

CHAPTER 3

HISTOCHEMICAL OBSERVATIONS OF THE ALDOLASE ACTIVITY IN THE
DEFINITIVE FEATHERS OF THE BLUE ROCK PIGEON,
COLUMBA LIVIA, DEVELOPING UNDER
THREE DIFFERENT CONDITIONS

The importance of Embden-Meyerhoff (EMP) pathway of glycolysis as a source of energy in vertebrate tissues has long been recognised. Enhanced activity of this pathway could be expected in living systems concurrent with increased energy requirements. During developmental processes, functional differentiation of organs demanding increased energy output would all the more necessitate an enhanced activity of this metabolic route. It is also known that ^{the} hexose monophosphate (HMP) shunt which is comparatively more active during the initial stages of development becomes subordinate to the EMP pathway of glycolysis in the later stages. Hence the comparative activities of these two closely related metabolic routes could provide an excellent example of metabolic regulations associated with the two major processes occurring during development, viz., cellular proliferation and cellular differentiation.

The presence of aldolase, which catalyses the reversible reaction between fructose-1,6 diphosphate and dihydroxyacetone phosphate is indicative of the existence

of glycolytic activity in the tissues concerned. The activity of this enzyme has been demonstrated in the nuclei of the liver cell (Roodyn, 1956; 1959), in the striated muscle (Long, 1961) and in the supernatant fraction of liver and other tissue homogenates (Duve et al., 1962). Literature on the activity of aldolase during developmental processes include the works of Gustafson and Hasselberg (1951) in sea-urchin development, of Eckhout (1955) in the anuran metamorphosis, and of Burt (1966; 1967) during avian neurogenesis. Apart from these, the activity of aldolase has also been studied using histochemical techniques in the normal and regenerating urodele limbs by Schmidt and Weidman (1964) and in the regenerating lacertilian tails by Magon (1970) and Shah and Ramachandran (1972).

However, a general lack of information on the pattern of aldolase activity during development of avian skin in general and feathers in particular, has been felt. Hence the present histochemical investigations were carried out to throw some light on the significance of this key enzyme of the EMP pathway of glycolysis, in the development of the definitive feathers of the blue rock pigeon, Columba livia, developing under three different conditions described earlier (vide introduction).

OBSERVATIONS

Normal Adult Skin: (Figs. 1)

The different components of the normal adult skin in the non-feather region, such as stratum germinativum, dermal fibrocytes and smooth muscle associated with the feather follicles, all showed a pronounced enzyme response. The intensity of the enzyme reaction, however, was slightly higher in the smooth muscle in comparison to the other two components and its localizations in muscle was both nuclear as well as sarcoplasmic. The adipose tissue showed only adsorption artifacts. Among the components of the feather follicle, cells of the stratum germinativum of the follicular wall as well as the cells of the resting germs were the ones which depicted the highest enzyme reactivity. Whereas the pulpa demonstrated only a moderate histochemical response for aldolase, the mesenchymal cells at the apical region of the pulp showed fairly high enzyme response.

Normal post-natal development: (Figs. 2 to 7)

A very weak, almost negligible level of enzyme activity in the various components of the skin was the feature on the day of hatching. However, during the immediate course of development, almost all the components appeared to acquire appreciable aldolase activity as could be visualized by the observation on the intensity of the enzyme during the 3rd

to 5th days post hatching. During this period, the epithelial cells, pulp cells, erector muscle of the follicles and stratum germinativum of the non-feather regions, all in that order showed high enzyme response. The keratinized outermost layers of epidermis revealed no histochemical reactivity for the enzyme. Along with the further progress of development, the enzyme activity reached its peak level in the feather forming tissues and the stratum germinativum of the non-feather epidermis at about the 10th day. However, in the smooth muscles and the fibrocytes of the dermis, the enzyme registered a fall in its activity at this stage, from the level noticed on the 5th day. Among the epithelial cells of the developing feather itself, enzyme activity appeared to be sequentially graded from a higher level in those cells lying adjacent to the pulp to a lower level in those lying farther away. In the already formed barb ridges, the more highly keratinized cells were, nevertheless, enzyme negative. This condition was retained in the epithelial cells hereonwards till the completion of post-natal development. The other components such as pulp cells, smooth muscle fibres and fibrocytes which had registered a slight decrease previously also gained the enzyme activity characteristic of the adult level by about ^{the} 15th day and remained so for the rest of the period of development and thereafter.

EXPLANATIONS FOR FIGURES

- Fig. 1 Longitudinal section of a normal adult feather follicle showing a pronounced aldolase activity. Note the comparatively higher enzyme activity in the smooth muscles and the components of the resting germ.

NORMAL DEVELOPMENT

- Fig. 2 Section of skin on the 3rd day post hatching, showing aldolase activity in developing definitive feathers.
- Fig. 3 Higher magnification of one of the developing feathers. Note the appreciable activity of aldolase in pulp and epithelial cells of barb ridges.
- Fig. 4 LS of developing definitive feathers on the 10th day post-hatching, depicting peak enzyme activity. Note the gradation in intensity of enzyme reactivity in the barb ridges.
- Fig. 5 TS (slightly oblique) of the feather on the 20th day ^{post} of hatching. The keratinised portions of barb ridges exhibit a lower degree of enzyme reactivity than those nearer to the pulp.
- Fig. 6 Oblique section of the feather on 25th day post-hatching showing the enzyme activity.
- Fig. 7 LS of feather on the 30th day. The cells of the regressing pulp and smooth muscles exhibit considerable enzyme activity.

INDUCED DEVELOPMENT

- Fig. 8 LS of feather follicle 15 hours after plucking. Note increased activity of aldolase in the germ as well as smooth muscles.
- Fig. 9 Section of skin on the 3rd day of induced development showing enzyme activity.
- Fig. 10 LS of an inducedly developing feather on the 5th day. Note decrease in aldolase reactivity in the distal parts of barb ridges.
- Fig. 11 LS of feather on the 10th day, passing through its dorsal aspect. Note the peak aldolase activity in the barb ridges.
- Fig. 12 LS of a 25 day old developing feather. The cells of the regressing pulp retain a high enzyme activity.
- Fig. 13 Oblique section passing through the base of a 30 day old feather, showing appreciable activity in the germ for next generation.

REGENERATION

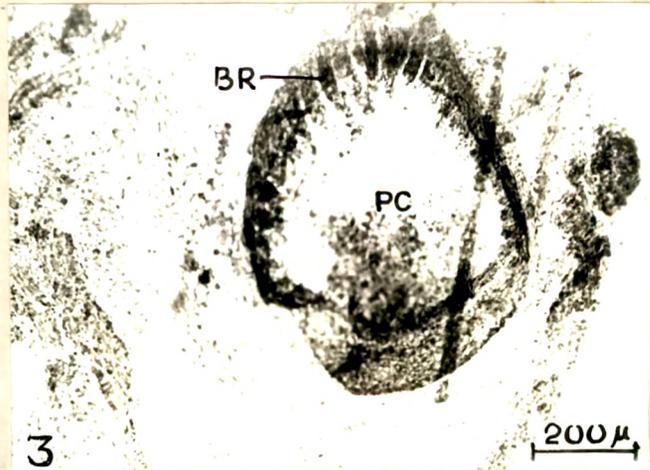
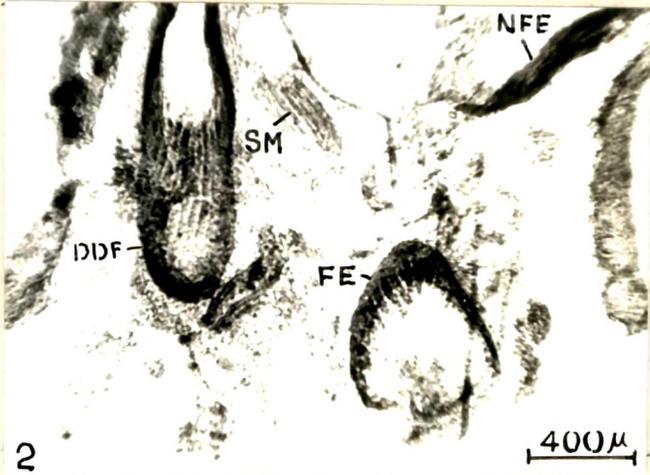
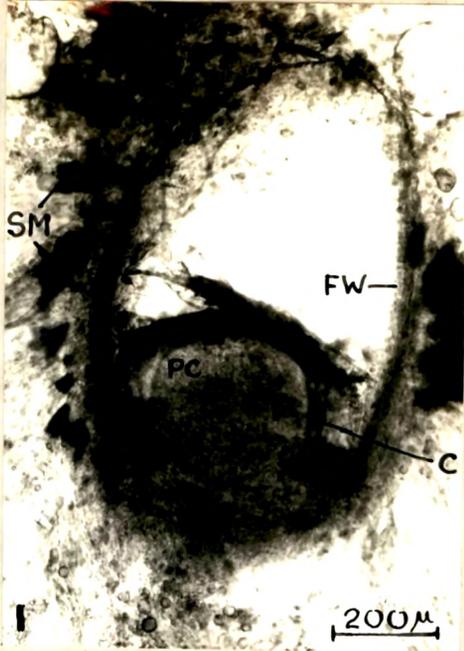
- Fig. 14 TS of feather follicle 24 hours after inducing feather regeneration, showing a decline in aldolase activity.
- Fig. 15 Section of skin showing non-feather epidermis and feather follicle. Increased activity of the enzyme is evident in the regenerating feather as well as the smooth muscles.

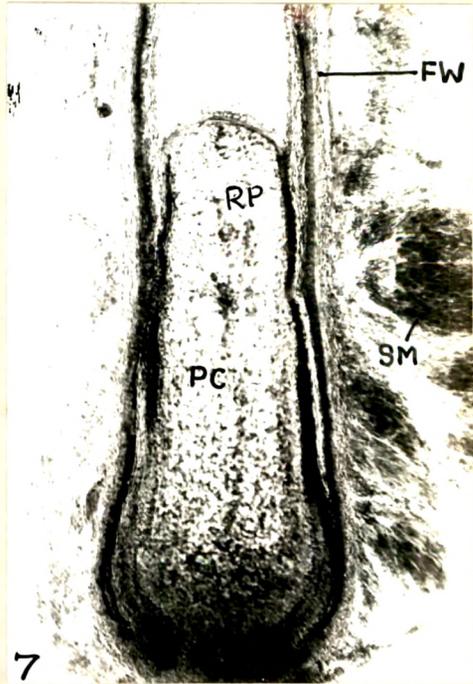
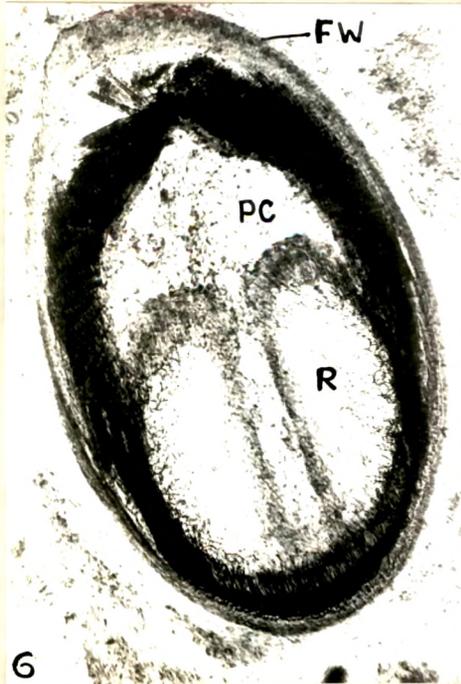
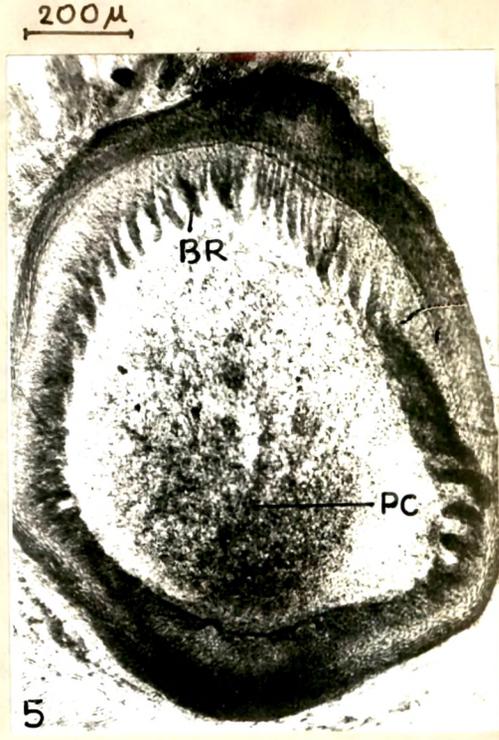
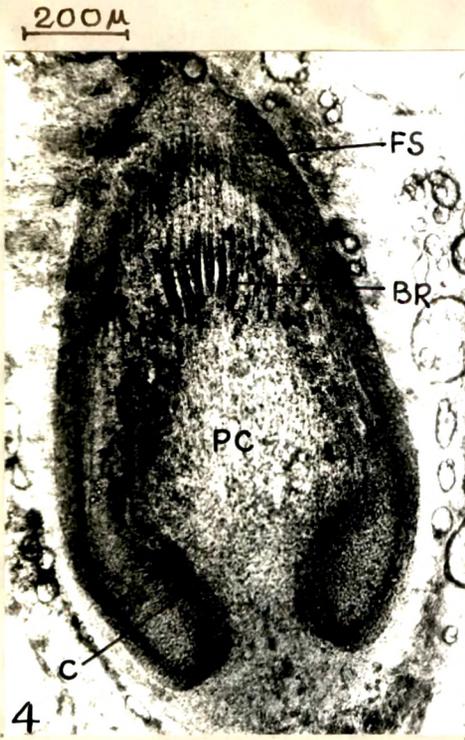
- Fig. 16 Regenerating feather on the 5th day depicting peak enzyme response.
- Fig. 17 Regenerating feather on the 5th day magnified to show aldolase response of the cells of barb ridges, collar and the mesenchymal pulp.
- Fig. 18 TS of a 7 day old feather regenerate showing aldolase activity.
- Fig. 19 LS of part of a 10 day old feather regenerate showing declined aldolase activity.
- Fig. 20 Oblique section of feather on the 15th day, showing reduction in enzyme activity in the barb ridges which are getting keratinised.
- Fig. 21 LS of feather on the 20th day, showing retention of a high aldolase activity.
- Fig. 22 TS of feather follicles on the 25th day after inducing regeneration. Note high aldolase activity in the pulp cells and smooth muscles.

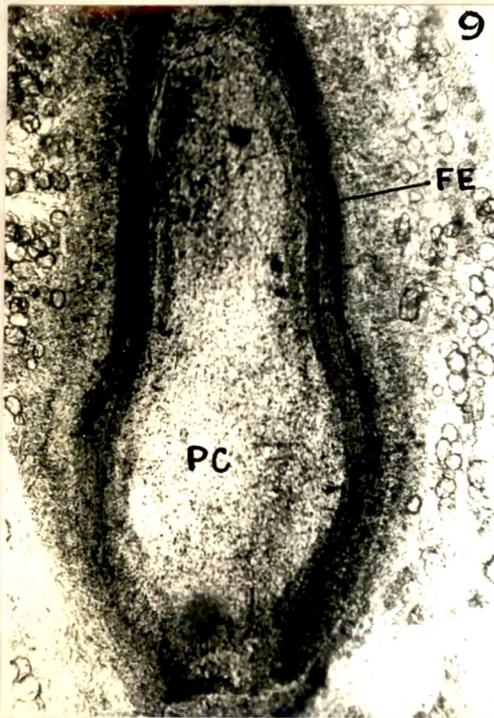
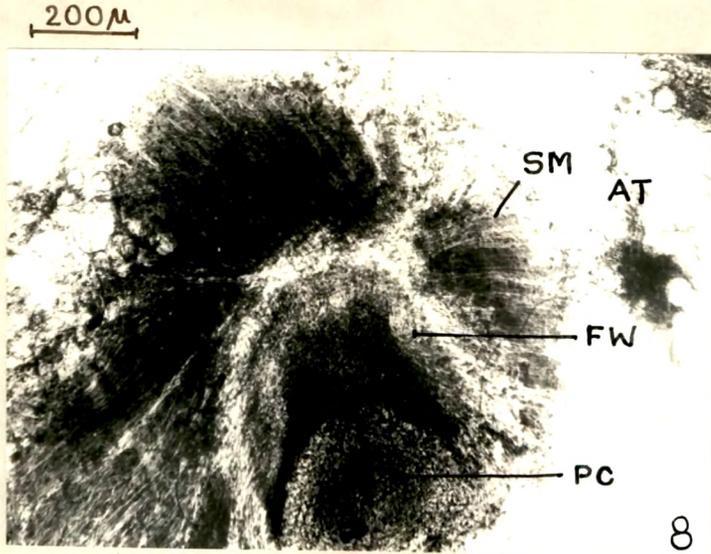
ABBREVIATIONS

- AA - Axial artery
AT - Adipose tissue
BC - Blood clot
BR - Barb ridges
C - Collar
DDF - Developing definitive feather
DP - Dermal papilla

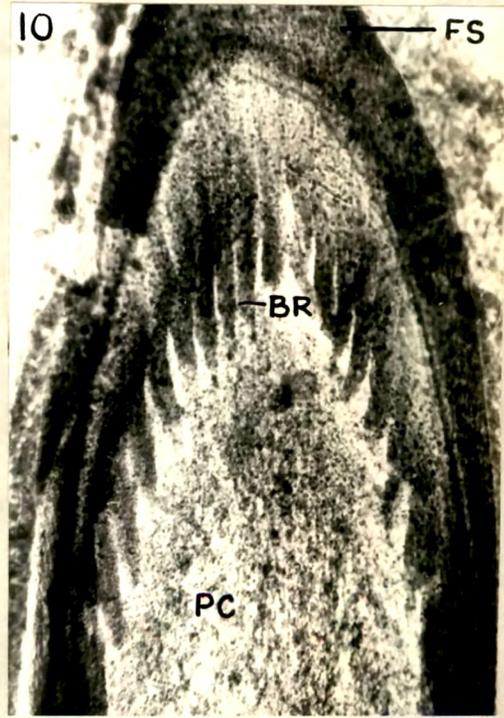
EC - Epithelial covering
FE - Feather epithelium
FKR - Fully keratinised region
FS - Feather sheath
FW - Follicular wall
MC - Melanocytes
NDF - Natal down feather
NFE - Non-feather epidermis
PC - Pulp cells
R - Rachis
RP - Regressing pulp
SM - Smooth muscles



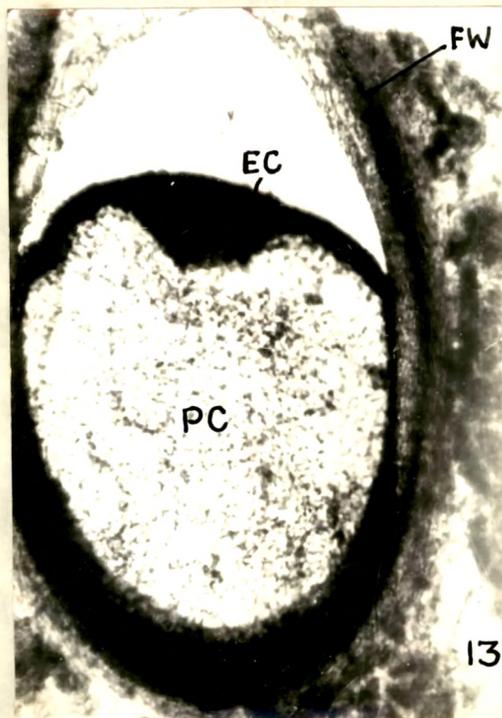
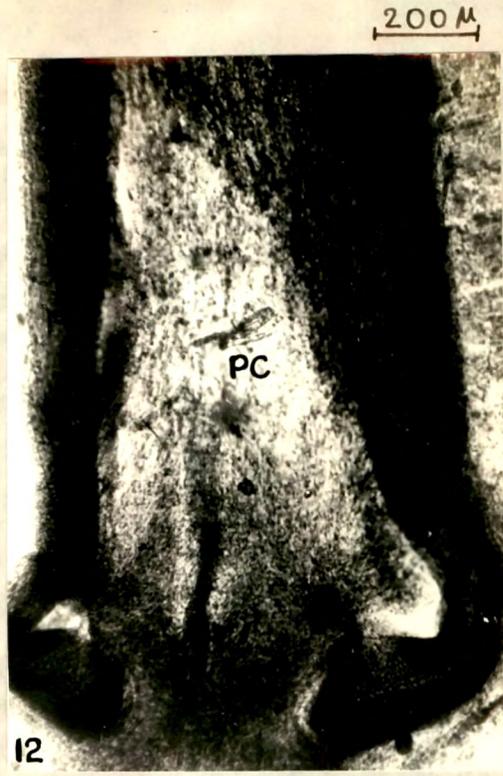
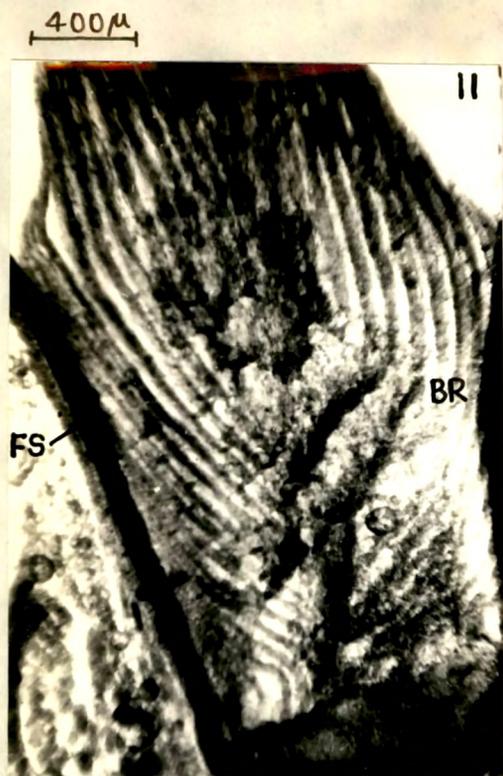




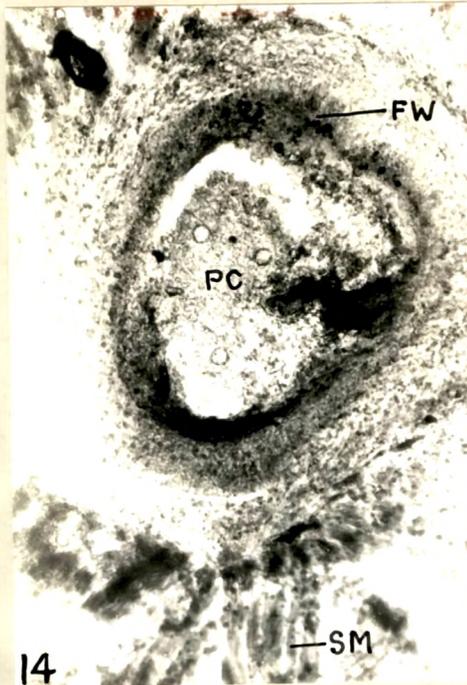
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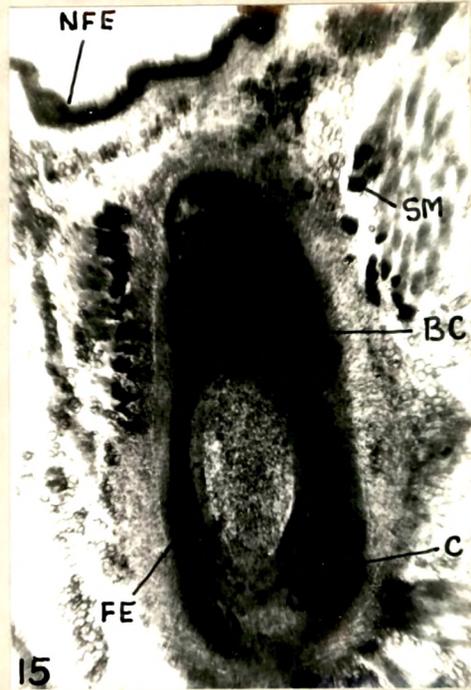
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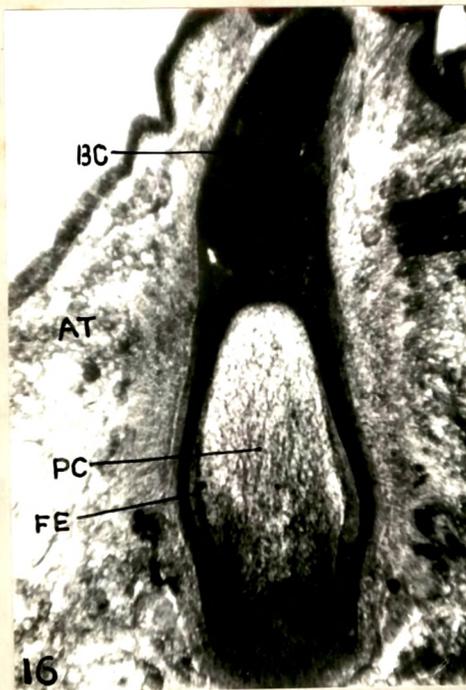
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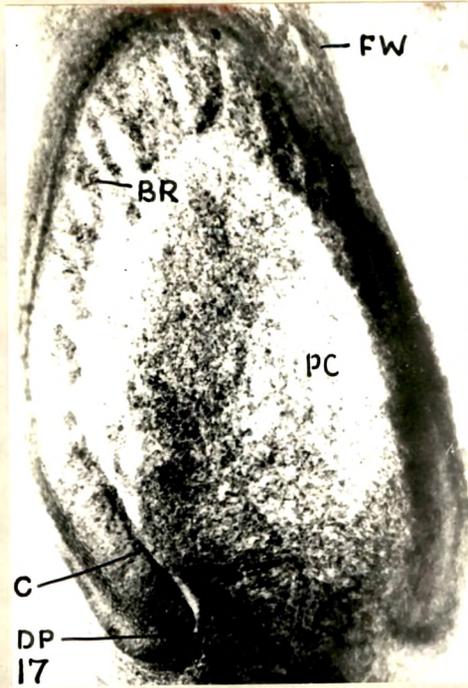
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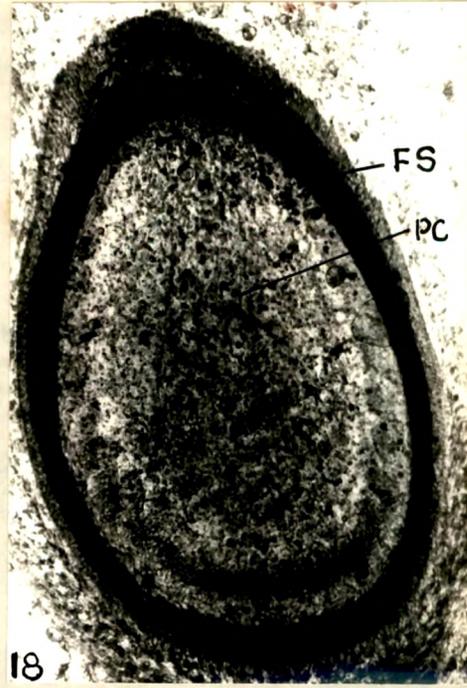
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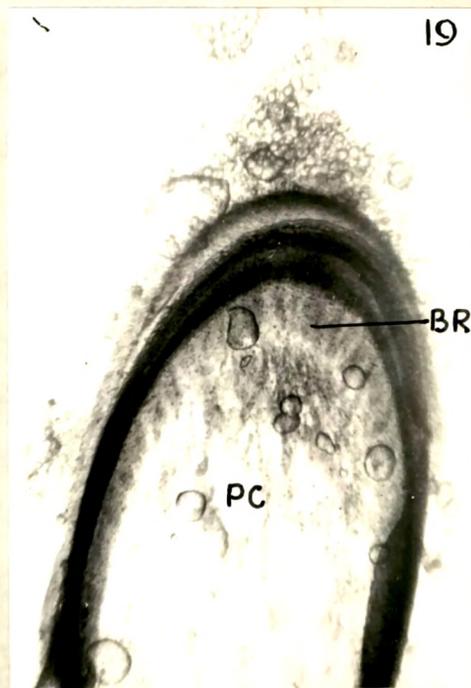
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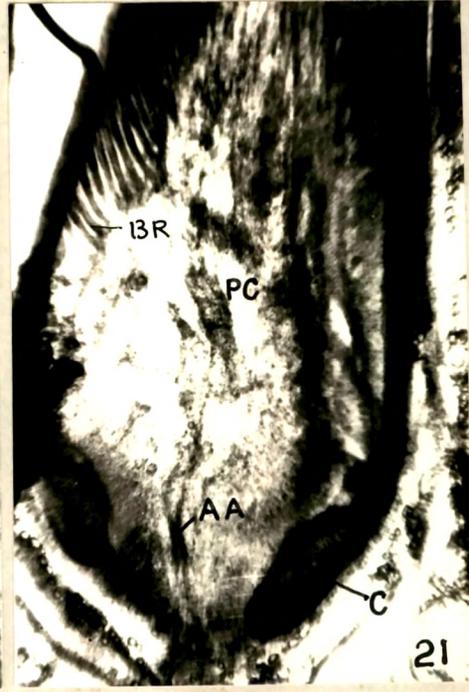
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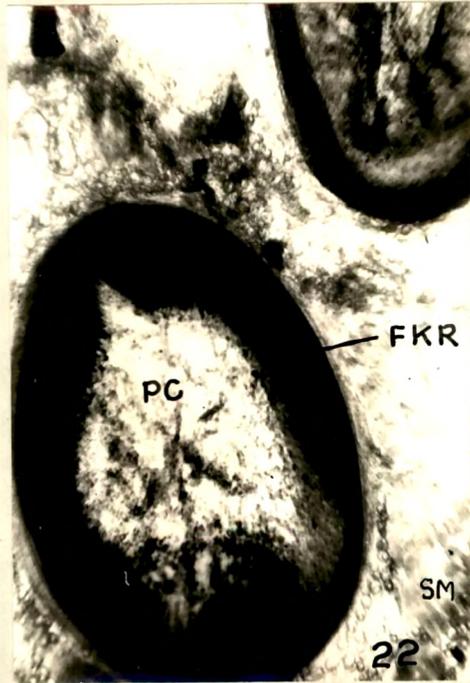
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Induced development: (Figs. 8 to 13)

15 hours after plucking, a sharp rise in the enzyme activity could be noticed in both the smooth muscles as well as the activated germ. An appreciably high level of enzyme activity could also be noted in the components of the feather follicle viz., the follicular epidermis, pulpa, pulp cells and epithelial cells covering the pulp. Blood vessels, fibrocytes and stratum germinativum of the non-feather epidermis were also appreciably enzyme reactive. This condition was soon reversed and by the 2nd day, the enzyme activity was very much reduced but to register an increase again to the same high level (observed 15 hours after plucking) on the 3rd day at which period however, there was a noticeable decrease in the enzyme concentration in the stratum germinativum of the non-feather epidermis and a slight decrease in the smooth muscle fibres. In the by now developing feather a fairly high activity of the enzyme was noticed in all the epithelial cells forming the barb ridges with the pulp cells next to them in the degree of enzyme reactivity. This condition was retained on the 5th day also, with the only difference that aldolase activity was slightly ^{higher} more in the cells of the basal parts of the barb ridges which are adjacent to the pulp than those which are away from it. In the epithelial and pulp cells, the peak activity of the enzyme was observed in the 10th day, though in the

smooth muscles and other components of the skin, aldolase registered a slight decrease. But, by about the 15th day the reactivity for the enzyme in the smooth muscles was found to be increased, while in the epithelial and pulp cells of the developing feather^x it tended to be slightly reduced, only to rise back to the high level on the 20th day. Thus on the 20th day all the components of the skin depicted a similar and equal level of aldolase activity as has been observed in the corresponding parts of the adult normal skin which was retained throughout the rest of the periods of feather development and thereafter.

Regeneration: (Figs. 14 - 22)

A sharp decline of enzyme activity in the various skin components was noticed 24 hours after plucking of the 10 day old inducedly developing feathers (a method adopted to induce regeneration). However, by the 3rd day after such plucking a considerably high aldolase activity could be noticed in the follicular wall, smooth muscle fibres and fibrocytes. Cells of the stratum germinativum of the non-feather epidermis registered the same moderate activity as seen in the normal skin. The first peak histochemical response for the enzyme was noticed in the components of the regenerating feather on the 5th day with the non-feather^x epidermis and dermis retaining the same moderate reactivity

observed earlier. Amongst the components of the regenerating feathers, highest activity of the enzyme was noticed in the epithelial cells of the barb and collar regions and the mesenchymal cells of the pulp in that order. Epithelial cells of the follicular wall almost equalled the epithelial cells of the developing feather in their enzyme concentration. At this stage, smooth muscle fibres also exhibited a peak enzyme concentration. This condition remained so upto the 7th day of regeneration after which the overall enzyme concentration of the skin was found to decline to a very low level by about the 10th day. This decline was most obvious in the components of the developing feather and the smooth muscles which were highly enzyme reactive in the previous stages. This phase of low aldolase activity was found to continue upto about the 14th day after which on 15th day, in the various components of the skin an increase in the enzyme activity appeared (reaching to the same level as was observed on the 7th day) which resulted in a second peak level of the activity of the enzyme. This increase was conspicuously marked in the pulp cells, the cells of the barb ridges, especially the basal ones in close proximity with the pulp, and the smooth muscles. Whereas the epithelial cells of the follicular wall registered a high enzyme reactivity, all the skin components of the non-feather regions showed only a moderate enzyme reaction. To a certain extent, this condition was maintained thereafter in almost all the

parts of the skin excepting for the barb ridges which showed a progressive reduction in the enzyme concentration relative to the degree of keratinization. Though the regression of pulp could be evidenced by the 25th day, its cells, however, retained their appreciably high enzyme activity though definitely lesser on a comparative basis than that observed on the 15th day. The same high enzyme concentration attained on the 15th day was maintained thereafter through the 30th day by which time the feather was fully regenerated.

DISCUSSION

The moderately high level of activity of aldolase, a key enzyme of the EMP pathway of glycolysis, noticed herein in the components of the developing feather, especially in the epithelial cells, from the 3rd day onwards until the 5th day after hatching, a period of peak HMP shunt operation (in the light of the study on G6PDH, chapter 2), is indicative of a close association of these two metabolic routes. Aldolase has been assigned a role in synthesising hexoses from trioses during the periods of high hexose monophosphate shunt activity (Gustafson, and Hasselberg, 1951). The presently observed appreciable activity of aldolase in the initial stages of post-natal development of definitive feathers makes it feasible to think that aldolase could be catalysing a hexose-forming

reaction during this period (3 to 5 days) of high HMP shunt activity. Since the reaction catalysed by aldolase is reversible, it could be easily visualised that hexoses are synthesised by condensation of trioses which are formed as a result of HMP shunt activity. Thus, through the mediation of aldolase, these products could be recycled so that a continuous supply of hexose phosphates is maintained and an optimal activity of HMP shunt is assured of. The significance of HMP shunt during the proliferative and differentiative phases of the developing feather is discussed in chapter 2. Such a function for aldolase seems to be quite in tune with the metabolic requirements of the developing feather at this stage, since HMP shunt is highly significant for all developing systems and its maximal operation depends on the availability of the hexose moieties. Further support for assuming such a role for aldolase could be ^{of its work} had from the works of Burt (1966) and Shah and Ramachandran (1972). In the other components of ^{the} skin, like the smooth muscles and fibrocytes, the enzyme activity could be interpreted along similar lines i.e., as aiding in hexose formation. The maturation of the skin involves the change from natal down feathers to adult or definitive feathers. It is noted that such a replacement of down feathers by definitive ones is accompanied by further growth and increase in the smooth muscles attached to the

follicles and also in the tensile strength of the skin, involving the elaboration of connective tissue fibres of the dermis. Thus the temporal relationship of attainment of functional differentiation of the feathers and smooth muscles of the follicles is reflected in the pattern of the enzyme activity of these two tissues. The functional differentiation of muscle also involves elaboration of tissue specific proteins. Enzymes are known to accumulate in the tissues long before the tissues become functional (Moog, 1965). Aldolase being an enzyme of the glycolytic pathway, the normal metabolic route in the muscle, its activity in an appreciable level, along with a high G6PDH activity (chapter 2) prior to functional differentiation is understandable. Fibrocytes involved in the elaboration of connective tissue fibres also exhibit an appreciable aldolase activity which also could be viewed in this light.

The peak histochemical response for the enzyme, aldolase, in the components of the developing feather by the 10th day after hatching when LDH activity also attained a peak level, and corresponding to a fall in the activity of G6PDH (chapters 4 and 2) is indicative of a predominantly anaerobic glycolytic metabolism at this time whence aldolase appears to catalyse the hexose splitting reaction. This

implies that aldolase has a dual function viz., hexose formation and hexose splitting. This contention is in agreement with that of Burt (1966) who postulated that aldolase has a hexose synthesising function in the early stages, while a catabolic function is discharged by the enzyme in the later periods of development of the chick spinal cord. Such a function of aldolase is more important at the stage of development (10th day) when energy demands are higher than during the other stages. The 10th day after hatching is a period marked by active growth of the feather as a whole, imposing increased energy demands which could be easily met with by tilting the balance of carbohydrate utilisation in favour of the EMP pathway of glycolysis as opposed to the HMP shunt. Significance of this pathway in growth processes of tissues has been recognised by study of the growth of the nerve fibre which is known to depend upon glycolysis for its energy requirements (Papaconstantinou, 1967). Thus it becomes evident that aldolase catalyses the hexose synthesising as well as hexose splitting reactions according to the physiological needs and requirements of the tissues concerned. Aldolase by its purported reversible reactions thus appears to play a pivotal role during feather development, bringing about either the forward or backward conversion between hexoses and trioses.

Further, apart from energy productions, aldolase could also be aiding in lipid synthesis through production of dihydroxyacetone phosphate which could be converted to α -glycerophosphate. The pioneering works of Kennedy (1954) have shown that lipid synthesis depends to a large extent on the availability of α -glycerophosphate, the most important precursor for triglycerides. By the 10th day, the high aldolase activity noted herein could be helpful in both the production of energy as well as ^{the} synthesis of lipids which are of importance not only in the laying down of ^{the} structural framework but also in the process of keratinization of ^{the} feather (Bell and ^hTathachari, 1963). Evidence of lipid utilisation at this time is obtained from the high levels of lipase activity at this stage (chapter 5). Concurrently, α GPDH also showed a considerable level of activity (chapter 4). Hence it could be surmised that at this stage, when the process of keratinization is in progress, an active lipid biosynthesis from carbohydrate intermediaries is also at work.

The period between the 15th and 20th days, whence aldolase activity remained at the same high level was also marked by a drop in LDH activity (chapter 4) and an increase to a second peak level by G6PDH (chapter 2). This could

imply that anaerobic glycolysis though active to a certain extent, becomes subordinate to the HMP shunt by about the 15th day after hatching and as such the function of aldolase appears to be tilted towards hexose formation. Such a shift in metabolic pattern towards ^{the} HMP shunt is not altogether surprising since differentiative processes like keratinization are in full swing at about this time and ^{as} this pathway is well implicated in such processes.

A constant level of aldolase activity during development, as has been observed in the developing definitive feathers has been reported in other developing systems also (Gustafson and Hasselberg, 1951; Burt, 1966). However, the differential activity of aldolase presently observed in the epithelial cells of the developing feathers deserves mention. It is significant that the high aldolase activity tended to show a gradual change from a high level in the basal layer of ^{the} epidermis which ^{is} proximal to the pulp to a negligible level in the apical cells which are distal to the pulp, with an intermediary level in the intervening cells. From this it could be postulated that even amongst the epithelial cells of the developing feathers, there could be differences in the metabolic pattern which are reflected in the activities of enzymes in these cells. The apical epidermal cells which are away from the pulp and are being keratinized may have a

prepondarent operation of ^{the} shunt pathway yielding NADPH_2 which may be useful in keratinization (Michael, 1965). The cells at the basal region of the barb ridges, as well as the epithelial cells of the collar region may have both glycolysis as well as HMP shunt pathways operating simultaneously providing ATP as well as NADPH_2 which are utilised for cell division. This finds support from similar observations of Michael (1965) on the activity of different dehydrogenases in the skin of the rhesus monkey. He found that the basal layers of the epidermis showed a higher activity of the glycolytic enzymes in comparison to the outer layers which are in the process of keratinization and showed NADP dependant dehydrogenases.

It has been suggested earlier that a differential availability of nutrients could initiate the differentiative activities. Michael (1965) correlated the differential distribution of various dehydrogenases in the epithelial cells of primate skin with their structural differences. He considers the higher activity of glycolytic enzymes in the lower layers of epidermis to be due to the proximity of blood capillaries and resultant availability of blood glucose which could be easily utilised through glycolysis. The higher activity of NADP linked enzymes at the site of ^{the} epidermis distant from the blood supply may be considered compensatory

for the reduced substrate level. Thus the increased activity of ^{the} shunt pathway in the epithelial cells of the developing feathers, away from the pulp, observed herein could be construed to be of utility in their differentiation, i.e., keratinization. Further evidence for the differential activity of enzyme can be had from the report of Novikova (1970) who observed that in the normal human skin the distribution of the various dehydrogenases varied in the different cells, reflecting the differences in intensity of biochemical processes in them depending on their functional state.

The high activity of aldolase and LDH along with a decline in G6PDH activity by the 25th day after hatching could be indicative of the establishment of anaerobic glycolysis as the chief metabolic route in these tissues since differentiative processes are almost complete by now. This contention appears to be in agreement with the generally accepted view that in the later stages of development, ^{the} pentose phosphate pathway becomes subordinate to the EMP pathway of glycolysis (Hermann and Tootle, 1964; Backstrom et al., 1960; Coffey et al., 1964). Such a change in the metabolic reactions at this stage of development when the feathers have attained their functional competence gains validity from the reports of Hermann and Tootle (1964) that increased catabolism

is a characteristic feature in many tissues during their functional maturation. The retention of high aldolase activity by the pulp cells even on the 25th day whence the regression of the mesenchymal pulp has commenced becomes interesting in the context that in the regressing tail of anurans, Eckhout (1965) has observed a decrease in aldolase activity during metamorphosis. The difference in the pattern of the enzyme activity noticed in these two instances might possibly lie in differences in the mode of cell death in these two cases. In this wake, the significance of the present observation of a high aldolase activity in the pulp even when it is in a process of regression remains enigmatic and difficult to assess at this juncture.

In the case of induced development of feathers the general pattern of enzyme activity during the various stages is very much similar to that observed during normal development. The sharp rise in aldolase activity, especially in the smooth muscles, to a peak level almost 15 hours after plucking could be attributed to the functioning of these muscles aiding in the shivering response to plucking. Apart from this, it may also be presumed that the increased rate of metabolism in the tissues of the skin soon after plucking could help considerably in the compensation of heat loss

from the exposed part of the skin. Further, as the resting germ is now activated to develop (as a result of the plucking of the feather), an increased rate of metabolism necessary for supporting cellular proliferation and the associated synthetic processes can well be assumed. However, the fall in enzyme activity noticed on the 2nd day remained enigmatic. Appreciable enzyme activity which was maintained from 3rd upto the 7th day of induced feather development concomitant with a high G6PDH activity (chapter 2) could be considered important for the continuous cyclic operation of the HMP shunt pathway. From the 7th day onwards the steadily increasing aldolase response coupled with a concomitant steady decline in G6PDH (chapter 2) could be suggestive of a preponderance of EMP pathway during this phase. The stepped up activity of aldolase denoting a high incidence of glycolysis appears to be more in tune with this phase of development as it is an active growth phase marked by the emergence of the growing feather stubs outside the follicle and above the skin surface. Considering that G6PDH is an important locus for the control of HMP shunt pathway (^{Brogles &}Strittmatter, 1973) and that in developing vertebrate tissues EMP pathway attains predominance over the HMP shunt in the later period, it seems feasible to think that at this phase of developing feathers carbohydrate metabolism is regulated in such a way that more of the

substrate ~~is~~ utilised for energy production rather than for the formation of the cofactors needed for synthetic activity. The increasing titre of aldolase activity could also be aiding in production of α glycerophosphate, an intermediary of very ^{high} ^{importance} much important for the synthesis of lipid molecules. Simultaneous peak activities of aldolase and G6PDH (10th day onwards) indicating optimal operation of HMP shunt could provide cofactors required in lipid synthesis. Thus the constant level of aldolase activity, after the 10th day of induced feather development till the complete formation of a new feather, resembles that observed during the process of normal development.

During regeneration, unlike other two developmental processes (i.e., normal and induced) aldolase was found to register two distinct periods of peak activity. The very low level of enzyme reactivity registered by all the components of the skin one day after inducing regeneration, could be related with the shock response of the skin as a result of the infliction of injury to the developing feather. However, the gradual rise in the enzyme concentration noted by the 3rd day could be regarded as a part of the metabolic adaptations for the wound healing processes. The first peak of aldolase activity during regeneration registered on the

5th day, a stage much earlier than that observed during normal and induced development, is probably indicative of an accelerated commissioning of the metabolic route in order to hasten the process of feather restoration. Due to the physiological importance of the feather in thermoregulation, the need for restoring these epidermal structures at a faster pace becomes important for the bird, and especially so during regeneration. Nevertheless, the fall in aldolase activity from 10th to the 14th day of regeneration denoted by a period of active growth could be explained in the light of simultaneous high cGPDH (chapter 4) activity indicating a period of lipid utilisation. Lipids could be considered as a more suitable source of energy during such processes when structural and functional restoration is progressing at a faster rate as during the process of feather regeneration (in the light of the study of lipids and lipase - chapter 5).

However, the second peak of aldolase activity registered by the components of the regenerating feathers from the 15th day and which remained constant thereafter resembles that found during the other two conditions of development of definitive feathers reported earlier.