CHAPTER I

EXPERIMENTAL PROTOCOL AND MATERIAL AND METHODS

Procurement and maintenance of chicks

Freshly hatched male White Leghorn breed of chicks (Euribrid, Hisex, Poland) procured from a local hatchery (Shakti Hatcheries, Sarsa, Gujarat) were used for the present experimental studies. The chicks were housed in a well ventilated aviary of the departmental animal house, with a continuous lighting regimen. A 100 W electric bulb was hung to provide lighting as well as to maintain a room temperature of 95± 5°F throughout the period of study. On the first day after hatching, the chicks were fed with 2% jaggery water. During the first week, the chicks were fed on carbohydrate diet (coarse maize flour) and thereafter maintained on a balanced diet of chick mash (obtained from the Government Poultry, Baroda, Gujarat) and water ad libitum. Batches of 60 chicks were maintained at a time so as to get 8 to 10 samples each by the end of 30 days of development for the determination of various parameters during the course of the present study.

Experimental set ups

The chicks were wing banded and divided into five groups. Group I, injected daily with 0.1 ml of 0.9% saline and served as controls. Group II and III which served as experimentals with adrenocortical insufficiency, received low and high doses of dexamethasone (DXM) as sodium phosphate (Dexona, Cadila Laboratories, Ahmedabad, Gujarat) in 0.9% saline. Dexamethasone a synthetic corticosteroid analogue is a well known inhibitor of ACTH synthesis and release thereby bringing

about adrenocortical insufficiency. Intraperitoneal (ip) injections were given every evening at 19.00 hrs in a graded fashion for 30 days (keeping in view the growth of chicks as well as increase in body weight) as follows:

Dexamethasone low dose [DXM(L)] - 10 µg/0.1 ml/bird/day for the first 10 days, 20 µg/0.1 ml/bird/day for the next 10 days, 40 µg/0.1 ml/bird/day for the last 10 days.

Dexamethasone high dose [DXM(H)] - 70 µg/0.1 ml/bird/day for the first 15 days, 120 µg/0.1 ml/bird/day for the last 15 days.

Group IV chicks served as experimental with adrenocortical excess. Corticosterone (Sigma Chemicals, U.S.A.) disolved in 0.9% saline with a few drops of alcohol as described by Abe and Critchlow (1977), was administered intraperitaneally (i.p.) in these chicks every morning at 7.00 hrs in a graded fashion as follows for 30 days.

5 µg/0.1 ml/bird/day for the first 10 days

10 µg/0.1 ml/bird/day for the next 10 days

15 µg/0.1ml/bird/day for the last 10 days.

These dosages were arrived at emperically based on previous studies from the laboratory. The time of administration of the hormones were based on the reported high plasma level of corticosterone in chicks during the early phase of photophase (Lauber et al., 1987) so as to induce corticosterone excess. Group V chicks were injected with vehicle (0.9% saline with a few drops of alcohol) and also served as controls. However none of the parameters studied presently showed any alteration between the vehicle treated and saline treated controls and hence only data of saline treated controls are presented throughout.

Parameters and methodology of evaluation

At the end of 30 days, the birds were weighed and sacrificied under mild ether anaesthesia taking maximum care to avoid any stress during handling. The viscera was cut open and organs were quickly excised, blotted free of blood and tissue fluids and morphometric evaluation of the organs was carried out using digital mettler balance. Organs were weighed accurately upto 0.01 mg. The absolute weights thus obtained were converted to relative weights and expressed in terms of per centage body weight.

Blood Glucose

Prior to decapitation of the chicks, 0.1 ml of blood was drawn from the jugular vein by a minute puncture. Blood glucose level was estimated by the glucose oxidase method of Trinder (1969). The glucose concentration was expressed as mg/100 ml blood.

Glycogen content in liver, muscle and testis

The glycogen content was estimated employing the method of Seifter et al. (1950). Small pieces of tissue were dropped in preweighed test tubes containing 2 ml of 30% KOH. Glycogen was precipitated with 95% alcohol. The diluted precipitates were treated with anthrone reagent and the colour intensity was read colorimetrically at 620 nm Glycogen content was expressed as mg/100 mg wet tissue weight.

Protein content in liver, muscle and testis

Tissue of known weight was homogenized in a prechilled mortar and diluted to a required concentration with chilled glass distilled water. The protein content was estimated by the method of Lowry et al. (1951)

using folin-ciocalteau as the colour reagent. The colour produced was read at 720 nm (red filter) on a colorimeter and protein content was expressed in terms of mg per cent of fresh tissue weight.

Ascorbic acid (AA) in liver, adrenal and testis

Total AA content in the tissues was estimated by the method of Roe (1954). The AA was extracted with 6% TCA and oxidized to dehydro-ascorbic acid by shaking with norit (activated animal charcoal) for 15 min. 4 ml of the filtrate was incubated with 2,4-dinitrophenyl hydrazine for 3 hr at 37°C to yield osazone. This was treated with 85% H_2SO_4 to form a reddish brown colour, which was read colorimetrically at 540 nm (green filter). AA content was expressed in terms of mg/100 mg tissue weight.

Lipids in liver, adrenal, testis and serum

Total lipid content was estimated employing the method of Folch et al. (1957) using a mixture of chloroform-methanol (2:1 v/v) as an extractant and measured gravimetrically. The total lipid content in liver, adrenal and testis was expressed as mg/100 mg fresh tissue weight. Serum lipids was estimated by the same method using 0.5 ml of serum. It was expressed as mg/100 ml serum.

Cholesterol fractions in liver, adrenal, testis and serum

Total, free and esterfied cholesterol contents were estimated by the method of Crawford (1950) using alcohol-ether mixture (3:1 v/v). Free cholesterol was precipitated with digitonin and estimated as the digitonide. $FeCl_3$ was used as the colour reagent. Colour intensity was

read at 540 nm on a colorimeter. Total, free and esterified cholesterol were expressed as mg/100 mg tissue in case of liver, adrenal and testis and as mg/100 ml of serum in case of serum.

Phosphorlyase in liver, muscle and testis

Total phosphorylase activity was assayed by the method of Cahill et al. (1957) using glucose-1-phosphate (Sigma chemicals, USA) as the substrate. The inorganic phosphate released was measured as per the method of Fiske and Subbaraw (1925). Enzyme activity was expressed as µg phosphate released/mg protein/30 minutes.

Gulcose 6 phosphatase (G-6-Pase) in liver

Homogenate for estimation of this enzyme activity was prepared in cold citrate buffer at 6.5 pH. Enzyme activity was assayed by the method of Harper (1960). Glucose-6-phosphate (disodium salt, Sigma Chemicals, USA) was used as the substrate. Inorganic phosphate released was measured as per the method described by Fiske and Subbaraw (1925) and the colour intensity was read at 660 nm (red filter) on a Klett-Summerson colorimeter. Enzyme activity was expressed as µg phosphate released/mg protein/10 minutes.

Fructose-1,6-diphosphate aldolase in liver, muscle and testis

The Fructose-1,6-diphosphate aldolase activity was estimated as per the method of Umbreit (1957). Fructose-1,6-diphosphate (Sigma chemicals, USA) was used as the substrate. The aliquot treated with substrate was incubated for an hour at 37°C and then terminated with TCA(10%). The supernatant was treated with 2, 4-dinitrophenyl hydrazine

and alkali and the colour intensity was read on a Klett-Summerson colorimeter at 570 nm. Enzyme activity was expressed as μ moles FDP cleaved/mg protein/60 min.

Lactate dehydrogenase (LDH) in liver, muscle and testis

Activity level of LDH was assayed by the colorimetric method of King as described by Varley (1975). The optical density of the colour developed was read of 440 nm on a colorimeter. The activity is expressed as μ moles lactate oxidised/mg protein/15 min.

Succinate dehydrogenase (SDH) in liver, muscle and testis

Activity level of SDH was assayed as per the method of Kun and Abood (1949) using indonitro-triphenyl-tetrazolium salt (INT) as the hydrogen acceptor. The formazan formed was extracted in 7 ml of acetone and the colour intensity was read at blue filter on a colorimeter. Enzyme activity was expressed as μ moles formazan formed/mg protein/30 min.

Acid and alkaline phosphatases in liver, muscle and testis

Biochemical assay of both the monophosphoesterases was carried out as per the method described in Sigma technical bulletin No. 104 using p-nitro phenylphosphate as the substrate. Enzyme activity levels were expressed as μ mole PNP released/mg protein/30 minutes.

Total, Na^+-K^+-and Ca^{++}-Mg^{++}-ATPase in liver, muscle and testis

ATPase activities were estimated as per the method described by Umbreit et al., (1957). Adenosine - 5 triphosphate sodium salt (Sigma Chemicals, USA) was used as the substrate. Ouabain was used as the inhibitor of Na^+-K^+-ATPase. Activity was terminated with TCA. Inorganic

phosphorous released was estimated by the method of Fiske and Subbaraw (1925). The colour intensity was read at 660 nm on a Klett-Summerson colorimeter and ATPase activity levels were expressed in terms of μg PO_4 released/mg protein/10 min.

Cyclic AMP Phosphodiesterase in liver, muscle and testis

cAMP specific phosphodiesterase activity level was measured employing the method described by Butcher and Sutherland (1962). cAMP was used as the substrate. Aliquots of the reaction mixtures were treated with vipera venom and were then analysed for inorganic phosphate by the method of Fiske and Subbaraw (1925) and the colour developed was read at 720 nm on colorimeter. Specific activity was expressed in units of μg PO_4 released per mg protein per 30 min.

Histochemical localization of steroid dehydrogenases in testis

The left testis from each bird was quickly excised after decapitation under mild anaesthesia, and transferred to a Cryostat microtome maintained at-20°C. Fresh frozen sections of 15-20 μ thickness were taken on a clean slide and thawed. 17B-hydroxy steroid dehydrogenase was localized employing the method of Kellog and Glenner (1966) using testosterone and estradiol as substrates. 3β-hydroxysteroid dehydrogenase was localized according to the method of Wattenberg (1958) using dehydroepiandrosterone and pregnenolone as the substrates while 3α-hydroxysteroid dehydrogenase was demonstrated histochemically as per the method of Balough (1966) using androsterone as the substrate. Incubation of the sections was carried out at 42°C. NAD was used as a coenzyme while Nitro blue tetrazolium salt was used as

hydrogen acceptor. The stained sections were washed thoroughly in distilled water and fixed in 10% neutral formalin for 15 min, washed again in distilled water and mounted in glycerine jelly. Fettrot 7-B and Sudan Balck 'B were utilized in demonstrating neutral and total lipids respectively (Pearse, 1968). Control sections for the enzymes were incubated in media devoid of the substrates.

Histological technique

Tissue to be processed for histological studies was fixed (immedaitely after decapitation) in Bouin's fluid and processed in the routine fashion and embedded in paraffin. Paraffin sections of 5 μ thickness were cut on a microtome and stained with haematoxylin-eosin. The stained sections were mounted in D.P.X.

Evaluation of adrenal cortico-medullary ratio

The ratio of cortex to medulla was determined by projecting areas at random on a 6 inch diameter screen using a 40X objective. The outlines of both cortex and medulla were sketched on thin paper, which were then cut and weighed. The percentage of cortex and medulla was determined and approximate cortex: medulla ratio calculated.

Glucose tolerance, insulin, glucagon and adrenaline response tests

A total of 280 chicks were procured out of which 40 chicks were used to study the various response tests on the day of hatch. The remaining chicks were used for the chronological glucose tolerance, insulin, glucagon and adrenaline response tests in normal as well as dexamethasone treated chicks and were divided into two batches. Bach I was injected with 0.9% saline and served as the controls. Batch II was

injected with the low dose of DXM. The chicks were maintained in the same fashion as described earlier, for chronological glucose tolerance, insulin, glucagon and adrenaline response tests on 10th, 20th and 30th days of treatment.

The glucose tolerance, insulin, glucagon, and adrenaline response tests were carried out as follows and the blood glucose was estimated by the method of Trinder (1969).

Glucose tolerance test (GTT)

Normal and DXM treated chicks were fasted overnight and infused intraperitoneally with glucose (70 mg/100 g b.w./bird). Blood samples were drawn from the brachial vein at 0 (prior to glucose loading) and 30, 60, 90, 120, 150, 180, 210 and 240 min post glucose loading for the evaluation of blood glucose.

Insulin response test (IRT)

Insulin response was assessed by injecting 0.5 IU insulin/100 g b.w./ bird in 0.1 ml of 0.9% saline and the resultant hypoglycemia and recovery to normal glycemic level were measured by drawing blood samples at fixed intervals of 30, 60, 90, 120, 150, 180, 210 and 240 min after insulin injection. The chicks were fasted overnight and the fasting blood glucose level (at zero level) prior to insulin injection was estimated. The insulin preparation used for the study was of Boots Company India Ltd. containing 40 IU/ml.

Glucagon response test (GRT)

Glucagon response was assessed by injecting a single dose of glucagon obtained from Sigma Chemicals, USA (30 µg/100 g b.w./bird in

redistilled water) to both normal and dexamethasonised chicks and the blood glucose levels were measured from samples drawn from the wing vein at regular intervals of 30, 60, 90, 120, 150, 180, 210 and 240 min after the injection. Prior to glucagon injection the birds were fasted overnight and the fasting blood glucose level (at zero level) was estimated.

Adrenaline response test (ART)

Adrenaline response test was carried out in terms of glucose levels in control and experimental chicks. A single injection of adrenaline (100 µg/100 g b.w.) was given to overnight fasted chicks and the glucose level estimated at zero hour (prior to adrenaline injection) and 30, 60, 90, 120, 150, 180, 210 and 240 min post injection.

Statistical analysis

For each of the parameters studied, eight to ten samples were used. The mean and standard error of mean were calculated and the statistical significance was determined by Student's 't' test.