitive inhibition of EMD with Km value of 0.8 mM (Fig.6). The Ki was
Fig. 4 Relative inhibition of AHH activity in hepatic PMS from 3MC-treated rats by 7,8-BF and piperine. Both the inhibitors were added in 10µl DMSO (1%, v/v) while the controls only received DMSO. Data are the mean±S.D. from three separate experiments conducted in duplicate. Percent inhibition was calculated from the control value taken as 100.

Fig. 5 Dixon plot of BP metabolism by AHH in the presence of various fixed concentrations of piperine using control hepatic microsomes.
Fig. 6 Lineeweaver-Burk plot of ethylmorphine metabolism by EMD in the presence and absence of various fixed concentrations of piperine.

Fig. 7 Dixon plot of ethylmorphine by EMD in the presence of various fixed concentrations of piperine using control hepatic microsomes.
35 uM (Fig. 7) which was close to that observed for AHH.

(f) **In vivo effect of piperine:**

A single oral dose of piperine (125 mg/kg) caused a maximal hepatic inhibition of AHH in control rats at 1 hr (Fig. 8). The enzymatic activity returned to the control value at 6 hours.

Since the dose used in the above experiments was very high and might have caused structural alterations at the molecular level, we observed the low-dose effect of piperine in vivo on the hepatic AHH and GT activities of control rats (Table 3). AHH and GT were inhibited 50% and 80% respectively at doses of 10 mg and 25 mg per kg b.w. Similarly GT activity was inhibited by 36% and 55% at these respective doses. This suggested that piperine at low doses is very effective.

(g) **Effect of piperine on hexobarbital sleeping time and zoxazolamine paralysis time:**

Piperine enhanced hexobarbital induced sleeping time in mice. Oral administration of 5 mg/kg b.w. increased sleeping time by about 50% and a 10 mg dose enhanced by 2.5-fold (Fig. 9).

Intraperitoneal administration (Table 3) of piperine 2.5 mg and 5 mg/kg enhanced hexobarbital sleeping time by about 70% and 220% respectively. Likewise the
Table 2: In vivo effect of piperine on AHH and GT from PMS of control rat

Each data point is geometric mean from three animals with less than 15% of variation. Piperine was given orally in coconut oil in empty stomach untreated control rats. Animals were sacrificed one hour after the piperine administration. Specific enzyme activities are expressed as described in Fig. 2.

<table>
<thead>
<tr>
<th>Piperine dose (mg/kg b.w.)</th>
<th>AHH</th>
<th>% Inhibition</th>
<th>GT</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>0.286</td>
<td>-</td>
<td>1.26</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>0.144</td>
<td>50</td>
<td>0.811</td>
<td>36</td>
</tr>
<tr>
<td>25</td>
<td>0.058</td>
<td>80</td>
<td>0.57</td>
<td>55</td>
</tr>
</tbody>
</table>
Fig. 8 Time course of inhibition and recovery of hepatic AHH after oral administration of piperine. Piperine (125 mg/kg b.wt.) was given orally in coconut oil to untreated control rats. Controls received the vehicle only. Restoration of enzyme activity was measured in PMS. The data are mean±SD from four rats.

Fig. 9 Piperine mediated enhancement of hexobarbital induced sleeping time in mice. Piperine in coconut oil was given orally at the indicated doses. One hour later hexobarbital was administered i.p. and sleeping time recorded.
Table 3: Effect of piperine and SKF-525A on hexobarbital induced sleeping-time and zoxazolamine paralysis-time in Swiss albino mice.

SKF-525A was dissolved in normal saline, piperine in small quantity of alcohol and mixed with refined coconut oil and zoxazolamine was dissolved in HCl/saline (Conney et al, 1960). Thirty min. after i.p. injection of SKF-525A or piperine, hexobarbital (60 mg/kg) or zoxazolamine (100 mg/kg) were i.p. administered.

<table>
<thead>
<tr>
<th>Modifier</th>
<th>Dose (mg/kg)</th>
<th>Sleep time (min)</th>
<th>Paralysis time (min)</th>
<th>Survivor/total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>-</td>
<td>36 (30-48)</td>
<td>72 (60-88)</td>
<td>5/5</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>2.5</td>
<td>110 (90-120)</td>
<td>N.D.*</td>
<td>5/5</td>
</tr>
<tr>
<td>SKF-525A</td>
<td>5.0</td>
<td>215 (212-227)</td>
<td>N.D.*</td>
<td>5/5</td>
</tr>
<tr>
<td>Piperine</td>
<td>2.5</td>
<td>60 (52-68)</td>
<td>116 (100-139)</td>
<td>5/5</td>
</tr>
<tr>
<td>Piperine</td>
<td>5.0</td>
<td>116 (104-130)</td>
<td>170 (145-190)</td>
<td>5/5</td>
</tr>
</tbody>
</table>

Five mice were used in each group. Data are geometric means with 2 S.E. limits. N.D. Not Determined.
zoxazolamine paralysis time increased by 50% and 140% with 2.5 and 5 mg doses of piperine. SKF-525A was as much effective at half its concentration of piperine.

4.4.2.2 Inhibition of Glucuronidation Rates:

(a) Influence of piperine on viability of intestinal cells:
Viability of the freshly isolated cells was more than 90% and this was not significantly altered when incubated in the presence or absence of 25 µM piperine over a period of 30 min (Fig. 10). Further, the effect of 50 µM piperine was not dissimilar to 25 µM of piperine (not shown).

(b) Rates of 3-OH-BP glucuronidation in the intestinal cells and the effect of piperine:
Rate of glucuronidation of 3-OH-BP measured in the intact cells was found to be substrate dependent and non-linear (Fig. 11). The non-linear formation of BP-3-glucuronide was similarly seen when cells were incubated with higher substrate concentration (100 µM) and variable cellular protein content (Fig. 12). The rate of formation of BP-3-glucuronide (per mg protein) was found higher at lower protein content, but the amount formed was higher at higher protein content. The appearance of BP-3-glucuronide either in the medium or in the cells did not show any lag phase. The cells appeared not to accumulate glucuronide and begin excreting into the medium soon the conjugate is formed within the cells. The double reciprocal analysis of the data indicated a Km value of about 25 µM with
Fig. 10 Viability of intestinal cells: Guinea pig intestinal cells (approx. 3 mg protein/ml) were incubated as described in section 3.17.1. After 5 min of pre-incubation, 25 μM piperine in dimethylsulfoxide was added (●). The controls received only the vehicle (●). Aliquots at indicated time periods were withdrawn and counted for viability (section 3.6). The data are means from two separate experiments.

Fig. 11 3-OH-BP dependent rate of glucuronidation in the isolated intestinal cells with different concentrations of 3-OH-BP. 100 ul aliquots were removed for glucuronide measurements. Details for the incubation of cells and determination of BP-3-glucuronide are given in section 3.17. (○) medium, (●) cells, (●) total. Insert: Line-Weaver-Burk plot of glucuronidation of 3-OH-BP in the isolated cells. Other conditions were same as described above *, total BP-3-glucuronide (1/v). The data are means from two separate experiments.
3-OH-BP substrate and maximal rate of glucuronidation of about 0.5 n moles per min per mg protein (Fig.11) was observed.

Piperine diminished the rate of glucuronidation at all protein concentrations (Fig.12) or incubation times of 1, 5, 10 and 15 min tested (data not shown) and caused a concentration related inhibition of 3-OH-BP glucuronidation described in the following. At about 50 μM piperine, the rate of glucuronidation was reduced to about 50 percent of the basal rate.

(c) Piperine-mediated modification of UDPGA in the intestinal cells and comparison with D-galactosamine:

After six minutes of equilibration in the incubation medium the epithelial cells were found to contain about 1.6 to 2.3 nmoles of UDP-glucuronic acid per milligram of cellular protein (equivalent to approx. 2.3 x 10^6 cells). The synthesis of UDPGA followed a linear increase at least for 20 min of incubation when the enhanced endogenous level of the cofactor observed was 25% above the zero minute control (Fig.13). Piperine at a concentration as low as 0.5 μM arrested this increase in the endogenous UDPGA content and further caused a concentration and time-dependent decrease in intracellular UDPGA content in the cells (Fig.13). Piperine at 1, 25, 50 and 100 μM lowered the intracellular UDPGA content by 20, 32, 42 and 57%. When we simultaneously determined the GT activity in these cells we observed a strong correlation between the intracellular UDPGA and BP-3-glucuronide formed.
Fig. 12  Rate of 3-OH-BP glucuronidation in the isolated cells and its dependency on protein content: Cells were preincubated for 3 min in 2 ml of incubation volume with indicated protein per ml. Cells in each vial were incubated further for 2 min with 50 μM piperine prior to the addition of 3-OH-BP (100 μM). 100 μl aliquots were removed after 5 min for silicon oil microcentrifugation and BP-3-glucuronide formed was measured. a—without piperine; b—with piperine: (•) cells, (○) medium, (*) total.

Fig. 13  Effect of piperine on UDPGA levels in isolated intestinal epithelial cells: After preincubation of the cells (3 mg/ml) for 5 min various amounts of piperine were added to the cell suspension. Final concentrations of piperine: (•) 0.0 μM, (•) 0.5 μM, (△) 1 μM, (○) 10 μM, (□) 25 μM, (△) 50 μM, (+) 100 μM. Details of cell incubation and determination of UDPGA are given in Materials and Methods (section 3.16.2 and 3.17.1). The data are expressed as percent of the UDPGA concentration present in the cells after an incubation time of 1 min in the absence of piperine. 100% amounts to 1.58±0.35 nmole UDPGA/mg protein. The values gave mean±SD from three different experiments.
(Fig. 14). Piperine at 50 μM caused about 50% inhibition of BP-3-glucuronide formed.

When we compared the UDPGA lowering effect of piperine with D-galactosamine, the former was found much more stronger than the latter; piperine at 1 and 25 μM produced similar effect as 1 and 2 mM D-galactosamine after 20 min of incubation with the cells. D-galactosamine caused a concentration and time-dependent fall in endogenous level of UDPGA (Fig. 15) which simultaneously reflected in the co-factor dependent rate of glucuronidation in the intestinal epithelial cells similar to the one observed in Fig. 14 (data not shown).

(d) **In vitro effect of piperine on UDPGA degradation:**

We considered perhaps the lowering of UDPGA content might have been the consequence of the in situ stimulation of pyrophosphatase activity towards UDPGA by piperine. Liver microsomes from male guinea pig contain negligible amount of this enzyme activity (Wong and Lau, 1970) and when the microsomal fraction was incubated with 50 and 200 nmoles UDPGA per ml for 5 and 10 min in the presence or absence of 25 μM piperine, the recovery of UDPGA was quantitatively similar, i.e. about 90% (data not shown). However, when the frozen-thawed intestinal cells were incubated with variable UDPGA content (50-400 nmoles) or with 50 μM piperine and 50 or 100 nmoles UDPGA a significant loss in the recovery of UDPGA was noticed (Fig. 16). This loss was, however, consistent whether the cells were incubated with or without piperine.
**Fig. 14** Piperine mediated lowering of UDPGA in cells and correlation of cellular UDPGA levels with glucuronidation activity. Cells were incubated with piperine as described in Fig. 13. After incubation of intestinal cells for 19 min with piperine, 200ul aliquots were taken for determination of UDPGA, one min later 3-OH-BP (100 μM) was added and glucuronide formation was assayed after 30, 60 and 90 seconds. Initial rates of glucuronidation were linear and are plotted versus the corresponding UDPGA levels. Data are from two separate experiments represented by open and closed circles.

**Fig. 15** Effect of D-galactosamine on UDPGA levels in isolated intestinal epithelial cells. Conditions were similar to that of Fig. 13. Final concentrations of D-galactosamine: (o) 1 mM; (*) 2 mM, (△) 3 mM, (□) 4 mM, (●) 0.0 mM. 100% amounts to 1.57±0.4 nmoles UDPGA/mg protein. The values give mean and ranges from three different experiments.
**FIG. 14**

![Graph showing the relationship between UDPGA (n moles/mg prot) and G-activity (n moles/mg prot).](image)

**FIG. 15**

![Graph showing the effect of D-galactosamine (mM) on UDPGA (% of untreated control) over incubation time (min).](image)
This may apparently preclude any possibility of piperine facilitated break-down of UDPGA enzymatically or non-enzymatically.

(e) In vitro inhibition of UDPGT and kinetics of inhibition:

Piperine caused a concentration related inhibition of GT activity of the intestinal cells and a hepatic microsomal fraction (Fig. 17). Fifty percent inhibition of the transferase activity was observed at 50-80 uM piperine. The apparent Km for GT of control guinea pig liver microsomes for 3-OH-BP as substrate was found to be 10 uM and the corresponding Vmax value was about 5 mmoles per minute per milligram protein (Fig. 18). A Dixon plot of the kinetics data (Dixon and Web, 1979) indicated the inhibition was non-competitive with a Ki of about 70 uM (Fig. 19).

4.4.3 Discussion

Among the three substrates utilized in the present study 7-ethoxycoumarin is a universal substrate metabolized by both forms of cyt.P-450 and cyt.P-448, ethylmorphine by cyt.P-450 and benzo(a)pyrene by cyt.P-448 (Lake and Paine, 1982). These three substrates metabolized by various isozymes of cyt.P-450 were taken to cover up a major proportion of drug metabolism for interaction with piperine and as well to evaluate any selectivity of piperine towards a particular inducer-specific monooxygenase form. The present studies demonstrated that piperine causes concentration related inhibition of 7-ethoxyvoumarin deethylase, ethylmorphine
**Fig. 16** Effect of piperine on the in vitro degradation of UDPGA. Different concentrations of UDPGA were incubated with frozen-thawed guinea pig intestinal cells (1 mg/ml) in the absence and or in the presence of 50 μM piperine. The reaction was terminated at indicated time intervals by immersing the tubes in boiling water bath for 3 min. The denatured protein was spun down and the supernatant was used for measurement of UDPGA as described in Materials and Methods (section 3.16.2). UDPGA (nmol/ml): (□) 400, (*) 200, (△) 100, (○) 50, (●) 50 μM piperine, 50 nmol UDPGA. The values give mean from two separate experiments and the variation between two experiments was not more than 10%.

**Fig. 17** Concentration related inhibition of GT activity in vitro in guinea pig intestinal cells and hepatic microsomes by piperine. Data are the mean and range of two separate experiments conducted in duplicate. Enzyme activity was measured in guinea pig liver microsome (○) and frozen-thawed intestinal cells (△) during the initial linear phase of the reaction in the presence of brij-58. The time of incubation was 3 min. Piperine at the indicated concentrations was added before initiation of reaction with 3-OH-BP. Other conditions were same as described in 'Materials and Methods' (section 3.14.4). 100% GT activity in liver microsomes amounts to 3.76±0.5 nmol/min/mg prot; in intestinal cells as 1.47±0.23 nmol/min/mg prot.
Fig. 18  Lineweaver-Burk plot of 3-OH-BP glucuronidation by GT in the presence of piperine using hepatic microsomes from male guinea pigs. Piperine was introduced in 10 μl of DMSO before the reaction was started with 3-OH-BP. The data are representative of two separate experiments.

Fig. 19  Dixon plot of 3-OH-BP glucuronidation in the presence of various fixed concentrations of piperine using hepatic microsomes from untreated male guinea pig. Other conditions were same as described in Fig. 18.
demethylase and benzo(a)pyrene hydroxylase activities in vitro. The concentration of piperine which caused about 50% inhibition was less than 50 μM almost for all the enzymes studied. Thus piperine caused similar quantitative inhibition of various forms of monooxygenases whether the enzymes were induced by PB or 3MC or existed in the native form. This is unlike 7,8-benzoflavone which inhibits strongly hepatic cyt.P-450 form (Wiebel, 1980). The way piperine caused common inhibition of the oxidative microsomal drug metabolism in vitro is not understood. From the kinetic studies of AHH and EMD, it is however, apparent that it causes non-competitive inhibition in the presence of piperine which indicated that the substrate affinity towards the enzyme is not affected, rather the enzyme-substrate complex is rendered inactive in the presence of piperine. The inhibition constant (K_i) i.e. the value of half maximal velocity of enzyme inactivation in the presence of piperine was found to be similar to both the control and 3MC-induced AHH. Similar kinetics of inhibition of EMD from control hepatic microsomes with soluble substrate ethylmorphine, was observed in the presence of piperine. These studies suggested that 1) piperine does not distinguish between the various monooxygenase forms and that 2) the biotransformation of both the soluble and insoluble substrates may be affected similarly in the presence of piperine.

The in vitro inhibition of hepatic UDP-glucuronyl transferase, both untreated and induced, suggests piperine's
general affinity towards microsomal drug metabolism, though further studies are needed in this direction. A strong correlation between the \textit{in vitro} and \textit{in vivo} inhibition of drug metabolism was found in the present study. This is evidenced from AHH and UDP-glucuronyltransferase inhibition of rat liver after oral administration of piperine. The \textit{in vivo} inhibition is further reflected in terms of its biological response in mice. Piperine enhanced hexobarbital induced sleeping time and paralysis time of the muscle relaxant zoxazolamine. These assays have been widely used as direct measures of hepatic drug metabolism (Conney, 1967; Wiebel \textit{et al.}, 1976; Workman \textit{et al.}, 1983). Further piperine required that of double the quantity of SKF-525A to arrive at the same enhanced sleeping time as achieved by SKF-525A alone, though the former was dissolved in coconut oil and the xenobiotics absorption rate through this vehicle is not known an yet. The \textit{in vivo} inhibition of AHH and its complete recovery within six hrs is considered desirable for the reasons that piperine binding to microsomes considering AHH inhibition, is reversible and apparently does not cause permanent damage to the drug metabolizing system at lower concentrations. Since the piperine-mediated inhibition potential of drug metabolism is sufficiently high at very low doses and that the reported LD_{50} values of piperine in mice, rats and hamsters are very high (Piyachaturawat \textit{et al.}, 1983), this natural product may, therefore, benefit adequately in improving the therapeutic indices.
Piperine has been shown in the present studies to inhibit the GT activity \textit{in vitro} and diminish the endogenous rate of glucuronidation in the isolated epithelial cells of the guinea pig small intestine. Similar to isolated rat hepatocytes (Singh and Schwarz, 1981), the intracellular UDPGA level registered a linear increase with time of incubation of cells. A tendency to rise with incubation has also been shown for UTP, UDP-glucose and UDPGA in the isolated rat hepatocytes (Hofmann \textit{et al.}, 1976; Singh and Schwarz, 1981). The present studies thus suggest that the intestinal cells are a convenient and suitable system for studying the \textit{in vivo} modification of glucuronidation activity.

The present studies have revealed that piperine modifies the rate of glucuronidation by lowering the intracellular level of UDPGA. This cofactor is a donor substrate for the enzyme GT and the \textit{in vivo} activity of GT has been found to depend on the endogenous concentration of this cofactor (Singh and Schwarz, 1981). Piperine has similarly caused concentration and time-dependent decrease in the UDPGA content and the rate of formation of BP-3-glucuronide in the cells. This indicated stronger dependency of \textit{in vivo} rate of glucuronidation on its intracellular level of UDPGA. Therefore, the decreased rate of glucuronidation \textit{in vivo} by piperine suggests that the alkaloid might be lowering the UDPGA content possibly by inhibiting its synthesis rather than facilitating its break-down. However, further in depth studies are required to prove these notions. The effect appeared analogous to D-galactosamine which has been
shown to inhibit UDPGA synthesis by trapping UTP and impairing
the UDP-glucose dehydrogenase activity (Keppler et al., 1970;

GT is a membrane bound microsomal enzyme whose
activity is prone to modulation by membrane perturbants
particularly those which interfere with the phospholipid
environment of the enzyme (Dutton et al., 1977). Piperine
being lipophilic might be binding at a site other than the
active site thereby rendering the enzyme-substrate complex
inactive. This is evidenced from the nature of in vitro
inhibition of the transferase activity. SKF-525A is a potent
inhibitor of drug metabolism and similar kinetics of non-
competitive inhibition of GT towards the substrate p-aminophenol
has been observed (Dvbing and Ruggstad, 1973). With the
microsomal in vitro system, it is not feasible to suggest if
piperine would inhibit the GT similarly in vivo. Nevertheless,
it seems to exercise its effect on GT activity by inhibiting
GT activity and by restricting the availability of UDPGA. The
latter mode of action appears more operative in view of the
high apparent Ki (70 uM) for GT and the in vivo modification
of UDPGA-dependent rate of glucuronidation by piperine.

UDPGA is synthesised by the action of cyto-
plasmic soluble enzyme, UDP-glucose dehydrogenases, on NAD-
dependent conversion of UDP-glucose to UDP-glucuronic acid.
It is quite possible that piperine might be inhibiting the
synthesis of UDPGA by inactivating the enzyme UDP-glucose
dehydrogenase. However, further studies are required to prove this notion. Regardless of the structural diversity of acceptor substrates and heterogeneity of the GT forms (Kasper and Henton, 1980; Bock et al., 1983), UDPGA is singularly a common donor substrate of all the GT forms. The inhibition of the transferase and decrease in UDPGA synthesis would consequently affect the metabolic conversion of numerous compounds which require glucuronic acid conjugation during intestinal first-pass or systemic circulation. This might also affect the steady-state level of these drugs and compounds which are directly glucuronidated in man without undergoing monooxygenase's action, for instance, drugs such as phenylbutazone used in the treatment of rheumatic diseases (Aarbakke et al., 1977) and tripelonnamine, an antihistaminic drug (Chaudhuri et al., 1976).

From the above discussion it may be concluded that piperine would exert dual effects in vivo: 1) at the level of enzyme-substrate (acceptor) complex and 2) by lowering the endogenous level of the donor substrate, UDPGA. The studies, therefore, indicate that piperine is a potent inhibitor of glucuronidation and could alter the disposition of a large number of drugs which require conjugation with UDPGA.

In conclusion, the present studies provide first evidence that piperine is a potent unspecific inhibitor of drug metabolism. Further studies are needed to delineate precisely its interaction with microsomes and evaluate its cyto/genotoxicity. Being a natural product considered here to
be of less toxicological consequences piperine might find useful applications in successful drug therapy and an important addition to the chemotherapist armoury for the quantitative enhancement of drugs bioavailability.
CHAPTER V

USE OF INTESTINAL EPITHELIAL CELLS IN ASSESSMENT OF CYTO-CUM-GENOTOXICITY OF XENOBIOTICS
4.5 Use of Intestinal Epithelial Cells in Assessment of Cyto-cum-Genotoxicity of Xenobiotics

4.5.1 Introduction

Various cell systems, from isolated cell suspension to established mammalian cell cultures, are increasingly used for studying the harmful nature of xenobiotics (Malik et al., 1983). Epithelial cells of the small intestine were explored in the present study to assess the cyto-cum-genotoxicity of chemicals. The primary target of chemico-biological interaction of xenobiotics with the cells is the plasma membrane and membranes of the subcellular organelles. This may allow the discharge of the intracellular material into the extracellular medium which may consequently result into the disintegration of the cellular functions if the primary damage is not repaired by the cells immediately. The other major risk of xenobiotics exposure is that some of the chemicals may directly or after activation through drug metabolizing enzymes alkylate DNA and consequently result in genetic damage. If the genetic damage is not repaired correctly it might lead to the development of malignancy (Section 1.13).

Intestinal cells were, therefore, used in the present study to understand such interactions and an attempt has been made to find out the possibilities and limitations of using this system. A number of procedures for evaluating...
membrane damage are available which involve the monitoring of either influx or efflux of marker molecules. The severity of membrane lesions may be estimated by determining the leakage of markers of varying molecular sizes ranging from $K^+$ (Medzhiradsky and Marks, 1975; Chen et al., 1977; Stacey and Klaassen, 1981), amino acids (Madoff et al., 1963; Thelestam and Möllby, 1975b, 1976, 1980a, 1980b; McClane and McDonel, 1980), nucleotides (Henney, 1973; Thelestam and Möllby, 1975a, 1976, 1980a, 1980b; McClane and McDonel, 1980) to macromolecules such as nucleic acids or proteins (Medzhiradsky and Marks, 1975; Thelestam and Möllby, 1975a, 1976; Miller et al., 1979; Thelestam and Möllby, 1980a, 1980b; Szinicz and Wegner, 1980; Stacey and Klaassen, 1981). Radioactive uridine, $\text{L}$-AIB, LDH and alkaline phosphatase were used as markers in this study. For interference with the integrated functions of the complete cells, measurement of the rate of $O_2$ respiration of the cells was considered as a more appropriate and sensitive parameter and necessary studies in this connection were also taken up.

For assessment of damage to genome, isolated intact intestinal cells may offer some advantage over the direct interaction with DNA in vitro. Because these cells have been shown to possess various levels and forms of drug metabolizing enzymes which may bear upon the genotoxic potential of chemicals. Therefore the effect of xenobiotics on the DNA of the same cell, which possesses a particular battery of phase I and phase II reactions, might predict damage of organ-specific nature because
it is due to the consequence of the balance of inactivation and activation potentials of the cells in converting a procarcinogen into a carcinogen. In the last decade a highly sensitive technique for detecting DNA strand breaks has been developed (Kohn et al., 1976). The technique took advantage of the fact that alkylation of DNA induces alkali-labile sites in DNA and the later falls apart into small single strands at higher alkaline pH. The technique called alkaline elution assay separates single strand DNA breaks on the basis of its length. It uses an inert filter of small pore size to mechanically impede the passage of long DNA strands while allowing shorter strands to pass through more rapidly. The elution of DNA, therefore, would be related to the extent of DNA damage by xenobiotics.

4.5.2 Results

4.5.2.1 Use of intestinal cells for assessment of cytotoxic action of xenobiotics in vitro based on the leakage of bio-chemical markers:

(a) Labelling conditions:

All these observations were made in the male guinea pig small intestinal cells (enterocytes). Variation in composition of the incubation medium, concentration of the radioactive marker, temperature, pH and time of labelling strongly varied the uptake and release of $^3$H-$\alpha$-AIB and $^3$H-uridine. A linearity in the $^3$H-$\alpha$-AIB uptake was observed for about 5 min which appeared saturated after 30-40 min (Fig.1). Cells in this case were incubated in Hank's basal salt solution supplemented with 10 mM Heps and 0.5% BSA. $^3$H-$\alpha$-AIB at 1 uCi/ml of
Fig. 1  Time-dependent uptake of $^3$H-Uridine (Δ—Δ) and $^3$H-α-AIB (○—○) in isolated guinea pig intestinal epithelial cells. The in vitro uptake studies were conducted as described in section 3.18.1 and 3.18.2. Each time point gives the mean±SD for replicate samples from at least three separate experiments.

Fig. 2  Time course of spontaneous release of $^3$H-α-AIB (○—○), $^3$H-Uridine (□—□), LDH (Δ—Δ) and AP (*—*) from guinea pig intestinal cells. The release of radioactivity from the prelabelled isolated intestinal cells and the maximal release was measured as described in Materials and Methods (section 3.18.2a,b).
incubation medium was found to be optimal for labelling the cells which strongly depended on the composition of the incubation medium (Table 1). The cellular uptake of $^3$H-$\alpha$-AIB was very low in Eagle's medium which might be due to the presence of other competing amino acids in the medium. The uptake was stimulated by glucose in PBS and was optimal in Hank's BSS containing 10 mM Hepes. In case of $^3$H-uridine labelling, a concentration of 1 uCi/ml was found to be optimal. Prewarming of $^3$H-uridine solution at 37°C before addition of cells produced better incorporation of uridine. Like $^3$H-$\alpha$-AIB, uridine incorporation was strongly dependent on the composition of the incubation medium (Table 1). Hank's BSS supplemented with 10 mM Hepes and 0.5% BSA appeared to be the most suitable cell incubation medium for the isolated enterocytes. A linear incorporation pattern of $^3$H-uridine was observed for about 5 min (Fig.1) in the acid insoluble fraction which was 10% of the total freely available $^3$H-uridine in the cells.

(b) Spontaneous release of $\alpha$-AIB, uridine, LDH and Alkaline phosphatase:

Spontaneous release of $^3$H-$\alpha$-AIB and $^3$H-uridine during standard incubations of 20 min at 37°C and pH 7.4, was studied in different media (Table-1). The lowest release of $\alpha$-AIB (30%) was in Hank's BSS supplemented with Hepes and glucose. The time-dependent release of $^3$H-$\alpha$-AIB was almost linear at least for 20 min (Fig.2). Addition of 1 mM cold $\alpha$-aminoisobutyric acid to the incubation system lowered the spontaneous release by about 10-15% of this low molecular weight
Table 1: Cellular uptake of $\alpha$-AIB and uridine incorporation into guinea pig intestinal cells in different media and their spontaneous release

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Uptake (cpm/mg prot/20 min)</th>
<th>Uptake (% of maximal release)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-AIB</td>
<td>Uridine</td>
</tr>
<tr>
<td>Hank's BSS</td>
<td>5323 ± 166</td>
<td>316 ± 26</td>
</tr>
<tr>
<td>Good's medium (Good et al, 1966)</td>
<td>4296 ± 153</td>
<td>346 ± 59</td>
</tr>
<tr>
<td>Dulbecco's PBS + 0.1% glucose</td>
<td>4870 ± 137</td>
<td>410 ± 62</td>
</tr>
<tr>
<td>Eagles medium</td>
<td>3266 ± 123</td>
<td>283 ± 38</td>
</tr>
<tr>
<td>Hank's BSS + 10 mM Hepes + 0.5% BSA</td>
<td>7550 ± 183</td>
<td>425 ± 45</td>
</tr>
</tbody>
</table>

The uptake and spontaneous release of $\alpha$-AIB and uridine in different extracellular fluids over the 20 min incubation at 37°C were measured as described in section 3.1.3. Values given are the mean±SD from 3 different experiments.
radioactive marker. The spontaneous release of \( ^3 \text{H} \)-uridine was of very low magnitude (Table 1 and Fig. 2).

Release of LDH and alkaline phosphatase (AP) were also monitored as indicators of severe membrane damage (Table 2). The spontaneous release of the two enzymes was studied under various conditions (Table 2 and Fig. 2). The rate of appearance of LDH and AP in the extracellular fluid in the radioactive labelled and washed cells is shown in Fig. 2. In the above standard incubation system a spontaneous release of LDH and AP was found to be negligibly small (6-15%). The percent spontaneous release of AP (15%) in all the cell incubations was almost double of that observed for LDH (6%) (Table 2).

For all practical purposes the release of the markers was followed for a 20 min period so that the viability of the cells is not affected. The release of all the four parameters was studied in the same cell preparation which has already been loaded with radioactive labels for 20 min, and washed off the free radioactivity. Moreover, the limited exposure period to potentially toxic agents was thought to be advantageous in that more likely the primary lesions of the membrane are assessed.

(c) Toxin cum xenobiotics-induced release of \( \alpha \)-AIB, uridine, LDH and alkaline phosphatase:

For evaluating the sensitivity and specificity of the 'leakage tests', the intestinal cells were exposed to three agents which are known to cause membrane damage
Table 2: Leakage of lactic dehydrogenase and alkaline phosphatase from intestinal epithelial cells in different media in vitro

<table>
<thead>
<tr>
<th>Incubation media</th>
<th>LDH (% of maximal release/60 min)</th>
<th>Alkaline Phosphatase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Modified DMEM</td>
<td>11 ± 4</td>
<td>20 ± 6</td>
</tr>
<tr>
<td>Good's medium (Good et al, 1966)</td>
<td>12 ± 3</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>MEM with Earle's salts</td>
<td>25 ± 3</td>
<td>42 ± 7</td>
</tr>
<tr>
<td>F-10-HAM</td>
<td>26 ± 5</td>
<td>30 ± 6</td>
</tr>
<tr>
<td>Eagles MEM with Earle's salts</td>
<td>21 ± 4</td>
<td>25 ± 5</td>
</tr>
<tr>
<td>Hank's BSS + 10 mM Hepes + 0.5% BSA</td>
<td>6 ± 1</td>
<td>15 ± 2</td>
</tr>
<tr>
<td>MEM Hank's</td>
<td>14 ± 2</td>
<td>20 ± 3</td>
</tr>
</tbody>
</table>

Isolated intestinal cells of guinea pig small intestine were incubated for 60 min at 37°C and the spontaneous release of LDH and AP in the different incubation media was measured as described in section 3.18.5. Spontaneous release is expressed as percentage of maximal release determined in 0.5% Triton-X-100 treated cell incubated for 30 min. Data are mean±SD from four different experiments.
of various types and degrees. Two of these, the non-ionic detergent Triton-X-100 and the anionic detergent sodium dodecyl-sulfate (SDS) disrupt the structural integrity of the membrane by insertion into the lipid phase of membrane (Helenius and Simons, 1975). The third compound, nystatin, a polyene antibiotic complex with membrane sterols to form minute hydrophilic pores increasing the ion permeability (Weissmann and Sessa, 1967). The damage was compared to various test xenobiotics.

Treatment of enterocytes with Triton-X-100 caused a dose-dependent leakage of all the four markers in a particular sequence. The maximal release of these cytoplasmic markers with 0.01% Triton-X-100 was 80% for $^3$H-$\alpha$-AIB, 40% for $^3$H-uridine followed by 30% of AP and 15% of LDH (Fig.3). With SDS the leakage of high molecular weight species LDH and AP was negligibly low. Leakage of $^3$H-uridine and $^3$H-$\alpha$-AIB appeared rapidly in the extracellular medium with SDS concentrations above 0.1 mM. The maximal release was almost comparable with 0.01% Triton-X-100 (Fig.4). Enterocytes exposure to nystatin (Fig.5) caused a significant loss in $\alpha$-AIB and uridine from the cytoplasm. Nystatin at a concentration of 50 ug/ml brought about a loss of 42% of intracellular $^3$H-$\alpha$-AIB and 12% of uridine while 30% of $^3$H-$\alpha$-AIB was lost even at concentration as low as 25 ug/ml. Leakage of LDH and AP, however, was not detectable.

The leakage of the various cytoplasmic markers produced by various xenobiotics are shown in Fig.6. Addition of aspirin brought about a significant loss of $^3$H-$\alpha$-AIB
Fig. 3-5 Leakage of $^3\text{H}-\text{L}-\text{AIB}$, $^3\text{H}$-Uridine, LDH and AP from guinea pig intestinal cells exposed to membrane-active compounds. The leakage of $^3\text{H}-\text{L}-\text{AIB}$ (○-○), $^3\text{H}$-Uridine (□-□), LDH (▲▲▲) and AP (•••••) was determined following exposure to various concentrations of Triton-X-100, CDS and Nystatin as described in Materials and Methods. (section 3.18.3c). Nystatin was dissolved in DMSO and further diluted with cell incubation medium (section 3.18.1). The maximum concentration of DMSO used (0.1% final) was without detectable effect on the plasma membrane.
Leakage of $^3$H-$\alpha$-AIB (o—o), $^3$H-Uridine (□—□), LDH (△—△) and AP (*) from intestinal cells exposed to xenobiotics. The leakage was determined following exposure to various concentrations of (a) aspirin, (b) Indomethacin, (c) DMH, (d) BQ, (e) B(a)P and (f) Endosulfan.
FIG. 5

NYSTATIN

PERCENT OF MAXIMAL RELEASE

5 25 50

CONCENTRATION µg/ml

FIG. 6a

ASPIRIN

PERCENT OF MAXIMAL RELEASE

0.01 0.1 1

CONCENTRATION (mM)
FIG. 6f

PERCENT OF MAXIMAL RELEASE

ENDOSULFAN

CONCENTRATION (mM)

0-01  0-1  1
from the cells which was 23% and 54% at concentrations of 0.1 mM and 1 mM (Fig. 6a). On the contrary, 1 mM aspirin produced only a 5-10% loss of $^3\text{H}$-uridine, LDH and AP into the extracellular medium. Ingomethacin produced similar but less marked effects (Fig. 6b) at concentrations comparable to aspirin. Dimethyl-hydrazine (DMH)-induced leakage of the four cytoplasmic markers was almost similar to aspirin-mediated effect. DMH at 1 mM produced 71% leakage of $^3\text{H}$-$\alpha$-AIB and 20% of uridine while the leakage of LDH and AP was negligibly small (Fig. 6c). Benzoquinone, a metabolite of benzene at 1 mM was relatively found less toxic as evidenced by its only significant leakage of $\alpha$-AIB as compared to other markers causing severe damage (Fig. 6d). Benzo(a)pyrene caused a concentration-dependent release of $^3\text{H}$-$\alpha$-AIB (Fig. 6e). At 1 mM B(a)P, 51% leakage of $^3\text{H}$-$\alpha$-AIB, 18% of uridine, 10% of LDH and 15% of AP was observed. The leakage of high molecular species predicts that the damage to the plasma membrane by B(a)P may be more of serious nature. Endosulfan caused a similar effect as obtained by B(a)P (Fig. 6f).

4.5.2.2 Measurement of respiration rate in small intestinal cells and rat liver mitochondria for assessment of cytotoxicity of xenobiotics:

(a) Effect of xenobiotics on intestinal cellular respiration:

Effect of various endosulfan concentrations on viable guinea pig intestinal cells was monitored polarographically. Endosulfan was found to lower the rate of $O_2$ consumption by the isolated enterocytes of both guinea pigs and
rats (Fig. 7; Table 3). Endosulfan at a concentration of 100 μM registered a 12% fall in O₂ consumption in the first minute which declined to 50% in the second min and by fourth minute total inhibition of respiration was evident. Endosulfan at 10 μM concentration had no significant effect on the cellular respiration (Fig. 7). When the sensitivity of cellular respiration in individual isolated cell fractions from villus-to-crypt surface was studied towards various xenobiotics (Table 3), it was found that villus cells did not distinguish between various xenobiotics action. However, the crypt cells were found much sensitive to damage by xenobiotics as evidenced by their xenobiotics concentration-related inhibition of respiration. Benzene inhibited significantly the rate of O₂ consumption of guinea pig intestinal cells (Fig. 8). Benzene concentrations as low as 3 mM caused a complete inhibition within 8 min of incubation. The metabolites of benzene also impaired the cellular respiration (Fig. 9). Benzoquinone at 100 μM concentration caused 30% inhibition within first min of addition which reached 83% within third min while 1 mM benzoquinone inhibited almost completely within the first min of respiration. Hydroquinone did not have any effect on the oxygen utilizing capacity of the cells even at concentrations as high as 1 mM (tracing Fig. 9).

(b) Mechanism of xenobiotics action of mitochondrial respiration:

Measurement of respiration in intact cells appeared to be a good indicator of integrated cell functions
Table 3: Percent inhibition of oxygen uptake in isolated cell fractions of rat small intestinal mucosa by xenobiotics

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fr.1</th>
<th>Fr.2</th>
<th>Fr.3</th>
<th>Fr.4</th>
<th>Fr.5</th>
<th>Fr.6</th>
<th>Fr.7</th>
<th>Fr.8</th>
<th>Fr.9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endosulfan</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>26</td>
<td>25</td>
<td>29</td>
<td>29</td>
<td>27</td>
<td>20</td>
<td>21</td>
<td>52</td>
<td>66</td>
</tr>
<tr>
<td>1 mM</td>
<td>85</td>
<td>81</td>
<td>89</td>
<td>86</td>
<td>92</td>
<td>80</td>
<td>82</td>
<td>84</td>
<td>100</td>
</tr>
<tr>
<td>Benzoquinones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>25</td>
<td>29</td>
<td>45</td>
<td>47</td>
<td>43</td>
<td>45</td>
<td>61</td>
<td>89</td>
<td>100</td>
</tr>
<tr>
<td>1 mM</td>
<td>93</td>
<td>94</td>
<td>96</td>
<td>95</td>
<td>98</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Hydroquinones</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>1 mM</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
<td>nil</td>
</tr>
<tr>
<td>Untreated control</td>
<td>43</td>
<td>51</td>
<td>65</td>
<td>57</td>
<td>51</td>
<td>44</td>
<td>36</td>
<td>19</td>
<td>8</td>
</tr>
</tbody>
</table>

Rat small intestinal cells were isolated as described in section 3.4. The cells were suspended in Hanks basal salt solution containing 10 mM glucose and 5 mM-L-glutamine and the respiration rate was measured (section 3.10.1). Benzoquinone and hydroquinone were introduced in 20 µl of normal saline and endosulfan in 10 ul of alcohol. The values represent percent inhibition in O₂ uptake (u atoms/min/gm protein) of respective untreated control fractions. The values obtained are calculated from polarographic tracings and are the mean of two separate experiments with variation of less than 10%.
Time dependent effect of various concentrations of endosulfan, benzquinone, benzene and hydroquinone on the respiratory activity of guinea pig intestinal cells. The values are plotted from the polarographic tracings given below the corresponding figure. Various concentrations of endosulfan in 10 μl ethanol were introduced into the incubation vessel. The basal rate of oxygen uptake was 42 u atoms/min/gm protein. The coefficient of variation was less than 5%. Other conditions were same as described in Materials and Methods (section 3.10.1).
FIG. 7

Endosulfan

- 10 µM
- 100 µM
- 200 µM
- 1 mM

Incubation Time (min)

Oxygen Uptake (% of control)

Cells

Endo 10 µM
Endo 100 µM
Endo 1 mM

10, 24 µM

1 min
FIG. 8

OXYGEN UPTAKE (% of control) vs. INCUBATION TIME (min)

BENZENE

- △ 3 mM
- □ 6 mM
- ● 12 mM
- ✗ 25 mM

[O₂] 24 μM

1 mM
FIG. 9

![Graph showing the effect of benzquinone on oxygen uptake in cells.](image)

- **Graph Title:** Benzquinone
- **X-axis:** Incubation Time (min)
- **Y-axis:** Oxygen Uptake (% of control)
- **Data Points:**
  - Δ 10 μM
  - 100 μM
  - 1 mM

**Legend:**
- Cells
- HQ 100 μM
- BQ 10 μM
- BQ 100 μM
- HQ 1 mM
- [O₂] 2 μM
- 1 mM
and hence a sensitive marker of cytotoxic action of chemicals. However, it offered limitations in understanding the mechanism of xenobiotics action because the transition states due to state-3 and state-4 respirations could not be worked out due to the presence of endogenous substrates and effector molecules coupling to cellular energy. For this purpose, it required to work with rat liver mitochondrial respiration in the presence and absence of xenobiotics. This would help to determine the mechanism of action of xenobiotics as described in the following.

(b-1) **Effect of endosulfan on \( \beta \)-hydroxybutyrate and succinate mediated mitochondrial respiration:**

The effect of varying endosulfan concentrations on substrates oxidation was examined in the presence and absence of ADP. The rate of oxidation obtained with substrates and prior to the supplementation of ADP was denoted as state-4 and after ADP addition as state-3.

The polarographic tracings of succinate oxidation on treatment with endosulfan at different concentrations are given in Fig.10. Endosulfan, upto 50ug per ml stimulated state-4 respiration with maximal effect of 50% at 33ug endosulfan. At 100ug state-4 respiration reduced to the endogenous level i.e. without the addition of substrate. State-3 respiration, however, registered a dose-dependent inhibition. The rate of oxidation during state-3 respiration was the same as obtained in state-4 upon addition of endosulfan at concentrations above 25ug/ml of incubation medium (Fig.11a). At 5-10ug endosulfan
Fig. 10  Polarographic tracings of the succinate-mediated respiration of rat liver mitochondria (RLM): Effect of variable concentrations of endosulfan on state-3 and state-4 respiration rates. The respiration rate was measured as described in Materials and Methods (section 3.10.2). Endosulfan in 10 μl of ethanol was introduced into the incubation vessel to obtain microgram concentrations per millilitre of the medium as indicated in each tracings.

Fig. 11  Relationship between endosulfan concentrations and mitochondrial respiration. The values were plotted from the data, representative of at least two separate experiments, of tracings obtained with B0HB and succinate as substrates. Procedures for determination of state-3 (●——●) and state-4 (○——○) respiration were according to Estabrook (1967). (a) Succinate oxidation (b) -Hydroxybutyrate oxidation. Other conditions were same as described under Materials and Methods (section 3.10.2). The coefficient of variation was less than 5%.
FIG. 10

[Diagram showing various concentrations and conditions with timepoints for oxygen uptake.]

FIG. 11

[a] Succinate

[b] Hydroxyl Butyrate

[Moles of substrate utilized/mg of protein vs. Endo Sulfan (μg/ml)]
the RCR ratio registered a significant decrease of 25-35% and ADP:O of 10-25% while at higher concentrations these criteria of coupled mitochondrial respiration were completely collapsed. Similar changes in bioenergetic parameters were observed during B-OHB oxidation (Fig. 11b; Table 4).

Endosulfan above 50ppm inhibited fully the CCCP-stimulated oxidation of either B-OHB or succinate. Similarly presence of endosulfan inhibited the rotenone-insensitive oxidation of succinate (Table 5) suggesting that the flow of reducing equivalents through the respiratory chain is impaired drastically by endosulfan.

(b-2) Effect of endosulfan on mitochondrial enzyme activities in vitro:

The data are recorded in Table 6.

Treatment with endosulfan in general inhibited the flow of electron transport through the respiratory chain, as evidenced by the dose related inhibition of respiratory chain linked enzymes in vitro. SDH and MAO exhibited a similar inhibition pattern. The concentration of endosulfan which caused 50% inhibition of the respiratory chain enzymes and SDH was found to be 50ug which remained generally unaltered at 100ug. In general MAO, SDH and enzymes of the respiratory chain elicited a comparable dose related inhibition i.e. 10-50% inhibition with 5-50ug of endosulfan. However, terminal oxidase was more sensitive to endosulfan treatment because at 100 ppm of endosulfan only one-tenth of cytochrome c oxidase appeared operating while
Table 4: Effect of Endosulfan Metabolites on the Rate of \( \beta \)-hydroxybutyrate respiration on Rat Liver Mitochondria

<table>
<thead>
<tr>
<th>Treatment (µg/ml)</th>
<th>Respiration rate*</th>
<th>Respiratory ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>State-4</td>
<td>State-3</td>
</tr>
<tr>
<td>Control</td>
<td>26</td>
<td>132</td>
</tr>
<tr>
<td>Endosulfan</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>26</td>
<td>124</td>
</tr>
<tr>
<td>10</td>
<td>26</td>
<td>114</td>
</tr>
<tr>
<td>25</td>
<td>40</td>
<td>102</td>
</tr>
<tr>
<td>33</td>
<td>61</td>
<td>61</td>
</tr>
<tr>
<td>50</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>100</td>
<td>17</td>
<td>17</td>
</tr>
</tbody>
</table>

Endosulfan at different concentrations were taken in 10 µl ethanol and introduced in 3 ml assay system containing 3 mg mitochondrial protein. The control received 10 µl ethanol. Other conditions are described in Materials and Methods (Section 3.10.2)

The data are from the polarographic tracings representative of at least two separate experiments. The coefficient of variation was less than five percent.

*) n atoms oxygen used x min\(^{-1}\) x mg\(^{-1}\) protein
Table 5: Effect of Endosulfan on $\beta$-hydroxybutyrate or Succinate mediated Mitochondrial Respiration in the presence of CCCP or rotenone

<table>
<thead>
<tr>
<th>Experimental regimens</th>
<th>$n$ atoms oxygen used x min$^{-1}$ x mg$^{-1}$ protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>B=OHB+CCCP</td>
<td>145</td>
</tr>
<tr>
<td>B=OHB+CCCP+ROT</td>
<td>2</td>
</tr>
<tr>
<td>B=OHB+endosulfan (33ug)+CCCP</td>
<td>67</td>
</tr>
<tr>
<td>B=OHB+endosulfan (100ug)+CCCP</td>
<td>12</td>
</tr>
<tr>
<td>B=OHB+CCCP+ROT+SUC</td>
<td>202</td>
</tr>
<tr>
<td>B=OHB+endosulfan (100ug)+CCCP+ROT+SUC</td>
<td>11</td>
</tr>
</tbody>
</table>

The data are from the polarographic tracings representative of at least two separate experiments conducted in duplicates. The coefficient of variation was less than ten percent. Endosulfan was introduced in 10 ul alcohol, controls received the vehicle. Respiration rate presented is from the last addition shown in the experimental regimens. Other conditions are similar as described in Materials and Methods (Section 3.10.2)

Concentration of the compounds:

5mm B-OHB; 3mm SUC; 16um CCCP; 10um ROT.
Table 6: Effect of Endosulfan on Mitochondrial Enzyme Activities In Vitro

<table>
<thead>
<tr>
<th>Endosulfan (μg/ml)</th>
<th>Succinate dehydrogenase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Succinate-Cyt. c reductase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>NADH-dehydrogenase&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cyt. c-oxidase&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase&lt;sup&gt;d&lt;/sup&gt;</th>
<th>Monoamine oxidase&lt;sup&gt;e&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>171±6.9</td>
<td>13.7±2.1</td>
<td>27.8±2.0</td>
<td>20.2±2.1</td>
<td>7.7±0.6</td>
<td>17.9±2.5</td>
</tr>
<tr>
<td>5</td>
<td>145±11.1**</td>
<td>11.5±1.9</td>
<td>24.8±2.7</td>
<td>19.7±2.7</td>
<td>51.2±7.0***</td>
<td>14.5±4.5*</td>
</tr>
<tr>
<td>10</td>
<td>132±10.2***</td>
<td>10.0±1.8*</td>
<td>22.0±2.2**</td>
<td>18.5±2.1*</td>
<td>132.5±10.2*</td>
<td>14.0±3.0*</td>
</tr>
<tr>
<td>25</td>
<td>112±8.0***</td>
<td>9.0±2.1**</td>
<td>18.5±2.5***</td>
<td>13.6±2.0***</td>
<td>190.4±12.1***</td>
<td>9.2±3.1***</td>
</tr>
<tr>
<td>50</td>
<td>92±6.5***</td>
<td>7.0±1.9***</td>
<td>14.8±2.3***</td>
<td>8.4±1.7***</td>
<td>76.1±6.7***</td>
<td>9.2±3.0***</td>
</tr>
<tr>
<td>100</td>
<td>73±4.1***</td>
<td>7.0±2.0***</td>
<td>14.7±2.9***</td>
<td>2.3±1.45***</td>
<td>31.4±3.6***</td>
<td>9.0±3.1***</td>
</tr>
</tbody>
</table>

Each value is the mean (+SD) of three experiments, each determined in duplicate. Endosulfan was dissolved in ethanol and introduced in 10 ul to the assay system, controls received the same quantity of ethanol. Procedures for the measurement of enzyme activities are described in Materials and Methods (Section 3.13).

Enzyme activities expressed:
(a) - n moles K<sub>3</sub>Fe(CN)<sub>6</sub> reduced/min/mg prot. (b) - n moles DCIP reduced/min/mg prot.
(c) - n moles Cyt.-c oxidase/min/mg prot. (d) - n moles pi liberated/min/mg prot. (e) - n moles benzenedehyde formed/min/mg prot. Benzenedehyde was freshly prepared by reacting benzenedehyde with HCl followed by crystallisation in cold ethanol. Significance of changes measured by student t-test: *P < 0.05; **P < 0.01; ***P < 0.001
other electron transport chain enzymes were available at 50% of their efficiency.

Mg\(^{2+}\)-ATPase of mitochondria was most dramatically affected by endosulfan treatment. There was 7, 17 and 25 fold activation of ATPase activity at 5, 10 and 25ug concentrations. A further increase in endosulfan resulted in gradual decline of the activated ATPase activity. It may be indicated that both activation of Mg\(^{2+}\)-ATPase and stimulation of state-4 respiration were achieved at comparable concentrations of endosulfan.

(c) **Measurement of alkali labile sites in DNA of intestinal cells:**

The results were plotted as the log of the fraction of DNA retained on the filter versus the elution time. DNA damage was quantitated by determining the percentage of the total DNA count retained on filter. Variation in the amount of H\(^3\)-DNA eluted from the filters was usually found to be very high in the control cells. The elution pattern of DNA from the intestinal cells incubated in vitro with the chemical carcinogens is described in the following.

Isolated rat small intestinal crypt cells labelled with H\(^3\)-thymidine were exposed to 250 rads and 500 rads of radiation (Co\(^{60}\) source) at 0\(^\circ\)C. The radiations caused extensive damage to DNA which was about 80% at 500 rads (Fig.12).

When the crypt cells were exposed to direct alkylating agent, MNNG, and procarcinogen B(a)P which required metabolic activation,
Fig. 12 Alkaline elution profiles of DNA from crypt cells of rat small intestine:

The cells (0.5 mg protein) were exposed to gamma rays (Co\(^{60}\)) at 0°C. Data are expressed as the fraction of \(^{3}\)H-DNA left on filter as a function of the elution time. Values give the results of one out of three experiments yielding similar results. Other conditions are described in Materials and Methods (section 3.19).

Fig. 13 Effect of B(a)P and MNNG on the rate of elution of DNA from rat intestinal crypt cells:

In case of B(a)P, the cells were incubated in HBSS containing 0.05% BSA with 100 uM B(a)P alone (●) or 100 uM B(a)P+microsomal activation system (△) or with or without microsomes (○) for 20 min at 37°C. Microsomes were obtained from 3MC-treated rat liver. The activation system in 1 ml of incubation contained 0.5 mg microsomal protein, 4 mg of glucose-6-phosphate, 0.03 mg glucose-6-phosphate dehydrogenase, 0.6 mg NADP and 10 mM MgSO\(_4\). The cells were washed in PBS and then loaded on filters.

In case of MNNG, the cells were incubated for 10 min at 37°C in HBSS containing 0.05% BSA. The cells were washed in PBS and loaded. Other conditions were same as in Fig. 12.
FIG. 12

% [3H] DNA remaining on filter

TIME OF ELUTION (minutes)

FIG. 13

% [3H] DNA remaining on filter

TIME OF ELUTION (min)

B(a)P

MNNG

contl

250 rads

500 rads

contl

25 μm

50 μm
Fig. 14 Effect of B(a)P on the rate of DNA elution from mid villus and crypt cells of rat small intestine:

Cells were incubated with variable concentrations of B(a)P and incubated at 37°C for 20 min in HBSS containing 0.05% BSA. The cells were washed in PBS and loaded on filters. Other conditions were same as in Fig. 12.

Fig. 15 Effect of DMH on DNA elution profiles from crypt and mid-villus cells of rat small intestine:

Cells were incubated in HBSS containing 0.05% BSA for 20 min at 37°C. Cells were washed in PBS and used. Other conditions were same as described in Fig. 12.
it was observed that MNNG induced single strand DNA breaks at
much lower concentrations of 25 and 50 uM while B(a)P was not
effective. When cells were incubated, with B(a)P and microsomes
of 3MC treated rats together with NADPH regenerating system,
the DNA damage was not still evident (Fig.13).

Because of the potential differences in
the drug biotransforming potentials between the crypt and mid-
villus cells, the sensitivity of the DNA damage in these two
population of the cells was studied. It was found that B(a)P
at the concentration of 50-200 uM was ineffective in inducing
DNA damage in either of the cell preparation from either 3MC-
treated or control rats (Fig.14). In case of DMH treatment of
cells, the frequency of DNA single strand breaks was much more
pronounced in mid-villus cells whereas there was hardly any
significant damage visible in crypt cells (Fig.15).

4.5.3 Discussion

Intestinal epithelial cells isolated from the
small intestine of guinea pig and rat have been utilized in the
present study as a possible system for short term studies to
detect membrane damage due to cytotoxic agents and DNA damage
due to genotoxic compounds.

The severity of membrane damage and cytotoxic
nature of compounds was detected by (1) estimating the leakage
of four markers e.g. $^3$H-$\alpha$-AIB, $^3$H-uridine, LDH and AP in the
extracellular medium based on their varying molecular sizes;
and by (2) measuring respiration rates. The genotoxic damage was estimated by measuring DNA single strand breaks in the intact cells \textit{in vitro} by xenobiotics. The results of these studies are discussed in the following which suggest the possibilities and limitations of using these cells for assessment of cyto-cum-

\textit{genotoxic nature of xenobiotics.}

4.5.3.1 \textbf{Leakage of biochemical markers:}

Leakage of \(\alpha\)-AIB has been an important marker for detecting minor damage induced by toxic agents to the cell membrane. The intestinal cells in our study actively accumulated \(\alpha\)-AIB as observed by others (Dickson, 1970; Mahoney and Rosenberg, 1970) and the efficient labelling was achieved in Hank’s BSS supplemented with 10 mM Hepes. The low uptake of \(\alpha\)-AIB into intestinal cells when incubated in Eagles medium might be due to the competition of other amino acids for \(\alpha\)-AIB (Thelestad and Möllby, 1975b; Christensen and Liang, 1966). The major advantage of using this marker is that it is not metabolized in the cells (Christensen \textit{et al.}, 1956) and its uptake and release can be accounted accurately. \(\alpha\)-AIB being a molecule of low molecular weight is comparable to \(K^+\) release in terms of its sensitivity to membrane alterations (Thelestad and Möllby, 1975b) and its leakage could be detected easily because of its rapid diffusion out of the cell after perturbation of the cell membrane.

Eighty percent release of \(\alpha\)-AIB caused by triton-X-100 may be due to its capability of producing small
functional holes in the cell membrane (Thelestaem and Möllby, 1975b). SDS may be causing direct or indirect effect on carrier proteins and therefore it might be altering the rate of $H^3$–$\alpha$-AIB release as the influx of $\alpha$-AIB is dependent on the sodium gradient across the plasma membrane (Christensen, 1975). Consequently higher leakage was observed at higher SDS and lesser leakage at lower SDS concentrations as also observed by Malik et al. (1983) in mammalian cell lines. Nystatin causes typical damage to cell membrane by producing small narrow transverse pores by stereochemically defined reactions with the membrane sterols (Van Hoogevest and de Kruijff, 1978; Thelestaem and Möllby, 1979). This causes damage to plasma membrane of lesser magnitude than SDS and triton–X–100 (Thelestaem and Möllby, 1979). Therefore, the leakage of $\alpha$-AIB was of low order.

The toxic effect of several xenobiotics on the membrane damage was monitored by observing $\alpha$-AIB leakage. The drugs and xenobiotics, e.g. aspirin and indomethacin (Lanza et al., 1979; Sohara, 1980), dimethylhydrazine (Sunter et al., 1981; Thomas, 1981), benzo(a)pyrene (Loquet and Wiebel, 1982), endosulfan and benzoquinone (Rickert et al., 1979; Raven and Chambers, 1982; Dubey et al., 1984) are known to cause toxic effects. The short-term effect of these xenobiotics on the leakage of $\alpha$-AIB from the intestinal cells may be related primarily to the initial perturbation of plasma membrane functions which is correlated with the efflux of $\alpha$-AIB. The maximal release of $\alpha$-AIB, registered on treatment with 1 mM of these xenobiotics was in the order, $DMH > aspirin > B(a)P >$
endosulfan > benzoquinone > indomethacin. Measurement of α-AIB leakage might offer limitation due to its spontaneous release on prolonged incubations in cases where the damage is negligible.

Cytoplasmic markers, having high molecular weight, are leaked out usually when a major damage to the cellular membrane occurs. $^3$H-uridine was effectively used as a second radioactive marker for detecting major membrane damage caused due to a wide variety of cytotoxic compounds. $^3$H-uridine is known to be rapidly incorporated into the RNA and nucleotides (Plagemann, 1971; Thelestam and Möllby, 1975a) and their rapid incorporation was also observed in intestinal cells in this study. Very low levels of spontaneous leakage of $^3$H-uridine-labelled RNA into the extracellular medium is evident from our studies which renders the use of this marker more effectively and accurately for detecting major and severe damage caused to the cell membranes. Thelestam and Möllby (1976) found nucleotide label to be a good standard marker for detection of membrane damage with a higher sensitivity than that of Cr$^{51}$ leakage. The severe damage caused by compounds known for such effects, e.g. Triton-X-100, SDS, Nystatin, is clearly reflected in the leakage of labelled uridine from the intestinal cells. The sensitivity of the assay could be increased by prolonged incubation with low concentrations of xenobiotics. However, due to the loss of viability over longer incubation the intestinal cells might be useful in rapid assessment of damage for shorter incubations with potent cytotoxic compounds. For instance the extent of damage caused by these known cytotoxic compounds to the
intestinal cell membrane in terms of labelled $^3$H-uridine leakage was, in the order of Triton-X-100 > SDS > nustatin. The potency of this damage was greater than produced by other xenobiotics in this study which was in the order of aspirin > endosulfan > DMH > B(a)P > benzoquinone > indomethacin. These observations were similar to that observed for $\alpha$-AIB leakage.

Lactate dehydrogenase and alkaline phosphatase are high molecular weight proteins which did not show any spontaneous release from the cells and proved to be a very insensitive marker for cellular damage because leakage of 15% LDH and 30% AP activity occurred at a maximum concentration of Triton-X-100 used. No significant release was observed on treatment with any of the other compounds except that of endosulfan and B(a)P. The findings suggest that leakage of these proteins due to cellular damage might occur at a point where the cells are already on the verge of disintegration.

Based on the leakage of biochemical markers it may be concluded that enterocytes in suspension might provide a useful system for detecting short term toxicity of test compounds. $\alpha$-AIB was a very sensitive marker for primary plasma membrane damage which is in agreement with the study of others using systems other than intestinal cells (Henney, 1973; Thelstam and Mölby, 1976). These workers stated that the sensitivity of the leakage test was, in general, inversely related to molecular size of the marker. However, the leakage of labelled uridine and high molecular weight proteins may
depict the potency of compounds in disrupting plasma membranes.

4.5.3.2 Rate of respiration:

The rate of oxygen utilization in the intestinal cells was impaired by the inhibitors of electron transport chain which shows that the respiration in intact cells is sensitive to its inhibitors as shown in mitochondria (Dubey et al., 1984). Most of the xenobiotics are lipophilic in nature and accessible to the subcellular organelles. Since cellular respiration represents the integrated functions of the cell, impairment of its respiration might, therefore, be a sensitive indicator for assessment of harmful nature of foreign compounds. This is supported by the impairment of respiration observed in cells with relatively higher amounts of endosulfan, benzene and its metabolites than in mitochondria. Moreover, if the metabolites of the compounds found in situ also are toxic, the respiration rate will also be affected. This is evidenced from the effect of benzene metabolite, benzoquinone. In other words, the effect on the respiration would be the consequence of the combined action of the parent compound and its metabolites generated through drug metabolizing enzymes. Low concentration of endosulfan was not much effective probably due to its easy dispositions while higher concentrations impaired bioenergetics. Crypt cells have low profile of drug metabolism, therefore, the cells appeared to be more sensitive to the parent compounds.

The intact cells, however, do not allow to understand the mechanism of action of xenobiotics on bioenergetics
for which a highly energy coupled mitochondrial preparation is deemed important. As a prototype the effect of endosulfan, organochloric insecticide on mitochondrial bioenergetics was undertaken. Such preparations would also suggest whether the effect on respiration is due to the parent compound solely or of its metabolites.

Endosulfan was found to possess dual properties of an uncoupler of oxidative phosphorylation and an inhibitor of electron transport chain. This is evidenced from the observations that upon endosulfan treatment of mitochondria in vitro the ADP-induced respiratory transitions were not manifested, ADP:O and respiratory control ratios decreased, mitochondrial oxidation of substrates got severely restricted with simultaneous inhibition of respiratory chain enzymes, the latent Mg$^{2+}$-ATPase was profusely activated while monoamine oxidase activity showed significant inhibition. Besides, endosulfan exerted biphasic effects on the in vitro respiration of rat liver mitochondria causing stimulation of state-4 respiration at lower concentrations and inhibition at higher ones. The reasons for such a biphasic effect of endosulfan and its derangement of mitochondrial bioenergetics is not understood at present. However, it is understandable that endosulfan being lipophilic and like other hydrochlorinated hydrocarbons (Haque et al., 1973; Ohyama et al., 1982) might be interacting primarily with the mitochondrial lipo-proteinous surface resulting in structural damage and changes in ionic permeability (Weinbach and Garbus, 1965; Fujita, 1966; Pritchard et al., 1982).
Inhibition of MAO by endosulfan would be indicative of such an altered outer mitochondrial membrane integrity, as indicated by others for certain compounds (Fowler et al., 1980; Byczkowski et al., 1981). However, this biphasic effect could not be seen in the intact intestinal cells.

The inhibition of mitochondrial respiration was found in parallel to the inhibition in enzyme activities of the respiratory electron transport chain of mitochondria. These studies demonstrated that endosulfan is not a specific inhibitor for a particular site of respiratory electron transport chain and the effect exercised by endosulfan on mitochondrial respiration is general one whether succinate or pyridine-linked substrate is used. The endosulfan thus diminished the availability of reducing equivalents to the terminal oxygen and hence the oxidation of substrates. Further lowered P:O ratio as well as the activation of latent Mg\textsuperscript{2+}-ATPase of mitochondria in the presence of endosulfan would be indicative of the uncoupling effect of this insecticide like known uncoupling effect of this insecticide like known uncouplers (Weinbach, 1956; Weinbach and Garbus, 1966).

From the foregoing observations it may also be considered that mitochondrial respiration might offer a simple in vitro system of choice for monitoring cytotoxicity of direct acting compounds. However, limitations of such a system are quite apparent where metabolic activation of test compounds is prerequisite to its biological action and in such cases
measurement of respiration in intact cells in vitro would be a system of choice.

4.5.3.3 Genotoxic damage

The major advantage of the technique described is that damage to DNA can be studied without using either radioactive carcinogen or radioactive DNA. Thus, those mutagens or carcinogens suspected of being able to react with DNA to produce alkali-labile sites can be tested cheaply in various tissues and cell preparations in vitro in a shorter period of time. The frequency of single strand DNA breaks measured due to genotoxic insult of chemicals in the crypt and villus cells of the small intestine suggested that cells can be used for studying the genotoxicity of xenobiotics. This is evidenced from the effect of 1,2-dimethylhydrazine which is a potent intestinal carcinogen (Pollard and Luckert, 1979) which required metabolic activation by monooxygenases to active alkylating agent. The mid-villus cells have higher monooxygenase activity while it is very low in crypt cells (chapter 4.2). B(a)P, a skin and lung carcinogen (Heidelberger, 1975; Mukhtar et al., 1984) on the contrary was not effective in inducing DNA damage which might be due to the higher inactivation of B(a)P metabolites formed in these cells by the strong conjugating enzymes (chapter 4.3). MNNG is a direct alkylating agent and does not require metabolic activation by monooxygenases (Kleihues et al., 1984), it, however is activated by sulfhydryl content of the (Wiestler et al., 1983) cells especially by GSH. Since the GSH content of
the intestinal cells is very high, the intestinal cells may, therefore, be amenable to such alkylating agents. This is also evidenced from the reported carcinogenic effect of MNNG on small intestinal epithelium (Kobori, 1984). In general the DNA damage elicited by carcinogens is rapidly repaired by the efficient DNA repairing system. And if the damage is repaired, the elution of DNA strands thus would not be seen. Therefore, the DNA damage measured in shorter periods of incubation would be a more sensitive parameter in assessing the genotoxic potential of chemicals in these cells. In intact intestinal cells both the activation and inactivation systems of xenobiotics are working simultaneously (section 1.6). Therefore, one of the major factors contributing to the damage to the genome would be the consequence of the balance of these opposing forces which have been shown at least to protect intestinal cells against B(a)P genotoxicity. The data appears quite predictive for intestinal carcinogens, DMH and MNNG; though the sensitivity might be low due to high elution of DNA in crypt and villus cells in untreated control channels.

In conclusion, the results indicate that intestinal cells offer an interesting system for understanding xenobiotics interaction with these cells in assessing their harmful effect. A number of criteria were selected for establishing the utility of these cells in assessing cytotoxicity and genotoxicity of xenobiotics which included leakage of biochemical markers, cellular and mitochondrial respiration, and measurement of DNA
damage. Though these studies are preliminary in nature, the measurement of these parameters definitely envisage the possibilities and limitations of using intestinal cells for assessment of harmful nature of xenobiotics, and provide openings to take up further work in depth and in greater perspectives.