CHAPTER 1

MATERIAL AND METHODS

For the present investigation, the domesticated variety of the blue rock pigeon, Columba livia, reared in a well maintained aviary of the department were used. Young ones of pigeons were selected at different intervals (in days), in order to study the normal post-hatching development of the first generation of definitive feathers of the ventral tract. The birds used were of different ages (in days), viz., 1, 3, 5, 7, 10, 15, 20, 25 and 30 days old. By the 30th day after hatching the definitive feathers were found to be fully developed. Healthy adult pigeons, maintained in the laboratory and fed on grains, weighing about 300 to 330 grams were used to study the enzymological pattern of the resting feather germ and the adult normal skin.

Induced development of the definitive feathers was initiated by plucking off the adult feathers, from the ventral tract extending from the pectoral tract upto near the region of the cloaca, at a time when the feathers are not to moult in the normal way. Pigeons which were in the normal moulting phase were not used for the experiment.

Regeneration of the feathers was induced by plucking off 10 day old inducedly developing feathers, as there are no preformed feather germs at this time (see introduction).

The birds, both young and adults, were decapitated under mild anesthesia and the skin was separated from the pectoral tract and cut into smaller pieces. The completely keratinised distal parts of the feathers were trimmed off before sectioning. For the histochemical demonstration of dehydrogenases, pieces of skin from the pectoral tract were removed immediately after sacrificing the animals, and fixed on the chuck of a cryostat microtome maintained at -20°C. Sections of 12 to 18 μ thickness were cut and transferred to chilled incubation media for the respective enzymes. The incubation media were then brought to room temperature and then the sections were incubated in a water bath maintained at 37°C, for 30 minutes.

The incubation media used for demonstrating the activity of lactate dehydrogenase (LDH), malate dehydrogenase (MDH), β-hydroxybutyrate dehydrogenase (BDH) and α-glycerophosphate dehydrogenase (αGPDH) were prepared according to the method of Ogata and Mori (1964) and contained the following ingredients in the given concentration.

1 M solution of the specific substrate (sodium salts of lactate, malate, β-hydroxybutyrate and α-glycerophosphate respectively)	4 ml
Nitro blue tetrazolium (5 mg/3 ml)	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

The activity of SDH was demonstrated using the modified method of Nachlas 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 (1 mg/1 ml)	2 ml

Glucose-6-phosphate dehydrogenase 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 Veronal buffer at pH 7.6	11 ml
Nicotinamide adenine dinucleotide phosphate (NADP)	8 mg

Incubation medium for demonstrating the activity of aldolase was prepared as described by Abe and Shimizu (1964) and contained the following ingredients in the given concentration:

0.02 M sodium fructose-1,6,diphosphate	10 ml
Nicotinamide adenine dinucleotide	5 mg
Nitro BT (1 mg/ ml)	10 ml
0.05 M Arsenate HCl buffer at pH 7.6	10 ml

The sections of skin were incubated in the respective incubation media at 37°C for 30 minutes as mentioned earlier, and washed in distilled water. The sections were then fixed for about 30 minutes in 10% neutral formalin, washed thoroughly in distilled water and then mounted in glycerine jelly.

Control: For control, sections were either incubated in media devoid of the respective substrates or heated to 80°C prior to incubation.

Fixed pieces of skin were used for the demonstration of alkaline and acid phosphatases, lipids and lipase. Pieces of skin were fixed in cold 10% formalin neutralised with KOH, for about 16 hours for both the phosphatases; Baker's calcium formol (Pearse, 1960) for lipids and cold 6% formalin neutralised with disodium hydrogen orthophosphate for lipase

(George and Ambadkar, 1963). Sections of 10 to 15 μ thickness were then cut on a freezing microtome and washed thoroughly in several changes of distilled water. For the histochemical demonstration of phosphatases, the sections were incubated in media prepared according to the method of Burstone (1962) and contained the following ingredients in the given concentration:

Ingredients	Alkaline	Acid
Napthol AS-MX sodium salt	1 mg	-
Napthol AS-BI sodium salt	-	1 mg
Acetate buffer at pH 5.2	-	5 ml
Tris buffer at pH 8.6	5 ml	-
Distilled water	5 ml	5 ml
Red violet LB (diazonium salt)	6 mg	6 mg
$MnCl_2$	-	3 drops

The incubation media were prepared and filtered using Whatman No.1 filter paper, before incubating the sections. An incubation time of about 6 hours was found to be satisfactory for the sections of the skin for optimal histochemical reactivity. After incubation, 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 substrate solutions.

Lipids were demonstrated in sections of tissues fixed previously in Bakers calcium formol. Sections were stained with fettrott 7B according to the method described by Pearse (1960) for neutral lipids, while for the demonstration of acidic lipids, the Nile blue sulphate method of Cain (1947) as cited by Pearse (1960) was employed. Sections treated with a mixture of methanol-chloroform (1:2 v/v) at 55°C for three hours prior to staining served as suitable controls.

The histochemical demonstration of lipase was carried out by the method of Gomori (1952), modified by George and Iype (1959), using 'Tween 85' (polyoxyethylene sorbitan trioleate) as the substrate as adopted by George and Ambadkar (1963). Sections were incubated at 37°C for 10 to 12 hours. Sections incubated in the medium devoid of the substrate and those incubated after treating them with warm distilled water (80°C) for 5 minutes served as controls.