MATERIALS AND METHODS
Fig. A: Wistar Strain Albino rat (*Rattus norvegicus albinus*)

Fig. B: Maintenance of the Rat Colony
Fig. C: Adrenal gland (in the circle)

Fig. D: Sutured Bilateral Adrenalectomized Albino rat
Procurement and maintenance of rats:

The selection of albino rats is based on its ability of survival, its withstanding capacity in a fairly wide range of stress conditions and its maintenance and handling is quite comfortable for study. Healthy Wistar strain male and female albino rats (*Rattus norvegicus albinus*) of the age of 120 days and body weight 220±10gms have been selected for present study (Fig. A). The stock of the litters was obtained from Indian Institute of Sciences, Bangalore. The rat colony was maintained in laboratory at 28±2°C and 12 hours light and 12 hours of darkness. Rats were fed on standard rat diet obtained from Hindustan Lever Ltd., Bangalore, and water was supplied ad libitum (Fig. B).

Experimental Design:

Rats were divided into 3 groups, each group consisted of 12 individuals, of this six were males and remaining six were females. First group of rats were called as sham operated (SO) in them the adrenal glands were kept intact and considered as control. The second and third groups of rats were bilaterally adrenalectomized (ADX) by the dorsal approach in a single stage of operation as followed by Bhaskar *et al.*, (1989) and Stith *et al.*, (1989) and these two group of animals were considered as experimentals (Fig. C and D). The rats were anaesthetized during surgery with ketamine (80mg/kg body weight) plus xylazina (12mg/kg body weight) administered intraperitoneally in a volume of 0.3ml. ADX rats were given 0.9% physiological saline as drinking water due
to loss of salts and SO rats were given normal tap water. All rats were housed and cared according to the “Guide for the care and use of laboratory animals” as mentioned by Devendra Naidu (2000).

The second group of experimentals was maintained for 15 days and the third group for 30 days. After the stipulated time, both the SO and ADX rats were killed by cervical dislocation and immediately the tissues of them, the liver, epididymis, penis and testis in males and liver, uterus, vagina and ovary in females, were isolated for biochemical and histological investigations. The blood was drawn for haematological and hormonal assays. The 15 and 30 days experimental duration were chosen in order to evaluate the short-term and long-term impacts of adrenalectomy on animals.

SOME ASPECTS OF CARBOHYDRATE METABOLISM

Levels of blood glucose, tissue glucose, glycogen, pyruvate and lactate and activities of glucose-6-phosphatase (G-6-Pase), lactate dehydrogenase (LDH) and succinate dehydrogenase (SDH) were estimated in the liver, epididymis, penis and testis of males and in the liver, uterus, ovary and vagina of females of ADX and SO rats under this study.

Estimation of blood glucose:

Glucose was determined by colorimetric method of Nelson and Somogyi (1952). To 0.1 ml of blood, 3.9 ml of deproteinizing solution (5% zinc sulphate and 0.3 N sodium hydroxide solution in 1:1 ratio) was added. The mixture was centrifuged at 3000 rpm for 10 minutes. To 1 ml of the
supernatant 1 ml of alkaline copper reagent was added. The mixture was shaken vigorously and heated in a boiling water bath exactly for 20 minutes. The mixture was cooled and added 1 ml of arsenomolybdate colour reagent. Made the entire solution to 10 ml with distilled water and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 540 nm. A blank and glucose standards were also run similarly. Glucose levels in the blood are expressed as mg %.

**Estimation of tissue glucose:**

Glucose in the organs was determined by colorimetric method as described by Nelson and Somogyi (1952). 5% homogenates of the organs were prepared in pure distilled water. To 0.5 ml of homogenate, 4.5 ml of 10% TCA solution was added and the mixture was centrifuged at 3000 rpm for 10 minutes. To 1 ml of the supernatant, 1 ml of alkaline copper reagent was added, shaken vigorously and heated in a boiling water bath exactly for 20 minutes. Then it was cooled and 1 ml of arsenomolybdate colour reagent was added. The entire solution was made to 10 ml with distilled water and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 540 nm. A blank and glucose standards were also run simultaneously. The glucose content is expressed as mg/gm wet weight of the organ.
Estimation of glycogen:

Glycogen was estimated using the anthrone reagent method as described by Caroll et al. (1956). A weighed piece of organs were digested with 3 ml of hot 30% potassium hydroxides (Hassid and Abraham, 1957). The digestate was cooled and 3.75 ml of absolute ethanol was added to it. The entire mixture was kept over night in a refrigerator. Then the mixture was centrifuged for 15 minutes at 2500 rpm. Decanted the supernatant and 10 ml of warm water was added to the residue to dissolve the precipitated glycogen. To 0.2 ml of this, 1.8 ml of distilled water and 0.5 ml of 2% anthrone reagent dissolved in 72% concentrated sulphuric acid were added and heated in a boiling water bath exactly for 10 minutes. The mixture was cooled and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 620 nm. A blank and glucose standards were also run similarly. The glycogen content is expressed as mg/gm wet weight of the organ.

Estimation of pyruvate:

The level of pyruvate in the organs was estimated using the method of Friedman and Hangen (1942). A 5% homogenate (W/V) was prepared in 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the estimation of pyruvate. 1 ml of the supernatant was taken and to it 1 ml of 0.001 M 2,4-Di nitrophyl hydrazine and 3 ml of 0.4 N sodium hydroxide were added. After 10 minutes, the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 540
nm against the reagent blank. Pyruvate standards were prepared alongside for comparison. The pyruvate content in the organs is expressed as mg pyruvate/gm wet weight of the organ.

**Estimation of lactate:**

The level of lactate in the organs was estimated using the method of Barker and Summerson (1941) as modified by Huckabee (1961). 5% homogenates (W/V) were prepared in cold 10% trichloroacetic acid and centrifuged at 3000 rpm for 15 minutes. The supernatant was used for the estimation of lactate. To 1 ml of supernatant, 20% copper sulphate was added and the mixture was made to 10 ml with distilled water. Then 1gm of powdered calcium hydroxide was added, shaken vigorously and kept for an hour at room temperature with intermittent shaking. The contents were centrifuged at 3000 rpm for 10 minutes and to 1 ml of the supernatant 0.5 ml of 4% copper sulphate was added followed by 6 ml of concentrated sulphuric acid. The contents were mixed by lateral shaking, kept in boiling water bath for exactly 6.5 minutes and cooled. When the contents were sufficiently cooled, 0.1 ml of 1.5% P-hydrophenyl (prepared in 0.5% sodium hydroxide) was added and the precipitate formed was kept at laboratory temperature for 30 minutes. Then the contents were placed in a boiling water bath for 90 seconds, cooled and the optical density of the colour developed was measured in spectrophotometer at a wavelength of 560 nm against reagent blank. Lactate
standards were prepared alongside for comparison. The lactate content present in different organs is expressed as mg lactate/gm wet weight of the organ.

**Estimation of glucose-6-phosphatase (E.C. 3.1.3.9):**

Glucose-6-phosphatase activity in the organs was estimated using the method of Yeung et al., (1968). A 5% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution. 0.5 ml of 0.2 M tris malate buffer, 0.2 ml of 0.05 M glucose-6-phosphate and 0.2 ml of distilled water were incubated at 37°C for 5 minutes and to this 0.1 ml of homogenate was added and incubated exactly for 10 minutes. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. A blank was also run similarly. Finally, the inorganic phosphates liberated were estimated by the method of Fiske and Subba Row (1925) at a wavelength of 660 nm and the activity is expressed as µg Pi liberated/mg protein/h.

**Estimation of succinate dehydrogenase (SDH) (succinate: acceptor oxidoreductase, EC: 1.3.99.1) activity:**

Succinate dehydrogenase activity in the organs was estimated using the colorimetric method of Nachlas et al., (1960). A 5% homogenate (W/V) was prepared in 0.24 M ice cold sucrose solution centrifuged at 3000 rpm for 10 minutes and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 0.2 ml of 0.4 M phosphate buffer (pH 7.7), 0.2 ml of 0.2 M sodium succinate, 1 ml of 0.004 M 2-(P-indophenol)-3P-nitrophenyl-tetrazolium chloride (INT), 0.1 ml of 0.005 M phenazine methosulphate and 0.5 ml of 5% enzyme preparation. The mixture was
incubated at 37°C for 30 minutes and the reaction was stopped by adding 6 ml of glacial acetic acid. The formozan formed was extracted into 6 ml of toluene overnight at 0°C and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank taking 0.5 ml of distilled water and control taking 0.5 ml of boiled enzyme was also run for comparison. INT standards were prepared alongside for comparison. The activity is expressed as µ M formozan/mg protein/h.

Estimation of lactate dehydrogenase (LDH) (L-lactate NAD oxido-reductase, EC: 1.1.27) activity:

Lactate dehydrogenase activity in the organs was estimated using the method of Srikantan and Krishnamoorthi (1955) as modified by Govindappa nad Swami (1965). A 5% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution, centrifuged at 2500 rpm for 15 minutes and the supernatant was taken as the source of enzyme. The incubation mixture consisted of 1 ml of 0.04 M phosphate buffer (pH 7.4), 0.5 ml of 0.1 M lithium lactate, 1 ml of 0.0001 M nicotinamide adenine dinucleotide (NAD), 1 ml of 0.004 M 2-(P-indophenol)-3 P-nitrophenyl-5-phenyl tetrazolium chloride (INT) and 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30 minutes and then added 6 ml of glacial acetic acid to stop the reaction. The formozan formed was extracted into 6 ml of toluene over night at 0°C. The optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank using 0.5 ml of distilled water and a control by taking 0.5 ml of boiled enzyme was also run similarly. INT standards were
prepared along side for comparison. The enzyme activity is expressed as \( \mu M \) formozan/mg protein/h.

**SOME ASPECTS OF PROTEIN METABOLISM**

The levels of soluble, structural and total proteins, free aminoacids, ammonia and urea and the activities of protease, alanine and aspartate aminotransferases and glutamate dehydrogenase were estimated in the liver, epididymis, penis and testis of males and in the liver, uterus, ovary and vagina of females of ADX and SO rats under this study.

**Estimation of soluble, structural and total proteins:**

The soluble, structural and total proteins in the organs were estimated using the folin phenol reagent method as described by Lowry *et al.*, (1951). A 1% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution. For soluble and structural proteins, 1 ml of homogenate was taken and centrifuged at 3000 rpm for 10 minutes. The supernatant was separated and to both the supernatant and residue 3 ml of 10% TCA was added and again centrifuged at 3000 rpm. The supernatants were discarded and the residues were taken for experimentation. For total proteins, 1 ml of homogenate was taken, to it 3 ml of 10% TCA was added and centrifuged at 3000 rpm. Discarded the supernatant and the residue was taken for experimentation. All the three residues were dissolved in 5 ml of 0.1 N sodium hydroxide and to 1 ml of each of these solutions, 4 ml of reagent-D (mixture of 2% sodium carbonate and 0.5% copper sulphate in 50:1 ratio) was added. The samples were allowed to
stay for 10 minutes, at the end of which 0.4 ml of folin phenol reagent (diluted with distilled water in 1:1 ratio before use) was added. Finally, the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 600 nm. A mixture of 4 ml of reagent-D and 0.4 ml of folin phenol reagent was used for blank. Bovine albumin was used for the preparation of protein standards. The protein content is expressed as mg/gm wet wt of the organ.

**Estimation of free amino acids:**

Free amino acid levels in the organs were estimated by the Ninhydrin method as described by Moore and Stein (1954). A 5% homogenate (W/V) was prepared in 10% TCA and centrifuged at 2000 rpm for 15 minutes. To 0.2 ml of supernatant 2 ml of ninhydrin reagent was added and the contents were boiled for exactly 5 minutes. They were cooled under tap water and the volume was made to 10 ml with distilled water. The optical density of the colour developed was measured in a spectrophotometer at a wavelength of 570 nm. A blank using distilled water and amino acid standards were also run similarly. The free amino acid levels are expressed as mg amino acid nitrogen released/gm wet weight of the organ.

**Estimation of protease activity:**

Protease activity in the organs was estimated using the ninhydrin method as described by Davis and Smith (1955). A 1% homogenate (W/V) was prepared in distilled water. To 2 ml of homogenate, 0.5 ml of 1% casein
and 2 ml of 0.1 M phosphate buffer (pH 5) were added. The contents were mixed well and incubated at 30°C for 30 minutes. The reaction was stopped by adding 2 ml of 2% ninhydrin reagent. Again the contents were mixed thoroughly and placed in a boiling water bath for 20 minutes. The solution was cooled and made to 10 ml with dilutent (distilled water and n-propanol in 1:1 ratio). The optical density of the colour developed was measured in a spectrophotometer at a wavelength of 570 nm. A blank taking 2 ml of distilled water and control taking 2 ml of boiled enzyme were also run similarly. Amino acid standards were prepared alongside for comparison. The protease activity is expressed as μ M amino acid nitrogen released/mg protein/h.

**Estimation of alanine (DL-alanine: 2-oxoglutarate, EC: 2.6.1.2) and aspartate (L-aspartate: 2-oxoglutarate, EC: 2.6.1.1) aminotransferase activities:**

Activities of alanine and aspartate aminotransferases in the organs were estimated using the method of Reitman and Frankel (1957). A 5% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution, centrifuged at 3000 rpm for 10 minutes and the supernatant was used as the source of enzyme. Two sets of incubation mixtures were prepared, the first set (for alanine aminotransferase activity) consisted of 0.5 ml of 0.2 M alanine, 0.5 ml of 0.005 M α-ketoglutaric acid (which was prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The second set (for aspartate aminotransferase activity) consisted of 0.5 ml of 0.2 M aspartic acid, 0.5 ml of 0.005 M α-ketoglutaric acid (which was
prepared in M/15 phosphate buffer and adjusted with 10% sodium hydroxide to 7.4 pH) and 0.1 ml of enzyme. The mixtures were incubated at 37°C for 30 minutes and then the reaction was stopped by the addition of 1 ml of 0.001 M 2,4-dinitrophenyl hydrazine (ketone reagent). Finally, the reaction mixtures were made to 10 ml with 0.4 N sodium hydroxide and the optical density of the colour developed was measured in a spectrophotometer at a wavelength of 545 nm. A blank taking 0.1 ml of distilled water and control taking 0.1 ml of boiled enzyme were also run similarly. Pyruvate and oxaloacetate standards were prepared alongside for comparison. The alanine aminotransferase activity is expressed as µM pyruvate formed/mg protein/h and the aspartate aminotransferase activity as µM oxaloacetate formed/mg protein/h.

Estimation of glutamate dehydrogenase (GDH) (L-glutamate: NAD oxalo-reductase, EC: 1.4.13) activity:

GDH activity was estimated in the organs using the method of Lee and Lardy (1965) with slight modification. A 5% homogenate (W/V) was prepared in 0.25 M ice cold sucrose solution and centrifuged at 2500 rpm for 20 minutes at 2°C to remove cell debris. The clear cell free extract was subjected to dialysis against 0.25 M sucrose at 2°C to 4°C for 24 hours. The incubation mixture in a final volume of 2 ml contained 40 µM of sodium glutamate, 100 µM of sodium phosphate buffer (pH 7.4), 0.1 µM of NAD (nicotinamide adenine dinucleotide) and 4µM of INT (2-P-indophenol-3-P-nitrophenyl-5-phenyltetrazolium chloride). The reaction was initiated by the addition of 0.5 ml of 5% enzyme preparation. The mixture was incubated at 37°C for 30
minutes in a thermostatic water bath and then the reaction was stopped by the addition of 5 ml of glacial acetic acid. The formozan formed was extracted into 5 ml of toluene overnight at 5°C. The optical density of the colour developed was measured in a spectrophotometer at a wavelength of 495 nm. A blank by taking 0.5 ml of distilled water and control by taking 0.5 ml of boiled enzyme were also run similarly. INT standards were prepared alongside for comparison. The enzyme activity is expressed as μM formozan formed/mg protein/h.

**Estimation of ammonia:**

Ammonia was estimated in the organs by the method of Bergmeyer (1965) with a slight modification. A 5% tissue homogenate (W/V) was prepared in cold distilled water and centrifuged at 2000 rpm for 15 minutes. To 1 ml of the supernatant, 2 ml of 15% perchloric acid was added and centrifuged again at 2000 rpm for 15 minutes. The supernatant was neutralized with 2 ml of 15% sodium hydroxide. To this, 0.5 ml of Nessler's reagent was added and the colour developed was read immediately in a spectrophotometer at a wavelength of 495 nm against a reagent blank. Ammonium sulphate standards were run alongside for comparison. The ammonia content is expressed as μM/gm wet wt of the organ.

**Estimation of urea:**

Urea was estimated in the organs by diacetylmonoxime method as described by Natelson (1971). A 10% tissue homogenate (W/V) was prepared
in 15% perchloric acid and centrifuged at 2000 rpm for 15 minutes. To 1.5 ml of supernatant, 1 ml of acid mix (3:1 orthophosphoric acid and concentrated sulphuric acid) was added and the contents were shaken well. To this 0.5 ml of 2% diacetylmonoxime was added and heated at 100°C in a boiling water bath for 30 minutes. The tubes were cooled and the colour developed was read in a spectrophotometer at a wavelength of 480 nm against a reagent blank. Standards of different urea concentrations were run simultaneously. The urea content is expressed as μM/gm wet wt of the organ.

**HAEMATOLOGY**

(a) **Red blood corpuscle (RBC) count:**

RBC count was made with a Neubauer Crystalling Counting chamber as described by Davidson and Henry (1969). Blood was collected in a vital containing 2% ethylene diamine tetra acetic acid (EDTA) as an anticoagulant. The blood was drawn from the vial into the RBC pipette upto 0.5 mark and immediately the diluting fluid is drawn upto the mark 101 (thus the dilution is 1:200). The solution was mixed thoroughly by shaking gently. It was allowed to stand for 2 to 3 minutes. The counting chamber and coverslip were cleaned and the coverslip was placed over the ruled area. Again the solution was mixed gently and the sternful of solution was expelled and a drop of fluid was allowed to flow under the coverslip, holding the pipette at an angle 40°. It was allowed to stand still (2 or 3 min) till the RBC settles. Afterwards the ruled counting area was focused under the microscope and the number of RBCs was
counted in five small squares of the RBC column under high power and the numbers of RBCs per sq.mm were calculated accordingly.

\[
\text{No. of cells} \times \text{Dilution factor} \times \text{Depth factor} \\
\text{Area counted}
\]

**Estimation of haemoglobin (Hb) concentration:**

The haemoglobin concentration was estimated by acid heamatin method (Sahli, 1966). N/10 hydrochloric acid (HCl) was taken upto 10.0 mark in the graduated tube. Blood was sucked into the Hb pipette upto the mark and was transferred into the graduated tube containing N/10 hydrochloric acid. It was allowed to stand in dark for 10 to 20 minutes after thorough mixing. Now N/10 HCl was added drop by drop, mixing between each addition, until the blood colour matched with the standard colour. And then the results were read from the scale on the graduated tube and the Hb concentration is expressed in grams percent.

**Estimation of packed cell volume (PCV):**

PCV was estimated by microhaematocrit method (Sachalm *et al.*, 1975). The blood was drawn into capillary tubes containing anticoagulant by capillary suction to 3/4 of their length. The outside of the capillary tubes were wiped free of blood and the index finger was placed over the moist ends to hold the column of the blood in place as the opposite dry ends were forced into the sealing material to form a tight plug. The capillary tubes were placed in the centrifuge with the sealed ends pointing outward and centrifuged at 12000 rpm for 2 min. PCV was determined by rolling the capillary tubes over a reader
card until the top of the plasma column was aligned with the 100% line and the bottom of the packed erythrocytes was on the zero line. The line that crossed the top of the packed erythrocyte column represented the PCV in percent.

**RED BLOOD CELL INDICES**

The derivation of the formulas for the calculation of red cell indices is followed as per Barbara and Brown (1980).

**Mean corpuscular volume (MCV):**

MCV expresses the average volume of red cells. For obtaining the mean corpuscular volume, the packed cell volume is divided by red cell count and 10 multiply the result. MCV is expressed as g/dl.

\[
MCV = \frac{\text{Haemotocrit (x) \times 10}}{\text{RBC in million/mm}^3}\ g/dl
\]

**Mean corpuscular haemoglobin (MCH):**

MCH represents the average weight of haemoglobin containing in each cell. MCH is influenced by the size of the cell and concentration of haemoglobin. For getting MCH the Hb concentration is usually divided by red blood cell count and the result is multiplied by 10 and is expressed as percent/gram.

\[
MCH = \frac{\text{Haemoglobin (g/100ml) \times 10}}{\text{RBC in million/mm}^3}\ = \text{percent/gram}
\]

**Mean corpuscular haemoglobin concentration (MCHC):**

MCHC refers to the average concentration of the Hb in the red cells. In contrast to MCH, MCHC is not influenced by the size of the cell. For getting
MCHC the haemoglobin content is divided by packed cell volume and the result is multiplied by 100. The MCHC value is expressed in terms of percentage.

\[
\text{MCHC} = \frac{\text{Haemoglobin (g/100ml)}}{\text{Haematocrit}} \times 100 = \%
\]

HORMONES

Testosterone:

The level of testosterone hormone in serum of male albino rats was analysed following the method of Jacob (1974), using a double-antibody (\(^{125}\text{I}\)) through Radio Immuno Assay (RIA). The level of testosterone hormone is expressed as pg/ml.

Estrogen:

The level of estrogen hormone in serum of female albino rats was analysed following the method of Badonnel and Coll (1994), using Enzyme Linked Fluorescent Assay (ELFA). The level of estrogen hormone is expressed as pg/ml.

HISTOLOGY

The histological sections of the liver, epididymis, penis and testis of males and the liver, uterus, ovary and vagina of females of ADX and SO rats were taken by adopting the procedure as described by Humason (1972). The tissues were isolated and gently rinsed with physiological saline solution (0.9% NaCl) to remove mucus and other debris adhering to them. They were fixed in Bouin’s fluid (75 ml saturated aqueous picric acid, 25 ml 40%
formaldehyde and 5 ml glacial acetic acid) for 24 hours. The fixative was removed by washing through running tap water for overnight. Then the tissues were processed for dehydration. Ethylalcohol was used as the dehydrating agent, as it is the most suitable and economical besides its hardening effect. The alcoholic transfer schedules were so arranged as to utilize both dehydration and hardening effect. The tissues were passed through successive series containing 30%, 50%, 70%, 80%, 90%, 95% and absolute alcohols. Then the tissues were cleared in methylbenzoate and embedded in paraffin wax. Sections of 5μ thickness were cut using “SIPCON” rotatory microtome. The sections were stained with Harris hematoxylin (Harris, 1900) and counter stained with eosin, dissolved in 95% alcohol. After dehydration and cleaning, the sections were mounted in Canada balsam. Photomicrographs of the section preparations were taken using Olympus (PM-6 model) photomicrographing equipment.

**STATISTICAL ANALYSIS**

All the results obtained in this investigation were subjected to statistical analysis. For this, the data was fed to the computer and ‘t’ values were derived at 5% level. As it becomes bulky to give all these values in the tables, the significance between SO and ADX rats was calculated by using these values and are represented in the respective tables. Further, the significance was also calculated between day 15 and day 30 and the deviations, if any, were represented in the respective tables.