MATERIALS & METHODS
3. **Materials and Methods**

3.1 **Animals:**

Adult male albino Charles Foster rats (200-250 g, b.wt.), male Swiss albino mice (25±1 g, b.wt.) and male guinea-pigs (400-450 g, b.wt.) were used in these studies. The rats and mice were fed a standard commercial pellet diet (Hindustan Lever, Bombay, India) and water *ad libitum*. The guinea pigs were maintained on grams and wheat bran concentrate with green vegetables and water *ad libitum*. The mice were used only for measurement of sleeping time and paralysis time.

3.2 **Animals treatment:**

The rats received a single oral dose of 1.0 ml coconut oil or 3MC (40 mg/kg b.wt.) in 1.0 ml coconut oil. PB (80 mg/kg b.wt.) in 1.0 ml normal saline was administered i.p. to the rats. The animals were further kept for 5 days on drinking water containing 0.1% of PB and 12 hrs before sacrifice the PB water was replaced by the tap water. Wherever required dexamethasone (10 ug/180 gm b.wt.) was administered orally in 0.1 ml coconut oil daily for 5 days. One day before sacrifice the rats from both the groups of PB or PB and dexamethasone were given oral dose of PB (120 mg/kg b.wt) in 1 ml of normal saline.

Wherever indicated piperine was given orally in 1 ml of coconut oil to rats for *in vivo* studies. The studies were routinely conducted on starved animals between
9-10 A.M. Other conditions are described under the respective tables and figures.

3.3 Hexobarbital induced sleeping time and zoaxazolamine-paralysis time:

The sleeping and the paralysis times were determined as the time required for the mice to regain their rightening reflex (Conney et al., 1960). The control animals received only the vehicle.

3.4 Sequential isolation of epithelial cells in gradient fraction from villus tip-to crypt surface of the rat:

Sequential cell isolation from rat small intestinal epithelium was accomplished by the method of Weiser (1973a). The animals were sacrificed by cervical dislocation and about a 50 cm segment of the small intestine, 10 cm beyond pylorus end of the stomach was quickly excised. The lumen was gently flushed with oxygen-saturated saline containing 1 mM dithiothreitol, at 37°C. The distal end of the intestine was clamped with a pair of spencer wells artery forceps and the lumen was filled with oxygen saturated solution A (1.5 mM KCl, 96 mM NaCl, 27 mM Sodium citrate, 8 mM KH$_2$PO$_4$, 5.6 mM Na$_2$HPO$_4$; pH 7.3). The proximal end was then clamped and the whole length was incubated in solution A for 15 min at 37°C. After incubation the citrate solution was drained from the intestine and discarded. The intestinal segment was then rinsed once and filled with oxygen saturated solution B (phosphate buffered saline) which contained 140 mM NaCl, 20 mM
Na$_2$HPO$_4$, 1.5 mM EDTA and 0.5 mM dithiothreitol. The gradient elution of the small intestinal epithelial cells was accomplished in 9 fractions of cells isolated by incubation of the intestine with solution-B for specified time intervals as follows: (1) 4 min (2) 2 min (3) 2 min (4) 3 min (5) 4 min (6) 5 min (7) 7 min (8) 10 min (9) 15 min. After each incubation, the cells were collected in plastic tubes containing 20 ml of ice cold phosphate buffered saline without EDTA and dithiothreitol by removing one clamp and allowing the intestine to collapse and drain the suspension of released cells into the plastic tube. The isolated cell fractions were finally washed twice in the same phosphate buffered saline by centrifugation at 100 x g for 2 min. The cell pellet was finally suspended in suitable cell suspension medium.

3.5 Isolation of total enterocytes from guinea-pig and rat small intestine:

The method of Weiser (1973a) as described above was used for isolation of total intestinal epithelial cells (enterocytes) with minor modifications (Hegazy et al., 1983). The intestine was incubated with solution-A for 10 min at 37°C to remove the mucus and food material, etc. The intestine was then filled with solution-B, and the enterocytes were isolated in 4 incubations of 4 min each at 37°C. The intestine was gently palpated with fingers after each incubation and the luminal content containing isolated cells were filtered through a mesh (pore size 0.25 mm x 0.25 mm) into a plastic container containing 200 ml of ice cold cell suspension.
medium (137 mM NaCl, 11.5 mM KH$_2$PO$_4$, 8 mM Na$_2$HPO$_4$, 2.2 mM KCl, pH 7.4, Reiser and Christiansen, 1971). The isolated cells were washed twice with cell suspension medium and by centrifugation at 70 x g for 2 min. The cell pellet was finally suspended in the same medium. Agitation was avoided except for gentle shaking for even dispersion before use.

3.6 **Cell viability by trypan blue**

Viability of the cells was checked by trypan blue exclusion frequency of the cells (Baur et al., 1975). Aliquots of the cell suspension were mixed with 0.4% Trypan blue (w/v) made in Hanks balanced salt solution, pH 7.4, so that the final concentration of trypan blue was 0.04%. The cells were counted immediately for total and stained cells in improved Neubauer chamber.

3.7 **Preparation of hepatic mitochondria**

Adult male albino rats were used for the isolation of liver mitochondria according to Schnaitman and Greenawalt (1968) with the following modifications. The isolation medium contained 220 mM D-mannitol, 70 mM sucrose, 2 mM Hepes buffer (pH 7.4), 0.05% BSA, and 1 mM EDTA. Ten percent whole homogenate of the liver in isolation medium was centrifuged at 500 x g for 15 min, and the resulting supernatant fraction of 7000 x g for 15 min. The pellet was suspended in isolation medium and recentrifuged at 7000 x g for 15 min. The pellet obtained was further taken into 20 volumes of BSA-free isolation medium and centrifuged at
8000 x g for 15 min, and the mitochondrial pellet was finally suspended in the same medium to obtain 30-40 mg protein/ml. The ADP:O ratio obtained with these mitochondrial preparations was close to the theoretical value, suggesting their "tightly coupled" nature. Mitochondria exhibited transitions in respiratory states with substrates and ADP; they were deenergized with the classical uncoupler carbonyl cyanide m-chlorophenyl hydrazone (CCCP) releasing the respiratory controls.

3.6 preparation of post mitochondrial supernatant (PMS) and microsomes:

Overnight starved animals were used for the preparations. Liver homogenate (25%, w/v) prepared in 0.25 M sucrose was centrifuged at 10,000 rpm (12000 x g) for 15 min. The supernatant was recentrifuged as before and the pellet discarded each time. The PMS thus obtained was stored in batches in the deep-freezer at -75°C and the preparations were stable at least for seven days for studying drug metabolizing enzymes. Liver microsomes were prepared by calcium aggregation method of Kamath and Rubin (1972). 20% homogenate in 0.25 M sucrose was centrifuged at 12,000 x g for 10 min. The supernatant was diluted 1:5 (v/v) with 0.125 M sucrose, which contained 8 mM CaCl₂ (pH 7.5). The solution was stirred for a few seconds and then centrifuged at 7000 x g for 10 minutes. The pellet was resuspended in 0.25 M sucrose. In certain studies the PMS was centrifuged at 105000 x g for 1 hr. to obtain the microsomal pellet which was washed with
0.25 M sucrose. The pellet was suspended in 0.25 M sucrose and stored in small portions at -70°C.

3.9 **Light and electron microscopy of cells:**

Cells were examined by phase-contrast light microscopy with a Carl Zeiss Jena microscope. For transmission electron microscopy, the intestinal cells were fixed at 4°C in a mixture containing 2.5% glutaraldehyde, in PBS pH 7.4 for 45 min. The cells were then washed free of glutaraldehyde with washing buffer and postfixed for 1 hr. in 2% OsO₄. The samples were again washed and subsequently dehydrated in acetone grades and propylene oxide and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate and examined in a Jeol-100 C x 2 electron microscope (Hayat, 1981).

3.10 **Respiratory measurements**

3.10.1 **Cellular Respiration:**

Oxygen consumption by isolated cells was determined polarographically using a Clark type oxygen electrode (YSI model), at 30°C in 3 ml of oxygen saturated incubation medium as described by Estabrook (1967). The incubation medium contained CaCl₂ free Hank's balanced salt solution and 10 mM Hepes (pH 7.4) and was supplemented with 10 mM glucose and 5 mM L-glutamine. The substrates and inhibitors were delivered in 10 ul of incubation medium or DMSO or ethanol. The respiratory rate was measured according to Estabrook (1967).
3.10.2 Measurement of Mitochondrial respiration:

Mitochondrial respiration employing succinate and \( \beta \)-hydroxybutyrate as substrates was measured as described in 2.9.1. The incubation medium contained 220 mM mannitol, 70 mM sucrose, 10 mM Hepes buffer, 2.5 mM \( K_2HPO_4 \), 5 mM \( MgCl_2 \), 0.05% BSA and 20 mM KCl. The test compounds were delivered in 10 ul of alcohol. Respiratory control ratio (RCR) and ADP:O ratio were also measured according to Estabrook (1967).

3.11 In vivo and in vitro incorporation of radioactive thymidine and DL-leucine:

Incorporation of thymidine in vivo along the intestinal mucosa was accomplished by injecting 100 uci of \( ^3H \)-thymidine (Sp. activity 6000 mci/m mole) intraperitoneally to rats. Three hours after the injection the animals were sacrificed and cell isolation accomplished. Thymidine incorporation for the acid insoluble fraction of each isolated cell fraction was followed. The cells were precipitated with chilled 10% trichloroacetic acid (TCA, w/v) and centrifuged. The precipitate was washed with 5% TCA followed by subsequent washings with 95% alcohol containing 5% K-acetate, alcohol: ether (3:1) and ether. The pellet was dissolved in 200 ul of 2% \( Na_2CO_3 \) in 0.1 N NaOH and radioactivity measured in Bray’s scintillation fluid (For 1 litre: 60 g naphthalein, 3.8 g PPO, 0.4 g POPOP, 100 ml methanol, 20 ml ethyleneglycol, 880 ml dioxan) using Beckman LS-3150 P scintillation counter.

Aliquots (3 mg cellular protein/ml) of isolated enterocytes prepared from guinea pig small intestine
were incubated in Hanks basal salt solution supplemented with 10 mM Hepes, pH 7.4 and BSA (0.5%). The incubation was carried out in small vials maintained at 37°C under a transparent hood provided with an inlet for oxygen gaseous phase. The cells were stirred gently over a magnetic stirrer. After preincubation of cells for 3 min, 1 uci of \(^3\)H-thymidine (Sp. activity 6000 mci/m mole) or \([\text{I}^1\text{C}]\)DL-leucine (Sp. activity 53.7 mci/m mole) per ml of incubation medium were added. Aliquots of 200 ul were drawn at different time intervals and added to 1 ml of chilled 10% TCA; the acid precipitated protein was washed and dissolved as described above and the radioactivity was counted in Bray's fluid.

3.12 **Intestinal brush border marker enzymes:**

To establish that the sequentially isolated cells are specifically from villi tip to crypt region as reported by Weiser (1973a), a systematic study of the pattern of distribution of brush border marker enzymes showing contrasting areas of activities were measured. Sucrase (E.C. 3.2.1.48) activity was assayed according to Messer and Dahlqvist (1966), alkaline phosphatase (E.C. 3.1.3.1) according to Weiser (1973a) and \(\gamma\)-glutamyl transpeptidase (E.C. 2.3.2.2) was assayed according to Boelsterli and Zbinden (1980).

3.13 **Mitochondrial enzymes:**

The following enzyme activities were measured during the initial linear phase of the reaction with respect to time and protein by standard procedures: succinic
dehydrogenase (EC 1.3.99.1) according to Slater and Bonner (1952); Mg\(^{2+}\)-adenosine triphosphatase (EC 3.6.1.3) by the method of Ohyama et al. (1982) and inorganic phosphorous liberated by the method of Taussky and Shorr (1953); Cytochrome C-oxidase (EC 1.9.3.1) according to Smith (1955); succinate-cytochrome-C reductase (EC 1.3.99.1) using 2,6-dichlorophenol indophenol (DCIP) as an electron acceptor as described by King (1967); NADH-dehydrogenase (EC 1.6.2.1) using DCIP as an electron acceptor as described by Mahler (1955) and monoamine oxidase (EC 1.4.3.4) by McEwen (1971).

3.14 Assays of drug metabolizing enzymes:

A number of studies presented in this dissertation were focussed on the determination of drug metabolizing enzymes. Therefore, the assay procedures used are described briefly in the following. All the enzymes were assayed during the initial linear phase of the reaction with protein and time. The assays were performed under subdued light.

3.14.1 7-Ethoxycoumarin deethylase:

7ECDE (EC 1.14.14.1) activity was determined fluorometrically according to Greenlee and Poland (1978). Briefly the reaction mixture in a total volume of 1 ml contained 40 mM tris-HCl, pH 7.6, 5 mM MgCl\(_2\), 0.5 mM NADPH, 0.5 mM NADH and 0.2 to 1 mg protein. The reaction was started by the addition of 25 ul of 10 mM 7-ethoxycoumarin in 50% methanol at 37\(^{\circ}\)C. After incubation for 30 min the reaction
was terminated with 0.125 ml of chilled TCA (15%, w/v) followed by 2 ml of chloroform. The tubes were stirred on vortex mixer and 1 ml of chloroform phase was transferred to another tube which contained 1.5 ml of 0.01 N NaOH and 1 M NaCl. After stirring thoroughly the upper phase was removed and the fluorescence was read at 368 nm excitation and 456 nm emission.


AHH was assayed by measuring the conversion of benzo(a)pyrene (BP) to phenolic products according to Wiebel et al. (1977). The reaction mixture in a total volume of 1 ml consisted of 50 mM Tris-HCl buffer, pH 7.6, 3 mM MgCl₂, 0.6 mM NADPH and protein (0.5 to 2.5 mg). The reaction was initiated by adding 50 ul of 2 mM benzo(a)pyrene dissolved in methanol. The tubes were incubated at 37°C for 60 min (if not stated otherwise) and the reaction was stopped by adding 4 ml of cold propanol:hexane (3:1, v/v). The tubes were mixed thoroughly and 2 ml aliquots of the organic phase were drawn and added to 4 ml of 1N NaOH, and mixed thoroughly. The fluorescence in the aqueous-alkaline phase was read at 396 nm excitation and 520 nm emission using 3-hydroxybenzo(a)pyrene as a standard.

3.14.3 Ethylmorphine-N-demethylase:

EMD was assayed as described by Mazel (1971) based on the methods of Anders and Mannering (1966). The reaction assay was modified at the termination step. Briefly, the incubation mixture consisted of 68 mM Na, K-phosphate buffer, pH 7.4, 0.6 mM NADPH, 6 mM MgSO₄ and 100 to 200 ug of
protein (if not stated otherwise) in a final volume of 250 ul. The reaction was initiated by the addition of 25 ul of 5 mM ethylmorphine-HCl. After incubation at 37°C for 15 min the reaction was terminated by the addition of 10% TCA (w/v) to give a final TCA concentration of 3.3%. The mixture was centrifuged at 2000 x g for 5 min. Five hundred microlitres of incubate were mixed with 500 ul of Nash reagent and heated to 60°C for 15 min (Nash, 1953). After cooling to room temperature the absorbance was read at 410 nm in Pye Unicam SP 8-100 U.V spectrophotometer, using formaldehyde as a standard.

3.14.4 UDP-glucuronyltransferase activity towards the substrate 3-hydroxy-benzo(a)pyrene:

GT activity (EC 2.4.1.17) towards 3-OH-BP as substrate was measured according to Singh and Wiebel (1979). The standard reaction mixture in a total volume of 0.2 ml contained 100 mM Tris-HCl buffer, pH 7.6, 0.025% brij-58, 5 mM MgCl₂, 3 mM UDP-glucuronic acid and 50 ug cellular protein. The reaction was started with 50 uM 3-OH-BP in 20 ul methanol at 37°C. The reaction was terminated after 3 min. of incubation with 6 ml of chloroform:methanol (2:1, v/v) and 0.8 ml of water. The mixture was shaken and the two phases were allowed to separate. An aliquot (1.8 ml) of the upper aqueous methanol phase was transferred to a tube and made alkaline by addition of 0.2 ml of 5 N NaOH. The tubes were centrifuged and the relative fluorescence intensity of BP-3-glucuronide was then read at 378 nm excitation and 425 nm
emission in a Kontron Spectrofluorometer.

3.14.5 **UDP-glucuronyltransferase activity with 4-hydroxybiphenyl as substrate**

GT activity (EC 2.4.1.17) towards 4-OH-biphenyl was assayed according to Bock *et al.* (1979). The standard reaction mixture in a total volume of 0.5 ml contained 0.1 M Tris-HCl, pH 7.4, Brij-58 (0.05%, w/v), 5 mM MgCl$_2$, 3 mM-UDP glucuronic acid and 0.5 to 1 mg cellular protein. The reaction was started by addition of 0.5 mM of the substrate 4-OH-biphenyl in 10 ul of methanol. In blanks UDP-glucuronic acid was omitted. After 30 min of incubation the reaction was terminated by the addition of 0.5 ml of 1 M TCA. Chloroform (1 ml) was then added and the tubes were stirred on vortex mixer. An aliquot of the aqueous phase (0.8 ml) was added to 1.6 ml of 1.6 M glycine buffer, pH 10.3 and fluorescence of glucuronide was determined at 325 nm emission and excitation setting at 290 nm in a Kontron spectrofluorometer. Calibration of glucuronide fluorescence was done by comparing the increase of fluorescence with the disappearance of the phenolic substrate measured with Folin ciocalteau reagent.

3.14.6 **Glutathione-s-transferases** (EC 2.5.1.18)

The transferase activity towards the major three substrates 1-chloro-3,4-dinitro benzene (CDNB), 3,4-dichloronitrobenzene (DCNB) and 1,2-epoxy-3-propane (Epoxy-prop) was determined according to Habig *et al.* (1974).
The cells were sonicated in PBS and centrifuged at 105000 x g. The supernatant was used for the determination of enzyme activity at 25°C in 1 ml glass cuvettes. The standard assay system in a final volume of 0.7 ml contained phosphate (88 mM)-EDTA (0.88 mM) buffer. The substrate concentrations and pH varied for each type of substrate used. The reaction was started with the 105000 x g supernatant as a source of enzyme. For CDNB, the assay was conducted at pH 6.5 with 1 mM GSH and 1 mM CDNB in 20 ul of ethanol. The reaction was started with enzyme and initial linear increase in optical density per unit time at 340 nm was recorded in Pye-Unicam Spectrophotometer. The non-enzymatic rate was measured without biological probe which was subtracted from the enzymatic rate.

For DCNB, the enzyme activity was monitored at pH 7.5 and 345 nm. The assay system contained 1 mM DCNB in 25 ul ethanol and 5 mM GSH. For epoxy-prop, the activity was monitored at pH 6.5 and 360 nm and the assay system contained 0.5 mM epoxy-prop in 25 ul ethanol and 5 mM GSH. The enzyme activity in each case was determined by using molar extinction coefficients of 9.6, 8.5 and 0.5 mM⁻¹ x cm⁻¹ for CDNB, DCNB and epoxy-prop, respectively.

3.15 Extraction and determination of glutathione content of isolated intestinal cells

3.15.1 Preparation of tissue extract:
Glutathione was extracted from isolated intestinal cells according to Tietz (1961). Aliquots (250 ul)
of cell suspension (2-4 mg of cellular protein) were taken in plastic centrifuge tubes and 100 ul of ice cold 35% perchloric acid containing 10 mM EDTA was added to it. The tubes were stirred immediately and placed in ice. The samples were sonicated (Soniprep-150.MSE) for 3-5 seconds and the precipitate was spun down in a Beckman microfuge. The clear supernate (300 ul) was transferred into another plastic tube and 50 ul of chilled 1 M triethanolamine containing 1.65 M K_2CO_3 and 30 mM EDTA was added to it slowly as gas bubbles are formed. The tubes were stirred gently over a vortex. Fifty microlitres portion of the above buffer was added similarly and the addition was repeated for the third time also. To expedite the complete precipitation of the K-perchlorate the tubes were chilled briefly in ice. The tubes were subsequently centrifuged and the supernate was transferred into another plastic vial. The pH of the clear supernate obtained was then measured (which should be between pH 5-6 as at pH 7 and above the GSH is rapidly oxidized to GSSG). The extract was stored at -75°C for not longer than 7 days or used on the same day.

3.15.2 Glutathione measurement:

Measurement of the total glutathione content (reduced and oxidised) was based on the reaction with 5,5'-di-thio-bis-(2-nitrobenzoic acid) measuring GSH equivalents, according to Tietz (1961). This is a kinetic test coupled with an enzyme reaction. It is based on the interaction of DTNB with GSH which is measured as an increase in 405 nm absorption. Glutathione reductase and NADPH are included into the reaction
to convert the oxidized glutathione into the reduced glutathione which in turn reacts with DTNB. The reaction will continue till all the DTNB or NADPH are used up. Under the conditions given, O.D. increase of more than approx. 1.2 O.D./min. was avoided. Glutathione content was measured spectrophotometrically in 1 ml glass cuvette at 25°C. The reaction mixture in a total volume of 1 ml contained 0.08 M Phosphate buffer, 0.8 mM EDTA, 0.05% NADPH, 1 ug glutathione reductase (Sigma Type III, 1.25 mg/2.5 ml) and 20-100 ul of the biological extract. The reaction was initiated by the addition of 50 ug (10 ul) DTNB dissolved in phosphate-EDTA buffer. The linear reaction rate was monitored by measuring the increase in absorbance as a linear function of time. The linearity of the test was established by a calibration curve using appropriate GSSG concentration (0-50 μM).

3.16 Extraction and determination of UDP-glucuronic acid from isolated intestinal cells:

UDPGA was extracted from cells and determined enzymatically according to Singh et al, (1980).

3.16.1 Extraction of UDPGA:

Extraction of UDPGA was followed immediately after removal of cell samples from the incubation or after fresh preparation of cells. Briefly 200 ul aliquots of cell suspension (1-3 mg protein) were cooked together with 300 ul of water in a boiling water bath for 3 min. The samples were cooled down and centrifuged at 3000 x g to sediment the denatured protein. The supernatant served as a source of UDPGA.
The material was either immediately used or stored at -70°C for later assays.

3.16.2 Determination of UDPGA:

UDPGA-dependent formation of benzo(a)pyrene 3-glucuronide catalysed by UDP-glucuronyl transferase of guinea-pig liver was determined as described in Section 3.14.4. Hepatic microsomal fraction from male guinea pigs was used as a source of UDP-glucuronyltransferase because the fraction contained negligible amount of pyrophosphatase activity directed towards UDPGA (Wong and Lau, 1970). The microsomal fraction prepared in 0.25 mM sucrose (section 3.8) was washed twice by resuspending in 0.25 M sucrose and centrifuging at 105,000 x g. The pellet was resuspended in 0.25 M sucrose and 0.5 ml proteins were stored at -70°C. The GT activity was stable at least for 1 month when stored at -70°C. For assay of UDPGA, 50 ul of tissue extract as a source of UDPGA and 50 ug of guinea-pig liver microsomal protein were used in a reaction mixture volume of 200 ul which contained 100 mM Tris buffer, pH 7.6, 5 mM MgCl₂, 0.025% brij-58 and 50 uM 3-OH-BP. The reaction mixtures were assembled in test tubes and immersed in ice. The tubes were incubated at 37°C for 60 min. with mild shaking. The reaction mixtures were extracted with 6 ml chloroform:methanol (2:1, v/v) together with 0.8 ml of water by vigorous shaking for 5-10 sec. Fluorescence of the product benzo(a)pyrene 3-glucuronide was measured at 378 nm excitation/425 nm emission. The amount was calculated from a standard curve obtained by incubating various amounts of UDP-glucuronic acid instead of tissue extract.
3.17  **Modulation of glucuronidation rate in intestinal cells**

3.17.1  **Cell incubation system**

In general, conditions for cell incubation varied with individual experiment and are described wherever required and in legends to their respective tables or figures. For modulation of glucuronidation rate in guinea-pig intestinal cells by piperine cells at a final concentration of 3 mg protein (equivalent to approx. $7 \times 10^6$ cells) per ml were incubated in MEM Eagle's medium with Earle's salts, 10 mM Heps, 26 mM NaHCO$_3$, pH 7.4 at 37°C under a transparent hood provided with an inlet for oxygen gaseous phase. The cells were stirred gently over a magnetic stirrer. After preincubation of cells for 5 min, different concentrations of D-galactosamine or piperine were added in saline or dimethyl sulphoxide (10 ul/ml medium) respectively. Aliquots of 200 ul were taken at different time intervals for the determination of glucuronide or UDPGA content. Wherever required, the cells (intracellular compartment) were separated from the medium (extracellular compartment) by silicon oil microcentrifugation (Klingenberg and Pfaff, 1967).

3.17.2  **Silicon oil microcentrifugation technique for rapid separation of intracellular compartment from extracellular compartment**

The rapid separation of cells from the incubation medium was done by utilizing the centrifugal filtration technique, originally developed for mitochondria, by Klingenberg and Pfaff (1967); and later employed for hepatocytes (Baur et al., 1975) and intestinal cells (Hegazy
et al., 1983; Schwenk et al., 1984) for desired measurements in intracellular and extracellular compartments (Fig.1). Briefly, a 400 ul centrifuge tube contained a layer of 100 ul of silicone oil (AR 20: AR 200, 3:1) on top of 50 ul layer of 3 M KOH, 3 M TCA or 6 M urea. Cell suspension (200 ul) was layered on top of the silicon oil layer. The tubes at time intervals were spun instantly in a Beckman microfuge, the cells together with a negligible amount of adherent medium passed through the silicon oil into the reaction termination medium (KOH, TCA or urea) where they are deposited in a form of pellet. The tubes were cut through the silicon oil and the extracellular compartment (medium) and intracellular compartment (cell pellet) were taken in separate vials for various measurements. The density of the silicon layer is adjusted higher than that of the medium and lower than that of the cells at working temperature which was accomplished by mixing AR 20 and AR 200 grades of silicon oil in desired ratios.

3.17.3 Determination of 3-hydroxybenzo(a)pyrene glucuronidation rate in intact cells:

The glucuronidation rate in the intact isolated intestinal cells towards the substrate, 3-OH-BP was determined according to Singh and Wiebel (1979). 3-OH-BP was added in DMSO (10 ul per ml incubation system) to a final concentration of 100 uM. At time intervals, 200 ul aliquots of the incubates were removed into 6 ml of chloroform:methanol (2:1) and 0.8 ml of water. The tubes were shaken vigorously on vortex mixer and the organic phase was allowed to separate
Silicon oil-Microcentrifugation in Cellular Transport Studies.

Fig. 1 A schematic representation of cell incubation and separation of intra-cellular compartment from the extra-cellular compartment.
from the upper aqueous phase. The BP-3-glucuronide formed was measured fluorometrically (Singh and Wiebel, 1979) in the aqueous phase at 378 nm excitation and 425 nm emission in Kontron Spectrofluorometer (Kontron Instruments Ltd., St.Albans).

Sulfation of phenols is also a major conjugation reaction of the intestinal cells which varies with the nature of phenol used (Dawson and Bridges, 1979) and under our experimental conditions of extraction BP-3-sulfate might interfere in quantifying BP-3-glucuronide. Therefore, in separate experiments with viable intestinal cells and 3-OH-BP, the aqueous phase obtained after extraction with chloroform:methanol (2:1), was dried under a stream of nitrogen. The residue was dissolved in a 0.5 M acetate buffer, pH 4.6. Aliquots in a total volume of 0.4 ml were incubated at 37°C for 2 hrs with 1 mg β-glucuronidase or with 5 mM D-saccharic acid 1,4-lactone and 150 μg arylsulfatase. After reextraction with chloroform:methanol (2:1), the BP-3-glucuronide was measured in the aqueous phase. BP-3-glucuronide was found to be the major product accounting for about 80% of the total conjugate formed and the remaining product(s) was hydrolysed by arylsulfatase under the above experimental conditions. This factor was taken into account while calculating the glucuronide content in the intact cells. Further, both the conjugates have been shown earlier to possess different fluorescence maxima i.e. 382/415 nm for the sulfate conjugate of 3-OH-BP and 378/425 nm for the glucuronide conjugate (Wiebel et al., 1980) and the relative fluorescence of both the conjugates was comparable at their wavelengths of interest.
3.18 Use of intestinal cells for assessment of cytotoxicity of xenobiotics:

3.18.1 Cell incubation system:

Enterocytes were isolated from guinea-pig small intestine as described (Section 3.5). They were washed twice with Ca$^{++}$, Mg$^{++}$-free Hank's basal salt solution, pH 7.4. The viability of the cells was checked by trypan blue staining and the cells were used when the viability was more than 90%. Aliquots of isolated intestinal cells (approx. 6 mg cellular protein) were incubated in 2 ml of Ca$^{++}$ and Mg$^{++}$-free Hank's basal salt solution containing 10 mM Hepes and 0.5% BSA, pH 7.4. The incubation was carried out in glass vials, at 37°C under oxygen gaseous phase. The cells were stirred gently over a magnetic stirrer.

3.18.2 Uptake of radioactive markers:

Cells were labelled according to Thelestam and Möllby (1975a, 1975b).

$^3$H-$\alpha$-aminoisobutyric acid (Sp. activity 10 ci/m mole) was added to the incubation system (1 uci/ml) after pre-incubation of cells for 3 min. At time intervals 100 ul aliquots of the cell suspension were drawn and centrifuged in the Beckman microfuge to separate the intracellular compartment from the extracellular compartment by silicon oil micro-centrifugation technique (Section 3.17.2). $^3$H-$\alpha$-AIB uptake was estimated in the intracellular compartment by assaying for the radioactivity in 5 ml of Bray's scintillation fluid.
For $^3$H-uridine incorporation aliquots of intestinal cells (approx. 6 mg cellular protein) were incubated in 2 ml of the cell incubation medium as described above. After preincubation, 2 uci of the $^3$H-uridine were added to the 2 ml incubation system. At different time intervals 100 ul aliquots of the cell suspension were drawn, precipitated in TCA and washed (Section 3.11). The pellet was finally dissolved in 100 ul of KOH and the radioactivity was determined in Bray's scintillation fluid.

3.18.3 **Release of radioactive markers**

(a) **Spontaneous release:**

The enterocytes were exposed to radioactive $\alpha$-AIB and uridine for 20 min as described above. The cells were washed twice with chilled Hank's basal salt solution, containing 10 mM Hapes, 0.5% BSA and 1 mM cold $\alpha$-AIB or uridine for their respective experiments. The labelled cells were incubated at 37°C in the same incubation medium supplemented with 1 mM cold $\alpha$-AIB or uridine. At time intervals 100 ul aliquots were removed and spun through silicon oil microcentrifugation. The radioactivity for $^3$H-$\alpha$-AIB in the medium was directly determined in Bray's scintillation fluid while for $^3$H-uridine labelled activity the supernatant was precipitated in 10% TCA, together with 0.25% BSA. The precipitate was processed for measurement of radioactivity as described above. The count determined in the supernatant represent 'spontaneous release.'
(b) **Maximal release:**

The maximal release of the intracellular amount of radioactivity was determined after the prelabelled cells were ruptured with 0.5% Triton X-100 in 2 ml of incubation medium at 37°C for 30 min. (Malik et al., 1983). This treatment released the intracellular radioactivity. The cell lysate was centrifuged in a microcentrifuge and 100 ul aliquots were removed for measurement of radioactivity.

(c) **Toxin treatment of cells:**

Prelabelled and washed enterocytes were incubated with various concentrations of the test substances in 2 ml of cell incubation medium with 1 mM cold L-arginine or uridine. After 15 min of incubation 100 ul of the supernatant was removed and centrifuged in a microcentrifuge. Radioactivity was measured in the medium as described (Section 3.18.3).

3.18.4 **Calculation of released radioactive markers:**

Released radioactivity was calculated as described by Thelestand and Mollby (1976). The sum of intracellular plus released radioactivity was taken as a measure of the "maximal release". The release induced by toxins "leakage" is expressed as % of maximal release:

\[
\text{Leakage} = \frac{\text{toxin induced release minus spontaneous release}}{\text{maximal release - spontaneous release}} \times 100
\]
3.18.5 **Measurement of the release of lactate dehydrogenase and alkaline phosphatase enzyme activities:**

LDH and AP activities were assayed in 0.1 ml aliquots of the supernatant removed simultaneously in experiments on release of radioactive markers (Section 3.18.3). LDH was assayed spectrophotometrically according to Bergmeyer and Berndt (1970) and alkaline Phosphatase according to Weiser (1973a). Total intracellular LDH and AP activities were assayed after lysing the cells with 0.5% Triton-X-100 at 37°C for 30 min.

3.19 **Determination of single-strand break frequency of DNA by alkaline elution assay:**

3.19.1 **Preparation and incubation of cells:**

Adult male albino rats were given \(^{3H}\)thymidine (6000 mci/m mole) at a dose of 100 uci/200 gm b.wt. intraperitoneally. The animals were sacrificed after 3 hrs, intestine removed and the small intestinal cells were eluted in the gradient fractions as described earlier (Section 3.4). Mostly cells from the crypt region and some times of mid-villus region were used for assessment of DNA damage. The cells (approx. 1 mg cellular protein) were washed in Hank's balanced salt solution (or otherwise stated) and incubated with or without variable concentrations of the test compounds in a total volume of 1.5 ml containing 0.25% BSA at 37°C under corbogen. All experiments were conducted under subdued light.
3.19.2 Alkaline elution assay:

A modification (Loquet and Wiebel, 1982) of the procedure of Kohn et al. (1976) was used to elute single stranded DNA breaks. Basically, the technique utilizes filters and alkaline solution to discriminate single strand sizes in cells (Kohn et al, 1979). The filters do not absorb DNA under the conditions employed, but rather act mechanically to impede the passage of long DNA strands. The assay is accomplished by depositing the cells on filters prior to lysis with a detergent solution. Most of the cell protein and RNA can be washed through the filter leaving the DNA in still double-stranded form (intact on the filter). The DNA can now be treated with proteinase to digest bound protein. An alkaline solution at pH 12 to 12.2 is then slowly pumped through the filter and the rate of elution of DNA due to single strand breaks is measured on the basis of an increase in DNA elution rate i.e. radioactive thymidine in this case.

For procedural details, briefly, cells after incubation were washed in cold Dalbecco's phosphate buffered solution deposited slowly on polycarbonate filter (pore size 0.2 μm, Bio-Rad Laboratories). The buffer was sucked through the filter using a peristatic pump (Colora 3600) at a flow rate of about 0.1 ml/min, and the cells were lysed for 1 hr in 5 ml of 2 M NaCl, 0.2% sarcosyl and 0.02 M EDTA, pH 10 containing 0.5 mg/ml of proteinase-K. After lysis the solution was sucked through the filters, they were rinsed with 2.5 ml
of 0.02 M EDTA, pH 10. Single stranded DNA was eluted with 15 ml of 0.02 M tetrapropyl-ammonium hydroxide, 0.02 M EDTA, 0.1% sodium dodecylsulfate, pH 12.2, at a flow rate of 0.1 ml/min. Fractions were collected at 20 min intervals. After elution the filters were removed, incubated at 70°C for 1 hr with 0.5 ml 1N HCl and treated with 2.5 ml of 0.4 M NaOH. After removing filters the pump channels were washed with 5 ml of 0.5 M NaOH. Aliquots (500 ul) were taken in 10 ml of Bray's scintillation fluid for measurement of radioactivity. The cells at 0°C in PBS were also exposed to different doses of radiation (Co^{60}, Gamma Chamber 900, Isotope Div, BARC, Bombay, India) and were used as external standard for producing single strand breaks in DNA.

3.20 Protein measurement:

Protein was determined by the standard procedures of Lowry et al. (1951) and by the modified biuret method as described by Szarkowsk and Klingenberg (1963) using bovine serum albumin as standard.

3.21 Chemicals:

The following chemicals were purchased from:

Sigma Chemical Co. (St. Louis, MO, U.S.A.): DL-β-hydroxybutyric acid, succinate, rotenone, carbonyl cyanide-m-chlorophenylhydrazone, tris(hydroxymethyl)-amino methane, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid-sodium salt, ethylenediaminetetraacetic acid, bovine serum albumin, antimycin-A, benzo(a)pyrene, 7-8-benzoflavone, 3-methylcholanthrene, uridine diphosphoglucuronic acid, Brij-58, 7-ethoxycoumarin, umbelliferone, D-saccharic
acid 1,4-lactone, D-glactosamine, dexamethasone, \( \gamma \)-glutamyl-p-nitroanilide, indomethacin, aspirin, triton-X-100, nystatin, 1-chloro-2,4-dinitrobenzene, reduced glutathione, N-methyl-N-nitrosoguanidine, 1,2-epoxy-3-phenoxy propane, 3,4-dichloro nitrobenzene, glucose oxidase from *Aspergillus niger*.

Boehringer (Mannheim, West Germany): 5',5'-Dithiobis-(2-nitrobenzoic acid), reduced nicotinamide adenine dinucleotide, reduced \( \beta \)-nicotinamide adenine dinucleotide phosphate, glutathione reductase-type III, aryl sulfatase from *Helix pomatia*.

Serva (Heidelberg, West Germany): \( \beta \)-glucuronidase from bovine liver, proteinase-K, dithiothreitol, sodium dodecyl sulfate.

Aldrich Chemical Co., Wisc.: Benzoquinone (p-benzoquinone), hydroquinone (p-dihydroxybenzene).


Loba Chemie (Bombay): 2,6-dichlorophenol-indophenol.

CSIR Center for Biochemicals, (V.P. Chest Institute, Delhi): Cytochrome-C, adenosine diphosphate, adenosine triphosphate, nicotinamide adenine dinucleotide phosphate.

HI-MEDIA (Hindustan Dehydrated Media, Bombay): Eagles MEM, Dulbecco's MEM, MEM with Earle's salts, MEM with Hanks salts, Dulbecco's modified MEM, Dulbecco's phosphate buffered saline.

Radioactive chemicals: (Me\(^3\)H)-thymidine (specific activity, 18800 mci/m mole), \((^3\text{H})\) uridine (specific activity, 15200 mci/m mole).
mole), (1-C^{14}) DL-leucine (specific activity, 53.7 mci/m mole) were obtained from Bhabha Atomic Research Centre (Bombay), and (^{3}H) L-aminoisobutyric acid (specific activity, 10 ci/m mole) from New England Nuclear Co. (Dreieich).

Chemicals obtained as Gifts:- Endosulfan was a gift from Hoechst, India, 3-hydroxy benzo(a)pyrene was a generous gift from Dr. Friedrich J. Wiebel and ethyl morphine from Dr. Thomas Wolff, both of the Department of Toxicology (GSF, D 8042 Neuherberg, Munchen, West Germany). Zoxazolamine and SKF-525A (β-diethyl-aminoethyl diphenylpropylacetate) were kindly provided by Dr. Paul Workman (MRC, Clinical Oncology and Radiotherapeutics Unit, Cambridge CBS 2CH, England). 1,2-dimethyl hydrazine-hydrochloride was a generous gift from Dr. Sidney D. Nelson (Department of Medicinal Chemistry, University of Washington, Seattle, WA 98195, U.S.A.).

All other chemicals used were of analytical grade available locally. Piperine was extracted from black pepper and purified in our laboratory (Atal et al., 1981). The purity of the product was determined by thin-layer chromatography and melting point.

3.22 Equipment:- Pye Unicam SP8-100 U.V. spectrophotometer, Kontron 810 spectrophotometer, Kontron spectrofluorometer, Carl Zeiss Jena phase contrast light microscope, Jeol-100 CX2 electron microscope, Soniprep 150-MSE sonicator, Beckman microfuge, Yellow Spring Instruments
biological oxygen monitor-53, (Colora-3600) peristaltic pump, Beckman L-S3150P β-scintillation counter, IEC (Indian equipment corporation) high speed refrigerated centrifuge, Sorvall RT6000 table top refrigerated centrifuge and Janetzki VAC 601 Ultracentrifuge.