CHAPTER II

MATERIALS AND METHODS

In order to achieve the objective mentioned earlier,manipulation of circulating levels of glucocorticoid is most essential to investigate its permissive action (basal levels) and under stress (elevated levels) condition.

For the studies of permissive role, animals are generally operated for bilateral adrenalectomy and kept for different time period postoperatively. Some of the adrenalectomized animals are also given hormone replacement for treatment to check whether the effects observed reversed when the animals are administered corticosterone.

To study the effects of excess of corticosterone which is often found with stress situation, generally such effects are studied by either subjecting animals to physiological or physical stress repeatedly or by administering exogenous corticosterone for different periods of time. In the present study the latter method i.e. administration of corticosterone was applied.

Since corticosterone levels are known to be higher in older animals and the same has been implicated in the aging phenomenon (Sapolsky *et al.*, 1986). One experiment was conducted in older rats with and without adrenalectomy to understand the role of high basal levels of corticosterone.

The first step involved selection of dose, period of treatment and specific ages to be studied *etc.* The following points were taken into consideration.

With respect to corticosterone administration it is very clear from the table given 2.1 that the amount of corticosterone injected varied from different laboratories as well as the duration of the treatment given.

Moreover some of these studies were carried out in adult rats. Since the concentration of basal levels of corticosterone are known to be different, the effects at younger ages were investigated.

Table: 2.1

List of reported with dose glucocorticoid used, duration and and parameters measured in rats.

Reference	Hormone	Dose	Duration	Parameter
Howard (1965)*	Cort.	0.5mg/day	3 days	brain growth
Gumbinas *et al.*,(1973)*	Pregn.	0.8mg	Once	myelin
Howard & Benjamins* (1975)	Cort.	1.6mg/kg b.wt.	10 days	Cell loss
Patel *et al.*,(1983a)*	Cort.	40mg/kg b.wt.	3 days	Glutamine synthetase
Dickinson *et al.*, (1985)	Cort.	50mg/kg b.wt.	4 days (twice)	Serotonin
Devenport & Devenport* (1985)	Cort.	7mg/kg b.wt.	19 days	brain growth
Duman *et al.*,(1986)	Cort.	15mg/kg b.wt.	14 days	cAMP
Duman *et al.*,(1989)	Dex.	4mg/kg b.wt.	33 days	cAMP
Saito *et al.*,(1989)	Cort.	100mg	7 days	G-protein.
Duman *et al.*,(1990)	Cort.	100mg	7 days	ARF
Gannon & McEwen (1990)	Cort.	50mg/kg b.wt.	21 days	cAMP
Iacopino & Christakos (1990)	Cort.	10mg/day	7 days	Calbindin protein

* Experiments on young animals.

In the earlier studies carried out in this laboratory a dose of 40 mg/kg body weight (s.c.) for three days was used for elevated corticos-

terone levels. This level and period of treatment was found to increase glutamine synthetase levels (Bhargava, 1988) and was very similar to what was reported by Patel *et al.* (1983a). The same dose and treatment was found to alter brain lipids such as total phospholipid and cholesterol (Bhargava *et al.*,1991), brain Na+ K+ ATPase (Saunderava, 1991) as well as hepatic mitochondrial energy metabolism (Jani *et al.*, 1991). These studies imply that three day treatment and the above dose is able to affect various biochemical parameters of the brain and membranal functions in young animals. Therefore the same dose and duration was selected for the present study.

In the present study the criteria for selecting 10, 20 and 40 day old rat was that at 10 days of age the circulating corticosterone is very low and is called stress non responsive period (Sapolsky and Meaney 1986). At this age, exogenous administration of hormone can cause deleterious effects to the brain. At 20 day old (weaning) the basal titers of circulating corticosterone reach the adult values (Hennings, 1978) and also cell division ceases at 20 day after birth (Howard, 1973). 40 day old rats were considered as young adult.

The depletion studies invariably have been carried out by bilateral adrenalectomy. Various studies reported in the literature vary in the duration of post surgery periods which is as short as 24 hours short to as long as 153 days (Table 2.2). Few studies have also been carried out using drugs which inhibit glucocorticoid synthesis such as metapirone (Robinson & Kendall, 1990).

Table: 2.2

Some studies of adrenalectomy with different duration and brain parameters studied.

Reference	Duration	Parameter
Meyer *et al.*,(1979)	21 days	GPDH activity
Mobely & Sulser (1980a)	15 days	cAMP
Devenport & Devenport* (1982)	65 days	Change in body size and brain weight
Meyer (1983)*	65 days	Brain growth
Preston & McMorris (1984)*	9 days	Myelin content
Meyer & Fairman (1985)*	63,153 days	Myelin content
Meyer (1987)*	60 days	Brain growth
Harrelson *et al.*, (1987)	6 days	cAMP
Yehuda *et al.*,(1989)*	7,14 days	Cell proliferation
Robinson & Kendall (1990)	21 days	cAMP
Spencer *et al.*, (1991)	6 days	Type I receptor
Lowy (1992)	24 hours	Corticosteroid receptor
Li *et al.*,(1992)	3 days	Gene expression

* Studies at various postnatal ages (weaning),rest of the studies are on adult animals.

In bilateral adrenalectomy, aldosterone levels would be decreased and therefore complete adrenalectomy would alter the plasma Na+ level. In the present study a few adrenalectomy animals were not given 0.9% saline just to monitor the plasma Na+ level as indicated by Gallagher and Glaser (1968).

Experiment I a:

It would have been ideal to monitor circulating levels of corticosterone under various conditions at different ages, however due to technical problems it was not possible to monitor the same. In the absence this information, it was thought essential to monitor those parameters which are a sure indication of alteration in the circulating levels of corticosterone. Therefore those parameters have been monitored all throughout in experiments to assert that, the mode used for manipulating the circulating levels of glucocorticoid indeed worked.

The parameters which can be used as an index of elevated corticosterone in the literature include growth retardation (Howard, 1968; Cotterrell *et al*, 1972; Kovacs, 1973; Meyer, 1986), increase in liver weight (Howard, 1973; Loeb, 1975), decrease in brain weight (Meyer, 1983; 1987; Devenport and Devenport, 1985) and decrease in thymus weight (Spencer *et al*., 1991) and blood glucose levels (Taylor and Howard, 1971). For depleted study apart from somatic growth and liver weight, the plasma levels of Na^+ has been the most appropriate.

The experiment Ia compiles all the data collected on these parameters under different experiments carried out in this thesis. The parameters include changes in body weight, liver weight, brain/region weight, thymus weight and plasma levels of Na^+.

Experiment I b:

Effects of corticosterone administration at different ages and various steroids at 20 day on the concentration of whole brain phospholipid frac-

tions and ^{32}P incorporation in the same:

For this experiment, animals of age 7,17, and 37 postnatal were injected corticosterone for 3 days for studying at ages 10, 20 and 40 postnatal days respectively. The parameters studied were phosphatidyl inositol, phosphatidyl inositol - 4 - phosphate, phosphatidyl inositol-4,5 - bisphosphate, phosphatidyl serine, sphingomyelin, phosphatidyl choline, phosphatidyl ethanolamine and ^{32}P incorporation in the same.

Studies were carried out by using other steroids such as dexamethasone- a synthetic glucocorticoid, testosterone- a gonadal hormone and ACTH- a tropic peptide hormone to identify the specificity of corticosterone on the concentration and ^{32}P incorporation into above mentioned phospholipids in rat whole brain at postnatal 20 days of age.

Experiment I c:

Effect of bilateral adrenalectomy on the concentration and ^{32}P incorporation in whole brain phospholipid fractions:

Experiment was conducted on animals which underwent bilateral adrenalectomy at postnatal age 20 and killed 15 days after postsurgery with subsequent replacement of corticosterone for the last 5 days (Mobely and Sulser, 1980a) on the phospholipid concentration and ^{32}P incorporation in the whole brain. Parameters measured are as mentioned above.

Experiment IIa:

Effects of corticosterone administration on lipid fractions in different brain regions of 10 and 20 day old rats:

To evaluate the above aspects studies were carried out by injecting corticosterone to 7 and 17 day old rats for three consecutive days and

killed 24 hours after the last injection. The parameters measured were the concentration of different phospholipid fractions, cholesterol and galactolipids in six brain regions. The regions studied were olfactory bulb, cerebellum, brain stem, hippocampus, striatum and cortex. The hippocampus,the highest, striatum and cerebral cortex have high steroid receptors whereas cerebellum and olfactory bulb have low steroid receptors and brain stem is a lipid rich (myelin) region.

Experiment II b:

Effects of bilateral adrenalectomy on the concentration of various lipid fractions in different brain regions:

40 day old animals were operated for bilateral adrenalectomy and were kept for 15 days post surgery. One group of these animals were given replacement for 5 days (10 mg/kg body. wt., S.C.) and sacrificed 24 hours after the last injection. The parameters measured were same as mentioned earlier.

Experiment III a & b:

In Vitro ^{32}P incorporation into phospholipids of different brain region slices in corticosterone administered, bilateral adrenalectomized and in hormone replaced animals:

Animals 17 days old were injected with corticosterone for three days and killed at 20 day postnatally and studied for the *in vitro* ^{32}P incorporation in cerebral cortex, brain stem and hippocampal slices. The parameters studied were all phospholipid subclass such as PIP_2,PIP, PI, PS, SM, PC, PE and PA. The rats were bilaterally adrenalectomized at 20 day and kept for 5 days, 15 days and 30 days postsurgery and half of these animals had re-

placement of corticosterone given throughout i.e., from 24hours after the surgery. In one experiment, metapirone (11-â-hydroxylase inhibitor) to deplete corticosterone was given to animals of 20 day old rats for 5 days and killed after the last injection. ^{32}P incorporation into above mentioned phospholipid fractions in the three brain region slices were studied.

Experiment IIIc:

The fatty acid composition of class of phospholipids of synaptosomes isolated from the control and corticosterone treated animals:

For this experiment the cerebral cortex was removed from the control and steroid treated (20 day) animals and synaptosomal fraction was isolated. These fraction were treated for lipid extraction, and separation of various phospholipids by TLC and subjected to fatty acid methyl ester preparation. The methyl esters of fatty acids were separated using gas chromatography.

Experiment IVa:

Studies on membrane bound enzymes:

Since lipid environment is required for the activity of certain membrane bound enzyme namely Na+K+ATPase, 5'Nucleotidase and acetylcholine esterase were studied in membrane preparation (Smith *et al.*, 1980) from six brain regions under different experimental conditions. Enzyme activities were measured in 10 and 20 day old rats after 3 days chronic corticosterone treatment. Study was also carried out in synaptosomes and myelin isolated from cerebral cortex and cerebellum of 20 day old rat after hormone treatment. To evaluate the permissive action of corticosterone on these enzymes, rats were adrenalectomzied at 20 day and after 15 day post surgery. Half of

the ADX animals were used for replacement studies. Above mentioned parameters were studied in membranes prepared from different regions. Additional studies were carried out in aged sham and aged ADX animals (22 months).

Experiment IIIb:

Effects on synaptosomal membrane fluidity in control and corticosterone treated animals:

The experimental design was similar to what has been described earlier. Synaptosomes were prepared and further used for membrane preparation and these membranes were used for membrane fluidity using DPH as a probe.

Experiment V:

Histology of the Hippocampus - Comparative study of young, young ADX and Aged and Aged ADX animals:

2 month and 17 month old animals were adrenalectomized and kept for 5 months. At the end of 5 months of steroid depletion some animals were killed, and histological studies were carried out in hippocampus for the selective cell loss.

Materials:

Reagent grade chemicals and solvents were used throughout the study and majority of the chemicals obtained from Sigma chemicals Co.- USA, BDH - India, E. Merck- Germany, Fluka- Switzerland. Solvents and other chemicals were obtained from SD chemicals -Bombay, Qualigens - Bombay, Allied chemicals - Baroda, SRL- Bombay *etc.* Radioactive chemical ^{32}P orthophosphate (Specific activity 20 mCi/mmol) was obtained from Bhaba Atomic Research Centre, Trombay, Bombay, India. Liquid nitrogen was obtained from IPCL,

Baroda.

Animals and treatment schedule:

Rats of Charles-Foster strain bred and maintained under standard laboratory conditions were used in the present study. Rats were given stock diet and water ad libitum. Corticosterone acetate suspended in 0.1% carboxy methyl cellulose was injected subcutaneously in neck folds for three days beginning from 7, 17, and 37 days. The animals were sacrificed 24 hours after the last injection. Littermate controls were injected with vehicle through out the study.

Replacement with corticosterone in ADX animals was done on one set of rats. For whole brain experiment, corticosterone was given from 9th to 14th day after surgery. In other adrenalectomy studies corticosterone was administered to rat from 2nd day after surgery to 24 hour before decapitation.

For aging experiments, litters were taken from stock colony and mothers were given stock diet till the pups were weaned. After weaning, pups were separated and male were retained for experiment. In the initial stages 3/4 weaning males were kept together and were fed stock diet and drinking water supplied ad libitum. Later on these rats were housed in single cage and periodically weighed and handled throughout the study. The healthy animals free from any infection were only used for the study.

Dose of Steroid treatment:

For chronic corticosterone studies, corticosterone acetate equivalent to 40 mg/kg body weight (Patel *et al.*, 1983a) was prepared as mentioned above, and injected subcutaneously in to the neck fold of the rats.

Adrenalectomized rats were replaced with a corticosterone dosage of 10 mg/kg body weight and were given s.c. for last 5 days of the studies (Mobely and Sulser, 1980a). Certain studies involving *in vitro* ^{32}P incorporation, replacement were started immediately 24 hours after surgery with the above mentioned dose till 24 hours prior to the killing. Dexamethasone phosphate was given 2 mg/kg body weight (S.C.). ACTH was given 50 IU/kg body wt. (S.C.) for three days (Duman *et al.*, 1989). Testosterone was given 4mg/kg body wt.(Bhargava, 1988). Metapirone drug was given to rats intraperitoneally 50 mg/kg body weight (Robinson and Kendall, 1990).

Adrenalectomy:

Male rats of Charles-Foster strain were selected of different age groups from the department animal house colony. These had been maintained on stock diet and allowed diet and water ad libitum through out the study. The animals were anesthetized with ether. In a prone position, a dorsal midline incision was made, extending from the 10th thoracic to the third lumbar vertebra of the rat. The skin was retracted, the facia cut through and muscle exposed. A lateral incision was made in this extending from the level of kidney towards the apex of an angle made by the lowest rib and the dorsal muscle mass. The fat tissue, connective tissue and the adrenals were loosely attached to the upper pole of the kidney. The adrenal was separated from the kidney and cut from the pedicle without rupturing the capsule. The gland itself is friable and was not touched. The blood vessels supplying the glands are very small and ligation is not necessary. The muscle was closed using half stitches with fine fiber and curved needle. The skin incision was closed by surgical suture. The survival of the adrenalectomized rats

depends on the post operative care. The operated rats were supplied with 0.9% saline in drinking water for its survival. The complete adrenalectomy was monitored by the body weight gain after surgery as well as examined for reminiscence and regeneration while killing animals.

Removal of frozen brain tissue:

In whole brain studies rapid freezing techniques was used. The head of the rats was frozen immediately in liquid nitrogen after decapitation. The time taken for the head to freeze properly appeared to depend on the thickness of the skull. The frozen head was removed from liquid nitrogen and allowed to thaw partially in cold conditions. The brain was removed by taking utmost care to maintain it at a low temperature as much as possible and then used for extraction of phospholipids and polyphosphoinositides. This method is routinely carried out in this department (Uma and Ramakrishnan, 1983a).

Dissection of brain regions and preparation of brain region slices:

On specified days, rats were sacrificed between 8.00 to 10.00am. After the removal of brain, regions such as olfactory bulb, cerebellum, brain stem, hippocampus, striatum and cerebral cortex were separated out on glass plate cooled on liquid nitrogen, according to the method of Glowinski and Iversen (1966).

For slices, rats were decapitated and the brain was removed. Cerebral cortex, brain stem and hippocampus were kept on a precooled inverted petri dish on ice. It was cross chopped with surgical scalpel into fine slices (Lalitha *et al.*, 1990). The whole slices from the three regions were incubated in Krebs-Ringer buffer as mentioned later.

^{32}P incorporation in to phospholipids:

In vivo

Chronic corticosterone treated rats as well as adrenalectomized and hormone replacement rats were injected with 5 µCi/g body weight of ^{32}P orthophosphate (Specific activity 20 mCi/mmol) intraperitoneally between 8.00 am and 10.00 am, i.e., 24 hours after the last injection of hormone. The rats were sacrificed after 2 hours and brain was removed for analysis by rapid freezing technique (Uma and Ramakrishnan, 1983a). Controls and experimental rats were killed simultaneously. A time curve of ^{32}P incorporation for control and treated rats were carried out for 1, 2, 4, 6 hrs. The ^{32}P incorporation was found to be increasing with time. However the pattern for experimental and control groups remained the same and therefore subsequently only two hour period was taken for all experiments.

In vitro :

The brain region slices were prepared as mentioned above incubated with 5 ml of Krebs-Ringer buffer* of pH 7.4. The slices were preincubated in centrifuge tubes (20ml capacity). After 5 minutes 0.4 µCi/mg tissue of ^{32}P was added and incubated for 2 hours in a shaking water bath maintained at 37°C (Harikumar, 1988). At the end of the time period the reaction was stopped by adding 1ml of cold KRB and the tubes centrifuged for 10 minutes at 650 g. After centrifugation the pellet was homogenized in 0.25M sucrose. Aliquots were taken for protein estimation and rest of homogenate for lipid extraction.

* Composition of Krebs Ringer Buffer: NaCl(118mM),KCl(4.7mM), $CaCl_2$

$2H_2O$ (0.75 mM), $MgSO_47H_2O$ 1.18mM), KH_2PO_4 (1.18mM), $NaHCO_3$ (24.8mM), Glucose (10mM), Myo-inositol (2.5mM), Eichberg *et al.*, (1981) modified by Harikumar, (1988).

Membrane preparation:

The crude brain membranes were prepared by the method of Smith *et al.* (1980). Animals were killed by decapitation and the brain of rats was removed and separated into regions. The brain regions from 10, 20 day old and 40 day old sham, ADX and ADX+H rats were chilled in ice cold 0.25M sucrose, quickly blotted on a filter paper and weighed. The tissue was minced and homogenized in 10mM Tris-HCL buffer (pH 7.8) with 25 strokes of tight pestle of glass homogenizer. The prolonged homogenization under hypotonic condition was designed to disrupt as far as possible the ventricular structure of the cells so as to release soluble protein and leave only membranes and nonvesicular particulate matter in a sedimentable form. The homogenate was centrifuged for 60 minutes at 100,000g in Sorvell ultracentrifuge (Rotor TFT 80.13). The clear supernatant was drained off and discarded. The sediment resuspended in the same buffer and used for different enzyme assays.

Preparation of myelin and synaptosomes & synaptosomal plasma membrane:

Subcellular fractionation was performed as described by Gray and Wittaker (1962). All steps were carried out at 4°C. A 10% homogenate of cerebral cortex or cerebellum from 20 day old rats was prepared in 0.32M sucrose using Potter homogenizer. A crude nuclear fraction (P1) was separated by centrifugation at 1000g for 10 minutes. The pellet was washed twice and the supernatant combined. Further centrifugation at 12,000g for

60 minutes yielded a crude fraction (P2). Pellet P2, resuspended in 0.32 M sucrose and was layered on top of a gradient consisting of 0.8 M and 1.2 M and centrifuged at 55,200g for 2 hours in SW-28 rotor. This procedure resulted in three fractions, between 0.32 M and 0.8 M - myelin, 0.8 M and 1.2 M is synaptosomes and pellet is mitochondria. The fractions were carefully diluted to approximately 0.32M sucrose with distilled water and finally centrifuged down at 12,000g for 30 minutes. Synaptosomal pellet was used for enzymatic analysis as well as lipid extraction and myelin fraction used for the 5'nucleotidase enzyme assay.

For fluidity measurement,synaptic plasma membranes were prepared as described below adapted from the method of Gray & Wittaker (1962). The synaptosomal pellet obtained was suspended in a small volume of 0.32M sucrose and osmotically shocked in 5 vol. of Tris buffer, pH 8.1 for 1.5 hours and centrifuged at 12000g for 30 minutes. The pellet was again layered on the top of 0.4M of discontinuous gradient. The discontinuous gradient consisted of successive 5 ml layers of 1.2 M, 1.0 M, 0.8 M, 0.6 M and 0.4M sucrose and centrifugation was carried out as mentioned above. The membranes obtained from the interphase of sucrose gradient 0.4- 0.6M, 0.6-0.8M and 0.8 and 1.0M were pooled together and centrifuged at 12000g for 30 minutes. The pellet membranes (SPM) are used for fluidity measurement. Isolated fractions were checked for purity.

Extraction of lipids:

The procedure of Folch *et al.* (1957) as modified by Suzuki (1965) was used for the extraction of lipids from rat brain. The tissue was homogenized with total 20 volumes of cold chloroform : methanol (2:1v/v)

mixture in a Potter-Elvejem homogeniser. The contents were filtered through Whatman filter paper. Twice the residue was re-extracted with small volumes of chloroform : methanol (2:1 v/v) mixture and then filtered again. The filtrates were pooled together and 0.2 volumes of glass distilled water was then added, mixed well by vortexing and allowed to stand for phase separation at room temperature (25 -30°C). The upper phase was collected with a pasture pipette and thrown out and the lower phase was washed with 0.4 volumes of Folch's pure upper phase solvent (consists of chloroform : methanol: water in a proportion of 3:48:47) and centrifuged at 3000 rpm for 10 minutes. The upper phase was removed and thrown out. The lower phase was used for the estimation of cholesterol, galactolipids and phospholipids.

Extraction of Polyphosphoinositides:

The tissue or the residue of the Folch's extraction was homogenised for two minutes with 25 volumes of chloroform : methanol (1:1v/v) contain 60 moles of $CaCl_2$ using mortar and pestle or homogenizer (Uma and Ramakrishnan, 1983a). It was filtered through Whatman no. 1 filter paper and made up to 2:1 of filtrate with chloroform. After adding 0.5% concentrated HCl to the filtrate and shaking it in a rotary shaker at 150 rpm for one hour, it was then filtered and the last drop allowed to pass through. The acid extract was mixed with 0.2 volumes of 1N HCL and the upper phase was removed. The procedure was repeated by washing first with 2/3 volumes of chloroform : methanol : 1N HCL (3:48:47 v/v) followed by the same proportion of each component except HCL, 0.01 N instead of 1N. The lower phase was made clear by the addition of 4-5 drops of methanol and the pH was adjusted by a drop of 25N ammonia solution. The extract was measured and stored

below 0°C and used for estimation.

Separation of Polyphosphoinositides:

Glass plates were cleaned with soap water,tap water and glass distilled water. The plates were allowed to dry and greasy materials were removed with acetone. It was coated to a thickness of 0.35 mm with Silica gel H containing 0.1% potassium oxalate. The coated plates were air dried and then activated for 10 minutes at 110°C before use. 80-120 µg of phosphorous containing aliquotes were applied on the activated plates. The plates were developed at room temperature in a TLC chamber saturated with n-propanol : water : ammonia (40:14:6 v/v/v). The plates were removed after 2.5 hours and dried. The dried plates were exposed to iodine vapors which revealed the spots of both PIP and PIP_2.(Rf values of PIP- 0.4 and PIP_2- 0.29)(Sharma *et al.*, 1980; Uma and Ramakrishnan, 1983a).

Separation of various Phospholipids:

The method of Horrocks and Sun (1972) was used for separation of phospholipids by two dimensional thin layer chromatography. Glass plates were cleaned as mentioned above and dried at room temperature. The plates were coated to a thickness of 0.5mm with Silica gel G in 0.01M sodium carbonate. The coated plates were dried at room temperature and then activated for 30 minutes at 110°C in an oven before use. The lipid extract containing 20-40 µg of phosphorous was spotted at the lower left corner of the silica gel coated plates. The plates were developed to a height of 13-15 cm in a glass chamber presaturated for one hour with chloroform : methanol: ammonia (65:25:6v/v/v). It took 30 minutes to develop. The plates were removed and air dried at room temperature. The second TLC development

was made at right angle to the first one in a solvent system containing chloroform : methanol : acetone : acetic acid : water (75:15:30:15:7.5) and allowed to run to a height of 10-12 cms. After this, the plates were removed and air dried for 10 minutes and the spots were visualized by exposing the plates to iodine chamber. The various phospholipid spots were scrapped either into test tubes and phosphorous estimated by Bartlett (1959) or into scintillation vials for radioactive counting.

Preparation of methyl esters of fatty acids:

Methanolysis of phospholipids is performed by the method of Morrison and Smith (1964). The spots of individual phospholipids were visualized quickly under iodine vapour obtained after separation of phospholipids by TLC, were scrapped and transferred into screw capped round bottom tubes. 2 ml of 14% Boron trifluoride in methanol (Fluka) and 0.5 ml of benzene were added, the tubes flushed with nitrogen and capped tightly. Susequently they were placed in a boiling water bath for 60 minutes. At the end of the methanolysis, the tubes were rapidly cooled under tap water and 3 drops of 3M HCL, 1.5 ml of water and 3 ml of hexane was added. The contents were mixed well by vortexing for 30 seconds. The tubes were then allowed to stand. The two phases are found to be clearly separated and the upper phase was removed with a pasture pippete. 3 ml of hexane was added to the lower phase, vortexed for 30 seconds and allowed to stand. The upper phase was removed. The combined upper phases are dried under nitrogen in a water bath maintained at 45^{o}C. The residue was suspended in a small volume (0.2 ml) of hexane. 2 µl of this was taken for fatty acid analysis.

Separation of methyl esters of fatty acids:

The fatty acid methyl esters prepared as mentioned above was separated by Variant, USA, gas chromatography. The gas chromatography used was equipped with 10 % cynosilicon (CS-10) column and a flame ionization detector. The column was kept programmed at a temperature of 170 ^{o}C (4 min.) 5^{o}C/min. increase and reach final 200^{o}C (5 min.). The injector port and the detector were kept at 240^{oC} and 250^{o}C respectively. The carrier gas used was nitrogen and a flow of 30 ml/min was maintained.

Estimation of Cholesterol:

The method of Bowman and Wolf (1962) was used for estimation of cholesterol. Aliquots of lipid extract were taken in duplicate test tubes and evaporated to a dryness at 60^{o}C in a water bath. 3 ml of distilled alcohol was added to the dried sample and contents were shaken in a vortex. 3ml of iron reagent* was added while shaking in a mixer. The colour developed was read after 30 minutes at 540 nm on a Carl Zeiss spectrophotometer against reagent blank which contained alcohol instead of the sample. A standard graph was prepared taking different concentrations of cholesterol (20 - 120 µg) and treated similarly.

* Iron reagent: 2.5 % of $FeCl_3$ in 80 % orthophosphoric acid. For working reagent 8 ml was diluted to 100ml with concentrated H_2SO_4.

Estimation of Galactolipids:

The method of Svennerholm (1956) modified by Neskovic *et al.* (1972) was used for the estimation of galactolipids. Aliquots of the lipids extract were taken in test tubes and evaporated to a dryness at 60^{o}C in a water bath. To this 0.3 ml of ethanol was added and the tubes were again kept

for 60°C for 3 minutes to dissolve the lipids in alcohol. After cooling to room temperature (30°C), 3 ml of *orcinol reagent was added. The tubes were shaken well on the vortex mixer and heated at 80°C for 20 minutes. The colour formed in the tubes was read at 505nm in a Carl Zeiss spectrophotometer against a blank which contains alcohol instead of lipid extract. A standard graph was prepared using different concentration of standard galactose (5-15 µg) and treated similarly. The values obtained for galactose was multiplied by 4.7 to obtain galactolipid content in the sample.

* Orcinol reagent: 100 mg of orcinol dissolved in 100 ml of 25N H_2SO_4. Prepared fresh before use.

Estimation of Phospholipids:

Lipid phosphorous was estimated by the method of Bartlett (1959). Aliquots of the lipid extract were taken in duplicates in test tubes and evaporated to dryness at 60°C in a water bath. One ml of 60% perchloric acid was added to each tubes along with small glass beads. The tubes were heated for one hour in a sand bath and maintained at 230°C. After the completion of digestion as judged by the colourless appearance of the solution, the tubes were cooled to room temperature (27 - 30°C) and volume was made up to 1ml with perchloric acid. To this was added 8.1 ml of glass distilled water, 0.5 ml of molybdate reagent * and 0.4 ml of ANSA reagent**. The tubes were shaken well and kept in a boiling water bath for 8 minutes. They were then cooled to room temperature (27 - 30°C) and the colour obtained was read at 820nm in a Carl Zeiss spectrophotometer against a reagent blank containing only perchloric acid.

A standard graph was prepared by taking different concentration of

phosphorous standard (2-8 µg) treated same way. The values obtained for phosphorous were multiplied by 25 to obtain the phospholipid content of the sample.

* Molybdate reagent: 5g of ammonium molybdate dissolved in 37.5ml of 10N H_2SO_4 and made up to 100ml with distilled water.

** ANSA reagent: 0.25% of 1-amino-2-naphthol-4-sulfonic acid (ANSA) in 97.5ml of 15% sodium meta bisulphate and 2.5ml of 20% sodium sulphite.

Radioactive measurement:

For radioactive measurement, the phosphoinositides and other phospholipid bands were scrapped after decolorisation was taken 10 ml of scintillation fluid containing 0.4% of PPO and 0.04% POPOP in toluene. The samples were counted in a Packard LS counter.

Estimation of Protein:

The method of Lowry *et al.* (1951) was used for the estimation of protein. Diluted membrane fraction aliquotes were taken in test tubes and to this 0.8 ml of 0.1N NaOH and 5 ml of Lowry C reagent* was added and allowed to stand. After 15 minutes 0.5 ml of 1N Folin Ciocaltein reagent was added and the contents were mixed well on a vortex mixer. Colour developed was read at 660nm in a spectrophotometer against a blank containing distilled water instead of sample. Different concentrations (20-200µg) of standard protein (bovine serum albumin) were taken and processed as above for standard graph.

* Lowry C reagent: 2 ml of 1% $CuSO_4$ (dissolved in 1% Na+K+tartarate) was mixed with 100ml of 2% Na_2CO_3 prepared in 0.1N NaOH.

Assay system for Na+ K+ ATPase:

Na+ K+ ATPase activity was measured in the brain membrane preparations by the method of Sawas and Gilbert (1981)

Details of assay system:	TotalATPase	Mg^{2+}ATPase
Incubation:		
Cocktail* (pH 7.4)	0.5 ml	0.5 ml
Water	0.2 ml	0.1 ml
Oubain (1mM)	___	0.1 ml
Enzyme	0.2 ml	0.2 ml
Preincubation	$37^{o}C$, 10 minutes	
Substrate- ATP (4mM)	0.1 ml	0.1 ml
Conditions of incubation	$37^{o}C$, 20 minutes	
Termination of reaction by	Addition of 1 ml of 5% TCA.	
Treatment of blank by	Addition of TCA before enzyme was added	

The tubes were centrifuged at 2000g for 10 minutes and the inorganic phosphorus released was determined from the supernatant. Enzyme unit expressed as µmoles of Pi liberated in an hour per mg of membranal protein. The difference in activity with and without oubain was taken as the Na+ K+ ATPase activity. The activity in the presence of oubain was taken as Mg^{+2} dependent ATPase activity.

* Cocktail- NaCl(240mM), KCl(20mM), $MgCl_2$10mM, EDTA (2mM), Imidazole (1.7mM) and the pH was adjusted to 7.4 with 0.1N HCl.

5'Nucleotidase:

Activity of 5'Nucleotidase (EC 3.1.3.5) in the membrane preparation

was measured by the method of Aronson and Trnuster modified by Smith *et al.* (1980). The assay was carried at pH 7.5 with and without 10mM Ni++ which is claimed to inhibit 5' Nucleotidase activity specifically in the plasma membranes and distinguished from non specific phosphatase. The reaction mixture consists of a final volume of 1ml, 50mM Tris-HCL, 10mM $MgCl_2$, 5mM AMP and membrane preparation (150-250 µg of protein). Reactions was started by adding substrate and incubated for 30 minutes at 37°C. The reaction was terminated with 2 ml of 8 %(w/v) of tricholoroacetic acid. Tubes were centrifuged at 2000g for 10 minutes and the inorganic phosphorus released was determined from the supernatant. Enzyme unit expressed as µ moles of Pi liberated in an hour per mg. of membranal protein. The difference in activity with and without Ni^{2+} was taken as the 5' Nucleotidase activity. The activity in the presence of Ni^{2+} was taken as total phosphatase activity.

Estimation of Inorganic Phosphorus:

The inorganic phosphorus was estimated by Fiske and Subbarow (1925) method. One ml of the supernatant was taken and the volume was made up to 8.6ml with glass distilled water. One ml of 2.5% ammonium molybdate reagent and 0.4 ml of ANSA I reagent was added. The colour developed in 8 minutes was read in a colorimeter using a blank containing water instead of sample at 660nm. A standard graph was prepared taking different concentrations of phosphorus standard (4-20 µg).

Assay of Acetylcholine Esterase activity:

This enzyme assay was carried out by the method of Ellman *et al.* 1961. The actual enzyme reaction was carried out in quartz cuvettes at 25°C. The assay system contaned

Phosphate buffer, 0.1 M,pH 8.0 ..... 3.0 ml

DTNB solution, 0.01 M ..... 0.1 ml

Enzyme (membrane preparation) ..... 0.1 ml

Acetylthiocholine iodide, 0.075M 0.02ml

The blank for such a run consisted of buffer,substrate, DTNB and water. The reaction was started by adding substrate solution and mixing the assay system. The change in the optical density per minute was recorded at 412 nm on the Shimandzu spectrophotometer.

The change in the optical density per minute computed from the graph of OD versus time is used for further calculations as follows.

Activity of the enzyme = μ moles of substrate hydrolyzed per minute per mg protein.

Fluorescence Polarization Studies:

Lipid fluidity was assessed by the steady- state fluorescence polarization of 1, 6 diphenyl-1,3,5,-hexatriene (DPH) according to the method described by Shinitzky and Barenholz (1978) modified by Deliconstatinos (1985). DPH was stored in tetrahydrofuran at a concentration of 2mM and, immediately before, use it was diluted to 1000 fold by injection into 5mM Tris- HCl containing 1.15% KCl, pH 7.4, then mixed in a 1:1 ratio with the SPM suspension to give a final concentration 40-50 μg SPM protein per ml. The mixture was then incubated at 0°C, 25°C and 37°C for 45 minutes each and the fluorescence was measured on Shimadzu specrophotoflurometer RL 5000 with polarization accessory for polarization measurement. Excitation wavelength was fixed at 360nm and emission wavelength at 430nm. The values of I_{VV},I_{VH},I_{HH} and I_{Hv} were measured with vertical(V) and horizontal(H)

position of the polarizers. Correction factor G = I_{HH}/I_{HV} was found out. Anisotropy of DPH = 0.362 (r_o).

$$\text{Calculation } r_s \text{ (anisotropy)} = \frac{I_{VV}/G - I_{VH}}{I_{VV}/G + 2I_{VH}}$$

$$\text{microviscosity} = \frac{2.4r_s}{0.362 - r_s}$$

$$\text{Fluidity} = 1/\text{microviscosity}.$$

Fixation and Processing of Brain for Histological studies:

Fixation and processing of rat brain was carried out by the method of Zoli *et al.* (1991). Rats of Charles -Foster strain of age 2 and 17 months were adrenalectomized as mentioned earlier. After 5 months of surgery these rats were used for histological studies. The rats (n = 5 per group) were sacrificed in the morning (between 8 am. and 10 am.) by intracardiac perfusion with 100 ml of warm saline followed by 100 ml of ice cold 4% paraformaldehyde + 0.3% picric acid in 0.1M phosphate buffer (pH 7.2) under deep sodium pentabarbital anesthesia (40 mg/ kg body weight- I.P). The brains were dissected out and postfixed for 48 hours in the same fixative.

Histology:

After 48 hours of fixation, brain was transferred to 70% ethanol. The brain was then cut longitudinally into two equal parts with surgical blade. These parts were dehydrated in ethanol grades in asending seris and cleared in chloroform and embedded in paraffin wax. Sections of 13 µm thickness were serially sectioned on a Leitz microtome in horizontal plane and mounted on albumin coated slides and dried overnight at 37°C. The

slides were stained with cresyl fast violet and mounted in Canada balsam. The sections were observed and desired areas were photographed in a Zeiss Photomicroscope. For morphometric studies of the hippocampal cells the sections were viewed under 40x magnification and cell measurements were taken with a calibrated occular micrometer.

Statistics:

The significance of difference between control and hormone treated as well as between adrenalectomy + hormone replaced, adrenalectomy and sham control samples were tested by Student's 't' test.

The results obtained in these experiments are reported and discussed with the literature studied in the chapter -III.