a. Primate injury model

Subjects: Macaca radiata monkeys of both sexes weighing 6-10 kg were used in this study. Approximately one half of the animals were female and the other half male to control for possible sex differences in response to spinal cord injury.

Anaesthesia: Surgical procedures were performed under general anaesthesia using intravenous (IV) injection of sodium pentobarbitone (30 mg/kg body weight). Occasionally additional amounts of pentobarbital (IV) were administered to maintain an adequate level of anaesthesia.

Surgical procedure: The anaesthetized animals were positioned in a stereotaxic frame in a prone position. Polyethylene cannulas were inserted into femoral artery and femoral vein. Systemic blood pressure and heart rate were monitored via the arterial catheter which was connected to a Statham transducer. The venous cannula was used to administer drugs. A laminectomy, extending from D3-D9 was carried out in all the animals. A Teflon impounder contoured to fit the dorsal surface of the exposed dura was placed at D7 which was the site for injury. Rectal temperature was maintained between 36°C and 38°C by a heat cradle.

Induction of injury: The injury apparatus (Fig. 3) consists
of a cylinder that is placed perpendicular to the surface of the spinal cord. The cylinder contains a loosely fitting Teflon impounder, which rests on the intact surface of the dura. The height of the cylinder is marked in centimeters, and a lead weight (50 g) is dropped through the cylinder for a determined distance, until it makes contact with the impounder resting on the dural surface.

In this model, a contusion injury to the spinal cord was produced by a 50 gm lead weight dropped from a height of 4 cm through a hollow glass tube onto the impounder. All the animals were maintained on spontaneous breathing in a heat cradle. The pH, PCO₂, PO₂ and blood pressure were intermittently monitored. This method had been standardized in our laboratory previously. (Abraham et al., 1986). Based on our previous study, this type of injury would be expected to result in paraplegia.

**Region of interest:** Regions of interest (ROI) consisted of two segments, segment I, the traumatized segment approximately 1 cm in length and situated over D7 and segment II, approximately 1 cm in length and centered 2 cm cranial to the traumatized area.

**Sham operation:** One group of animals had laminectomy performed, and at the end of 1/2, 4, 24, and 48 hour and 1
week following surgery, the standard regions of interest segments were removed and were used as controls. Cerebrospinal fluid (CSF) was tapped by a lumbar puncture before sacrificing the animal.

**Normal controls:** To determine the effect of anaesthesia alone, the segments were removed in one group of monkeys at 1/2, 4, and 8 hour after anaesthesia.

**Experimental group:** Contusion injury was produced in a group of animals and spinal cord segments were removed. At variable time periods after trauma, animals were reanaesthetized (pentobarbital 30 mg/kg) and the regions of interest were removed.

**Post operative care:** The animals were sedated for nearly 20 hours after the experimental procedure. When the animals awakened from anaesthesia, they were returned to their cages. Proper post-operative care such as care of the wound and administration of antibiotics (intramuscular doses of procaine penicillin G) was done by the investigator.

**b. Rat injury model**

**Subjects:** Adult rats (Wistar) weighing 180-200 g of either sex were used for this study.
Anaesthesia: Rats were anaesthetized with pentobarbitone sodium (nembutal) 5 mg/kg body weight and supplemented with anaesthetic ether as and when needed.

Method of injury: A laminectomy was performed from C7 to T2. The spinal cord at T1 was compressed by the extradural clip compression technique (Rivlin, 1978). This technique consisted of placing a modified Kerr-Lougheed aneurysm clip around the cord extradurally. The clip used in these experiments had two arms connected by a steel spring. The arms of the clip were 1 mm wide, 0.5 mm thick, and 8 mm long, and were curved to facilitate extradural placement around the cord.

Normal controls: A group of animals were killed under anaesthesia with sodium pentobarbitone and the spinal cord segments were removed.

Sham controls: Laminectomy was done from C7 to T2 in a group of rats. The animals were sacrificed after one week and the traumatized (segment I) and an adjoining upper segment 1 mm length (segment II) were removed.

Experimental group: Spinal cord compression injury was produced at the level of T1 by placing a modified Kerr-Lougheed aneurysm clip around the cord extradurally for 30 seconds. Both segments of interest (segment I and segment II)
were removed after one week.

Post-operative care: Immediately after surgery, all animals received 0.2 mg/100 gm body weight, intramuscular injection of gentamicin. The urinary bladders of all operated were manually compressed every 12 hours for one week following surgery. Water and food were made available ad libitum.

DRUG STUDY

The drugs studied were, Verapamil ( Isoptin R, Boehringer Knoll Ltd. India), Nifedipine, Dexamethasone (Wymesone Wyeth laboratories, India.), Dipyridamole, Dimethyl sulfoxide, and Naloxone Hydrochloride (Sigma Chemical Co., St. Louis, MO).

Drug details:
Dexamethasone: Treatment was begun 30 minutes post injury. Dexamethasone was given 0.5 mg/kg intravenously 30 minutes after injury. Systemic blood pressure was monitored for 2 hours after treatment. Dexamethasone was administered for 7 days in the following schedule:-

- 0.500 mg/kg (IM) 2nd day
- 0.250 mg/kg (IM) 3rd day
- 0.250 mg/kg (IM) 4th day
- 0.125 mg/kg (IM) 5th day
- 0.125 mg/kg (IM) 6th day
- 0.125 mg/kg (IM) 7th day
The animals were sacrificed after one week. Both segments were removed for biochemical and pathological analysis.

**Dexamethasone + sham:** To ascertain the effect of dexamethasone on sham animals, a group of animals were given the drug in the same regimen.

**Verapamil:** Verapamil was given (0.2 mg/kg) intravenously 30 min after injury. Blood pressure was monitored for 4 hours after treatment. The drug was administered for one week in the following regimen:

- 0.2 mg/kg (IM) 2nd day
- 0.2 mg/kg (IM) 3rd day
- 0.4 mg/kg (IM) (divided doses) 4th day
- 0.4 mg/kg (IM) 5th day
- 0.8 mg/kg (IM, divided doses) 6th day
- 0.8 mg/kg (IM, divided doses) 7th day

The animals were evaluated for motor function for one week. After one week both segments were removed for biochemical and pathological studies.

**Verapamil + sham:** To ascertain the effect of verapamil on sham controls, a group of animals were given verapamil in the same regimen.
Nifedipine: Nifedipine was dissolved in ethyl alcohol and propylene glycol. The drug was given 30 min post injury in the dose of 1 mg/kg (IM). The animals were followed for one week and both segments of interest were removed.

Naloxone: Naloxone was given in a bolus injection of 4 mg/kg (IV), 30 min post injury. Blood pressure was monitored for 4 hours after treatment. The animals were sacrificed after one week.

Dipyridamole: Dipyridamole was administered 1 mg/kg intravenously 30 min post injury, followed by intramuscular injection of graded doses (2-4 mg/kg) for 1 week.

Dimethyl sulfoxide (DMSO): 2.5 gm/kg (40% solution in normal saline) was given 30 min after trauma intraperitoneally and 2.5 gm/kg daily for 1 week in divided doses.

Saline controls: A group of experimental animals received normal saline for one week and served as controls to the treated groups.

EVALUATION OF NEUROLOGIC RECOVERY

Motor function:

Primates: In primates motor testing was done by the method of Tarlov (Green, 1980). A 0-4 rating scale was used for the
Fig. 4. The inclined plane apparatus consists of two wooden boards connected at one end by a hinge. The ridged rubber mat is seen on the movable plane.
neurological testing. Neurological testing was blindly scored by an unbiased observer, once in two days during the 1 week post injury period studied. The scores were given as follows:

- 0 - Hip movement only
- 1 - Knee movement
- 2 - Ankle and/or toe movement
- 3 - Spastic or imperfect gait
- 4 - Normal gait

Rats: Motor testing was carried out on an inclined plane once in two days for 7 days. A blind testing method was used whereby the observer was not aware whether a given animal was a control or a treated animal. The inclined plane consists of an adjustable inclined plane covered by a rubber mat constructed after the apparatus described by Rivlin & Tator (1977) (Fig. 4). The angle of the plane was increased from 0° until the rat could not maintain its position for 5 sec. Rats were placed on the inclined plane and the maximum angle at which the rat could maintain its position for at least for 5 sec without falling was carried out.

STUDIES ON BIOCHEMICAL CHANGES

Acetylcholinesterase (AChE): Both segments (I & II) and cerebrospinal fluid (CSF) were used for AChE assay. Tissues were cleaned of blood and weighed and homogenized in 8 volumes
of 0.05 M Potassium phosphate buffer in a Potter Elvehjem homogenizer at 1200 rpm. Diluted homogenates were used for enzyme assay. CSF was tapped by lumbar puncture before sacrificing the animals. Acetyl cholinesterase was assayed by the method of Ellman et al., (1961) using acetylthiocholine iodide as substrate. 50 mmole potassium phosphate buffer, pH 8.0, 15 mmole acetylthiocholine iodide, 0.1 ml of a 5, 5, dithiobis (2-nitrobenzoic acid) reagent and 0.05 ml of the enzyme in a total volume of 0.5 ml, and incubated for 10 minutes at 37°C. The reaction was stopped with 0.5 ml of 1 mM eserine sulphate and the absorbance of liberated product read at 412 nm. Butyl cholinesterase (BuChE) activity was also done using butyl thiocholine as substrate and in the presence of BW 284 C51, a specific inhibitor of acetylcholinesterase to ascertain any activity due to BuChE. The unit of AChE is the change in absorbance of 1/min at 412 nm under standard assay conditions. The specific activity of the enzyme was expressed as units of enzyme activity per mg protein.

The protein was determined by the method of Lowry et al., (1957).

\[ \text{Na}^+, \text{K}^-\text{ATPase:} \] \text{Na}^+, \text{K}^-\text{ATPase was measured after 1 week injury. Both segments were used for assay. Spinal cord tissues were homogenized in sucrose Tris-imidazole buffer. ATPase activity was determined by the rate of liberation of phosphate} \]
Inorganic Pi was determined by the method of Taussky and Shorr (1953). The activity was expressed as nmole Pi/mg protein/min.

**Lysosomal Enzymes:** All of the following steps were carried out at 4°C unless otherwise indicated. Both segments were cleaned of blood and homogenized in 7 volumes of 0.15 M NaCl in a Potter Elvehjem homogenizer at 1200 rpm. Homogenates were then centrifuged at 12000 g for 1 hour in a Servall RC-2B refrigerated centrifuge. Supernatants were separated and kept frozen at -20°C until assayed.

α-L-Fucosidase was assayed as described (Alam, 1978) except that bovine serum albumin was omitted. β-D-hexosaminidase was assayed at pH 4.5, using 0.2 nmol of N-acetyl β-D-glucosaminide as substrate in the absence of bovine serum albumin, as described earlier (Alam, 1975). One unit of enzyme activity was defined as 1 nmol of p-nitrophenol liberated per hour at 37°C. The specific activities of the enzymes were expressed as units of activity per mg protein. The activities in CSF were expressed in terms of units per ml.

**Verapamil:** Both sham and experimental animals were treated with intravenous verapamil hydrochloride for one week. Blood samples were collected at 30 minutes, after treatment each
day. The animals were sacrificed after one week and blood, CSF, and spinal cord tissues were taken for verapamil assay. Fluorescent verapamil concentrations were estimated in sera, CSF, and spinal cord tissues flurometrically (McAllister, 1976). To 5 ml. of homogenate in a 45 ml. centrifuge tube were added 1.0 ml. of 1N NaOH and 10 ml of heptane (analytical grade). Each sample was shaken vigorously for 15 min in the upright position and then centrifuged for 10 min at 1800 rpm in a clinical centrifuge. After centrifugation 7 ml of the heptane layer was transferred to a clean 45 ml centrifuge tube containing 5 ml of 0.1N HCl. Each tube was vigorously shaken for 15 min and centrifuged at 1800 rpm for 10 min. The supernatant organic phase was aspirated and discarded. Two milliliters of the aqueous phase was used for spectrophotometric analysis at an excitation wave length of 275 nm and an emission wave length of 310 nm. Concentrations of unknown samples were calculated by comparison to the readings obtained for internal standards.

**Phospholipids:** The concentration of total and individual phospholipid in spinal cord segments were determined in chloroform-methanol extracts. Total lipid extracts were prepared by the method of Folch et al. (1957) by homogenizing spinal cord tissues in 5 ml chloroform-methanol-12M HCl (200 : 100 : 0.8 by volume) with a motor driven (Tri-R instruments) Teflon pestle and glass homogenizer tube. Each extract was
washed three times with two volumes of 1M MgCl₂, dried under vacuum and dissolved in a small volume of chloroform : methanol (2:1 by volume). Phospholipids were isolated by two dimensional thin layer chromatography (Tou, 1978). Separated phospholipids were visualized by exposure to iodine vapors and identified by comparison with the positions of standards chromatographed in the same system. Each iodine positive spot was carefully scraped from the plate and quantitated by Pi determination after acid digestion (Bartlett, 1959). The reduced phosphomolybdate complex was read at 820 nm.

HISTOPATHOLOGICAL CHANGES

One week after the injury the spinal cords were removed and subsequently embedded in paraffin blocks. Sections were cut and stained with haematoxylin and eosin. Histological section were taken from sham controls, experimental and drug treated animals.

STATISTICAL ANALYSIS

Statistical analysis was done by Student's t-test. The values obtained from segment I were compared to the values obtained from segment I of sham operated controls. Drug treated groups were compared to experimental group.
Table I. Changes in blood pressure during spinal injury.

<table>
<thead>
<tr>
<th>Surgical details</th>
<th>No. of monkeys</th>
<th>Blood pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Before laminectomy</td>
<td>20</td>
<td>117.70 ± 2.365</td>
</tr>
<tr>
<td>After laminectomy</td>
<td>7</td>
<td>120.85 ± 5.95</td>
</tr>
<tr>
<td>After weight drop.</td>
<td>13</td>
<td>144.50 ± 6.708*</td>
</tr>
</tbody>
</table>

Values are mean ± SEM. *P<0.001

Table II. Effects of various drugs on mean arterial pressure.

<table>
<thead>
<tr>
<th>Drug</th>
<th>No. of Monkeys</th>
<th>Mean arterial BP (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Before</td>
</tr>
<tr>
<td>Verapamil (0.2 mg/kg, IV)</td>
<td>4</td>
<td>113.75 ± 3.3.</td>
</tr>
<tr>
<td>Dexamethasone (0.5 mg/kg, IV)</td>
<td>3</td>
<td>107.5 ± 8.77</td>
</tr>
<tr>
<td>Naloxone (4 mg/kg, IV)</td>
<td>3</td>
<td>110.83 ± 4.4</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM. * P < 0.001.
Table III. Effect of anaesthesia on monkey AChE activity (units/mg protein).

<table>
<thead>
<tr>
<th>TIME INTERVALS (HOURS)</th>
<th>NO. OF MONKEYS</th>
<th>SEGMENT I</th>
<th>SEGMENT II</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>4</td>
<td>0.235 ± 0.0051</td>
<td>0.2516 ± 0.020</td>
</tr>
<tr>
<td>4.0</td>
<td>3</td>
<td>0.2404 ± 0.0027</td>
<td>0.2494 ± 0.020</td>
</tr>
<tr>
<td>8.0</td>
<td>3</td>
<td>0.2369 ± 0.0014</td>
<td>0.2962 ± 0.019</td>
</tr>
</tbody>
</table>

Values are Mean ± SEM. SI: Traumatised segment. SII: non-injured segment.