Chapter 1

MATERIALS AND METHODS

SECTION I

COLLECTION OF THE BIRDS AND EXPERIMENTAL METHODS

Painted Storks (Ibis leucocephalus) :

Nestlings, at various stages of post-hatching development as well as adults were collected from the natural nesting colony at the Delhi Zoological Park, New Delhi, India. Stages were selected according to the type of feathers present on the crown region, viz., protoptiles, mesoptiles, juvanal feathers etc., as described by Desai (1975). One year old storks with bare forehead and partial loss of feathers from the crown, and adult storks, where transformation of the crown feather tracts into aptarium is complete, were trapped and used for studying the permanent suppression of feather development. On hatching, the head of a nestling is covered sparsely with gray natal down feathers (protoptiles) which get subsequently replaced with white mesoptiles (after about the 10th day of post-hatching development). With the subsequent moult, the mesoptiles are replaced by contour feathers (juvanal feathers which impart a grey colour to the plumage of the head which is clearly discernible by about the 45th day post-hatching). These in turn are replaced by white definitive feathers once the birds undergo

post-juvanal moult. The frontal tract and anterior parts of the coronal region become bare in a one year old bird, whereas the occipital region bears white definitive feathers. After the second annual moult, the coronal region and anterior parts of the occipital region also become bare and the skin of the face and head turn bright yellow in colour, almost similar to that of the adult painted stork. However, at this stage the birds are not sexually mature. Sexual maturity is attained only when they are three years old (Desai, 1975). Feather loss from the forehead and crown regions is observed in both the sexes. Biopsies of the skin from the head region were taken under local anaesthesia. After taking biopsy material, the skin was stitched and the necessary post operative care taken, following which birds were later released. The biopsy material was always taken from the same region, and the skin was used for histological and histochemical studies, employing the methods described in Section II of this chapter.

Adult feral Blue Rock Pigeons (Columba livia), captured from the University Campus, and acclimated to laboratory conditions were used for the study on retardation of feather development under experimentally induced athyreosis. To induce feather development, contour feathers from the ventrum were plucked during the non-moulting period and the birds were divided into three groups; A, B and C.

Group A :

Birds of this group were rendered functionally athyroidic by providing a 0.2% acqueous solution of thiouracil (pH 8.2-8.4) in the drinking water, starting from three weeks prior to plucking of the feathers to one month afterwards. A few of these birds were maintained on the same schedule for a duration of two months for morphological observations.

Group B :

Birds of this group which served as controls to those of Group A, received no drug but the feathers were plucked.

Group C :

Birds of this group were treated like those of Group A, but received a daily injection of 1 mg/0.5 ml of thyroxine (L-thyroxine BDH, England) starting from 3 days prior to plucking of feathers uptill a month afterwords.

Birds from all the three groups were sacrificed under mild anaesthesia at selected stages viz., 1,5,7,10,15,25 and 30 days post-plucking, at a fixed time of the day. Skin from the ventrum was immediately excised and used for histological and histochemical studies (see Section II). In all the birds used for experiment, functional status of the thyroid glands was histologically evaluated.

Adult female house sparrows (Passer domesticus), collected from the nest-boxes installed in the M.S. University Campus, were used to study the cyclic suppression of feather develop-

ment in the ventral skin, during incubation patch formation. Female House Sparrows were selected during both the non-breeding as well as breeding seasons; and the incubation patch formation during the breeding period was arbitrarily staged as follows : (1) Nestbuilding, (2) Initial stage of defeathering, (3) Defeathered and Vascularized, (4) Fully formed patch (Defeathered, vascularized and edematous), (5) Regression and (6) Refeathering.

The birds were decapitated under mild anaesthesia after trimming the feathers from the ventrum, (wherever required) to facilitate easy sectioning of the skin. The ventral skin was excised immediately and used for gross morphological, histological and histochemical studies (See Section II).

Female house sparrows, with fully formed incubation patch, were captured during September 1978 in late evenings from nest-boxes kept for regular observation in the M.S. University campus, Baroda (Long 73° 13' Lat 22° 18'N) India. This being an intensive breeding period of the sparrows, all the captured females were found to be incubating a full clutch of 4-5 eggs. Those collected on the same evening were grouped into three groups. The first group received a subcutaneous injection of thyroxine (BDH make, England) on alternate days. The second group received injection of saline on alternate days, and the third group was kept untreated under captivity. The saline injected birds served as controls for those administered with thyroxine. The

untreated birds were maintained as an additional control group, so as to avoid the risk of misinterpreting the changes if any that may occur in response to mechanical stimulation (injection of saline). The dose of thyroxine administrated was 1 mg/ 0.3 ml of normal saline (pH 8.4). The hormone as well as saline were administered subcutaneously in the incubation patch region of the first and second group of birds respectively. When thyroxine injections were given daily, the birds died on the 4th day after starting of the injections and their pectoral muscles were found to be greatly wasted. The sparrows, however, survived without any apparent ill effects when injections were given on alternate days. Some of the birds of Group A were given only a single dose of hormone injection, after which they were kept in captivity for about three weeks. Morphological observations were made on all the birds regularly. The birds of Group A and Group B were sacrificed under mild anaesthesia at regular intervals (24 hours after 1st, 2nd or 3rd injections, as the case may be), and the ventral skin from them was excised immediately. After the 1st injection of the hormone and subsequent treatment by two additional injections of thyroxine, feather development in the patch was observed by the 7th day.

Certain aspects of secretory activity of avian integument were studied in Sarus Crane (Grus antigone antigone) and Indian Ibis (Threakiornis malanocephala). These birds were provided by the authorities of the Sayaji Zoo, Baroda, immediately after

their natural death. The bare skin from the head and neck regions was taken and used for histological and histochemical studies (vide- Section II).

SECTION II

MORPHOLOGICAL, HISTOLOGICAL AND HISTOCHEMICAL METHODS

For the gross morphological study of the incubation patch skin of the sparrows, the skin pieces excised from them were placed in a 0.2 M acqueous solution of sodium bromide (NaBr 20-21% w/v) for about an hour and a half. The material was then transferred to distilled water and the epidermis then teased loose and lifted away with forceps. The dermis was used for observations after staining it with Haematoxylin.

For the general and specific histological studies of the skin of the birds under investigation, the material was fixed in Bouin's fluid and Paraffin sections of 6 to 7 μ thickness were cut and stained with Haematoxylin-Eosin (HE), Haematoxylin-Picro ponceau S (HP-S) and Mallory's triple stains respectively as per the methods described by Gurr (1956).

Paraffin sections were also stained with Alcian Blue (8GS) and Alcian Blue-PAS procedures of Lillie (1965) and Pearse (1968), for sulphated (AB positive) and non-sulphated (PAS positive) mucopolysaccharides (Glycosaminoglycans i.e. GAG) respectively.

For the study of the histochemical profile of different enzymes and metabolites, the tissues were removed immediately after sacrificing the birds and fixed on the chuck of a cryostat microtome maintained at -20°C. For histoenzymology, sections of 10 to 12 μ thickness were cut and transferred to the respective chilled incubation media. An incubation time of 30 minutes at 37°C was found to be satisfactory for optimal histochemical demonstration of dehydrogenases.

For the demonstration of acid and alkaline phosphatases, the sections were first transferred to chilled 10% neutral formalin for about 15-20 minutes at room temperature. They were then washed thoroughly in several changes of distilled water and incubated for 6 hrs. in the media prepared according to Burstone (1962) as given below :

Ingredients	Ac Pase	Alk Pase
Napthol AS-MX sodium salt	-	1 mg
Napthol AS-BI sodium salt	1 mg	-
Acetate buffer at pH 5.2 (·2M)	5 ml	-
Tris buffer at pH 8.6 (·2 M)	-	5 ml
Distilled water	5 ml	5 ml
Red violet LB (diazonium salt)	6 mg	6 mg
10% $MnCl_2$	3 drops	-

The incubation media were prepared and filtered using

Whatman No.1 filter paper, before incubating the sections. After incubation (for 6 hours as mentioned earlier), the sections were thoroughly washed in distilled water and mounted in glycerine jelly.

Control : For control, sections were incubated in media devoid of the respective substrates.

The incubation media used for demonstrating the activities of lactate, malate, β-hydroxy butyrate and α-glycerophosphate dehydrogenases (LDH, MDH, BDH and α-GPDH) were prepared according to the method of Ogata and Mori (1964) containing the following ingredients.

LDH, MDH, BDH and α-GPDH :

1 M. solution of sodium salts of lactate, malate, β hydroxy - butyrate and α-glycerophosphate - as the case may be :	4 ml
Nitro blue tetrazolium (NBT)	3 ml
0.1 M phosphate buffer at pH 7.6	11 ml
Nicotinamide adenine dinucleotide (NAD)	2.5 mg
0.1 M potassium cyanide (adjusted to pH 7.6 with 0.5 M hydrochloric acid)	2 ml

SDH :

The activity of succinic dehydrogenase (SDH) was demonstrated using the modified method of Nachals et al (1957) and the incubation medium consisted of :

0.2 M sodium succinate	1 ml

0.2 M phosphate buffer at pH 7.6	1 ml
Nitro blue tetrazolium (NBT) (1 mg/1 ml)	2 ml

G-6-PDH :

The glucose-6-phosphate dehydrogenase (G-6-PDH) activity was demonstrated employing the method described by Ogata and Mori (1964). The incubation medium had the following constituents in the given concentration :

0.02 M Disodium glucose-6-phosphate	4 ml
Nitro blue tetrazolium (5 mg/3 ml)	3 ml
0.1 M Vernol buffer at pH 7.6	11 ml
Nicotinamide adenine dinucleotide phosphate (NADP)	8 mg

The sections of the skin that were incubated in the specific incubation media at 37°C for 30 minutes were washed in distilled water after incubation. They were then fixed for about 20 minutes in 10% neutral formalin, washed thoroughly in distilled water and then mounted in glycerine jelly.

For control, sections were incubated in media devoid of the respective substrates.

LIPIDS :

Neutral and acidic lipids were demonstrated in the skin sections which were previously fixed in Baker's calcium formol. Sections were washed thoroughly with distilled water and stained with Fettrot 7 B as well as acqueous Nile Blue Sulphate for demonstration of neutral and acidic lipids

respectively (Pearse, 1968). Control sections were treated prior to staining, with chloroform - methanol mixture (1:2 v/v). Finally, the sample as well as control sections were mounted in glycerine jelly.

In the case of Painted Storks, the biopsy material was fixed in chilled neutral formalin, and Baker's calcium formol, for the histochemical demonstration of phosphatases and lipids respectively. A PELCOOL FREEZING microtome (MSE) was used for cutting frozen sections.

For histochemical localization of the phosphatases, Sigma kit No.85-L (USA) was used (Sigma Bulletin No.85-1971).