# CHAPTER 2

## HISTOCHEMICAL OBSERVATIONS ON THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE IN THE DEFINITIVE FEATHERS OF THE BLUE ROCK PIGEON, COLUMBA LIVIA, DEVELOPING UNDER THREE DIFFERENT CONDITIONS

The increasing evidence in favour of a high dependancy of proliferating and differentiating cells upon the hexose monophosphate (HMP shunt) pathway of carbohydrate utilisation and the fact that during the early developmental stages of vertebrate tissues, this pathway is the major metabolic route identifiable (Papaconstantinou, 1967) are suggestive of the pivotal role played by HMP shunt during morphogenesis. The production of ribose sugar for nucleic acid synthesis, generation of $NADPH_2$, a cofactor extensively implicated in a wide variety of synthetic reactions in cells (Langdon, 1957; Cohen, 1959) and providing a means for interrelation of the metabolism of hexoses, pentoses and trioses are the chief functions of this pathway which would aid in the proliferative and differentiative activities of the tissues concerned. $NADPH_2$ generated by the activity of HMP shunt plays a unique role in

lipogenesis (Siperstein, 1958), nucleic acid synthesis (Beaconsfield & Capri, 1964a; Beaconsfield & Reading, 1964b) and synthesis of some amino acids (White, Handler and Smith, 1954). The existence of HMP shunt activity in the vertebrate skeletal muscle has been established by Hoskin (1959) and Rossi et al. (1963). The activity of G6PDH, which diverts glucose-6-phosphate towards HMP shunt from the Embden Meyerhoff pathway of glycolysis, is considered to be an indicator of the operation of this metabolic route in tissues. Histochemical demonstration of this enzyme has been reported in a number of tissues and organs such as, avian skeletal muscle (George and Nene, 1966); fish skeletal muscle (Bokdawala, 1965); choroid plexus of vertebrates (Shanta and Manocha, 1968); mammary gland (Abraham and Chaikoff, 1959); mammalian adrenal gland (Hopwood, 1968); corneal epithelium of mammals (Ehlers, 1970); developing teeth (Fullmer, 1963); regenerating limbs of adult newts, Triturus viridescence (Wolfe and Cohen, 1963) and Diemictylus viridescens (Schmidt and Weidman, 1964); normal and regenerating tail of the lizards, Hemidactylus flaviviridis (Magon, 1970) and Mabuya carinata (Shah and Ramchandran, 1973); developing avian nervous system (Burt, 1965, 1966); the

significance of which has been discussed in relation to the physiological processes of the tissues concerned. However, apart from the observations of Magon (1970), Shah and Ramchandran (1973) and Wolfe and Cohen (1963) on the epidermal reactivity of G6PDH in the regenerating appendages, there is a general lack of relevant literature on the role of this enzyme in the developing epidermal derivatives of vertebrates. Hence, keeping in view the multifarious implications of HMP shunt activity in the various metabolic activities and the importance of G6PDH in the regulation and control of the shunt pathway (Mckerns, 1966; Anstall and Trugillo, 1967; Criss and Mckerns, 1968), it was deemed worthwhile to investigate this enzyme during the different stages of definitive feather development in pigeon under the three conditions mentioned earlier.

## OBSERVATIONS

Normal adult skin: (Fig. 1)

In the resting feather germ, an appreciably high concentration of the enzyme was observable, especially in the cells of stratum germinativum and those in the upper region of the pulp. Amongst them, the epidermal

cells of the collar region were the highest in enzyme reactivity. The papilla was comparatively less enzyme reactive than the other components of the feather germ. Smooth muscles of the feather follicles were the next in order of enzyme reactivity. In the non-feather region of the skin, the epidermis was rather poor in enzyme concentration. The adipose tissue cells did not register any appreciable histochemical response for this enzyme.

Normal post-hatching development: (Figs. 2-9)

Different concentrations of the enzyme were noticed in the various tissues of the skin on the day of hatching. Stratum germinativum from the non-feather region was found to show maximum enzyme activity while the outer keratinised layers were almost enzyme negative. The follicular epidermis had registered an appreciable response for the enzyme. The cells of the pulp and epidermis of the developing definitive feathers, though showing appreciable response for the enzyme, was nevertheless noticeably lower than that shown by the epidermis of the follicular wall. In the dermis, fibrocytes, adipose tissue cells as well as the smooth muscles were all moderately enzyme reactive. Excepting for the adipocytes, fibrocytes and smooth muscles, all the other components of the skin registered a gradual

increase of the enzyme activity thereafter and attained the first peak level on the 3rd day which was so retained uptil the 5th day especially well marked in the pulp cells, epithelial cells of the collar region and the barb ridges. However, at this stage, the epidermis of the non-feather areas showed a slightly reduced level of the enzyme concentration. By about the 10th day, a decline in the G6PDH activity from the level observed on the 5th day could be observed in the skin, especially in the epidermis of non-feather regions, adipose tissue, fibrocytes and smooth muscles of the dermis. However, in the developing feather, the declined enzyme activity was evident only in the pulp cells, with the epithelia of the collar region and the barb ridges remaining as highly enzyme reactive as in the previous stage and with the papilla depicting no noticeable change. This declined activity of the enzyme in the skin lasted only for a short while, and soon registered an upward trend in the next three days to culminate in a second peak of enzyme response in all the components of the skin, especially in the pulp and barb ridges by about the 15th day post-hatching. This peak enzyme activity remained so until the 20th day, after which there was a gradual decline reaching ultimately on the 30th day a level characteristic of the resting germ in a fully grown adult feather.

## EXPLANATIONS FOR FIGURES

Fig. 1 LS of a resting feather germ. Note appreciable activity of the enzyme in collar, pulp cells, and smooth muscles.

## NORMAL DEVELOPMENT

Fig. 2 Section of skin of one day old pigeon with developing definitive feather showing enzyme activity.

Fig. 3 Enlarged portion of Fig. 2 showing enzyme activity in feather follicle.

Fig. 4 Definitive feather on the 3rd day of post-hatching development exhibiting G6PDH activity.

Fig. 5 LS of definitive feather on the 5th day post-hatching. Note enzyme activity in the barb ridges.

Fig. 6 LS of the feather on 10th day showing enzyme activity.

Fig. 7 LS of 15 day old feather showing attainment of peak activity. Note the differentiating barb ridges.

Fig. 8 Oblique section of feather on 20th day after hatching. Note the enzyme activity in regressing pulp.

Fig. 9 Oblique section passing through base of the 30 day old feather showing enzyme activity in the cells of the germ for next generation which is being laid.

INDUCED DEVELOPMENT

Fig. 10 LS of follicle 15 hours after plucking adult feathers. Note increased enzyme activity in the epithelial and pulp cells of the germ.

Fig. 11 LS of developing germ on the second day depicting high G6PDH activity.

Fig. 12 Section of inducedly developing feather on the 5th day showing peak enzyme response in barb ridges and pulp.

Fig. 13 LS of feather on 7th day showing the enzyme activity.

Fig. 14 Section of a 10 day old feather showing enzyme activity.

Fig. 15 LS of 15 day old feather exhibiting peak activity in epithelial and pulp cells.

Fig. 16 Section of feather on the 20th day of induced development, depicting high G6PDH activity.

Fig. 17 Oblique section of a 25 day old feather showing declined enzyme activity in the regressing pulp.

Fig. 18 Section passing through the base of a feather on the 30th day of induced development showing the enzyme reactivity in the pulp and epithelial cells of the germ for next generation.

REGENERATION

Fig. 19 LS of a healed feather on the 3rd day of regeneration exhibiting a considerable enzyme activity in the pulp and epithelial cells.

Fig. 20 LS of a 5 day old regenerate pushing out the blood clot and cellular debris. Note the peak enzyme activity in pulp and epithelial cells.

Fig. 21 Section of skin showing G6PDH activity in smooth muscles and TS of regenerating feathers, on the 7th day.

Fig. 22 Magnified picture of TS of a 10 day old feather regenerate showing high enzyme activity in barb ridges and pulp cells.

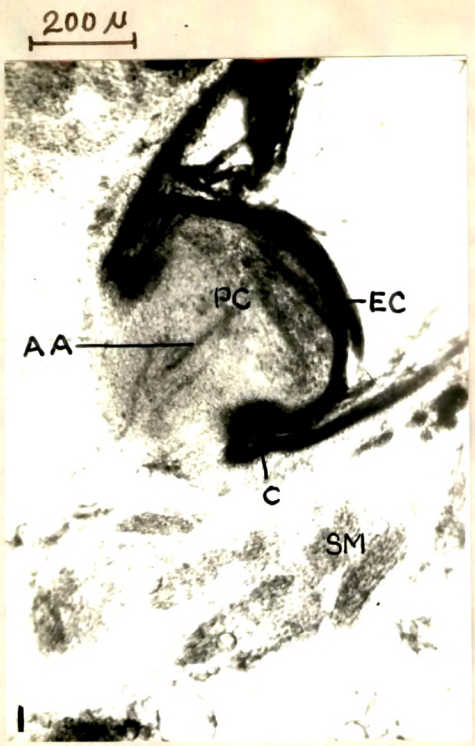
Fig. 23 Oblique section of regenerate on the 15th day showing high enzyme reactivity in parts of barb ridges.

Fig. 24 Section of a 25 day old regenerate showing a reduced level of G6PDH activity.

ABBREVIATIONS

AA - Axial artery
AT - Adipose tissue
BC - Blood clot
BR - Barb ridges
C - Collar
DP-Dermal papilla
EC - Epithelial covering
DDF - Developing definitive feather
FF - Feather follicle
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 muscle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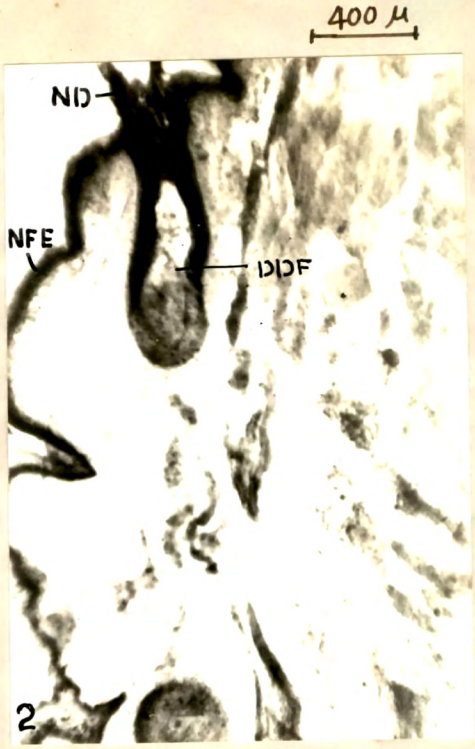
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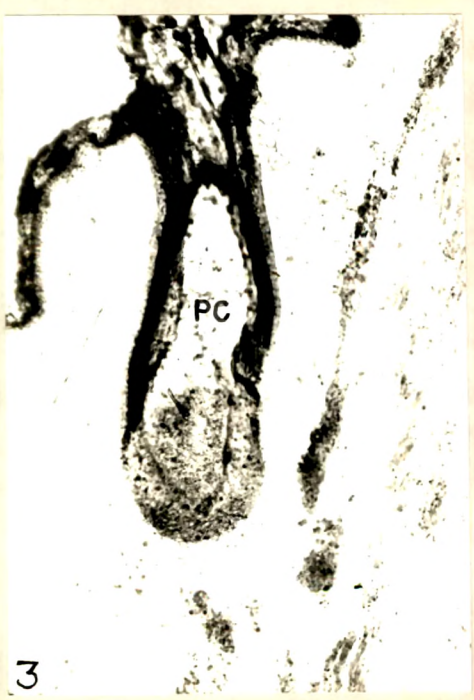
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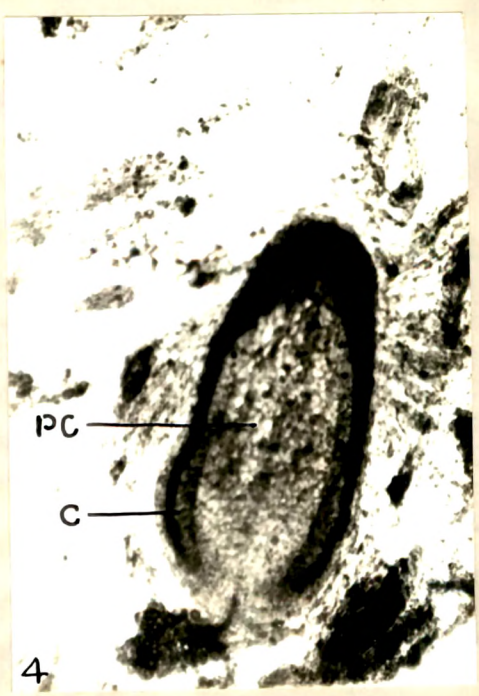
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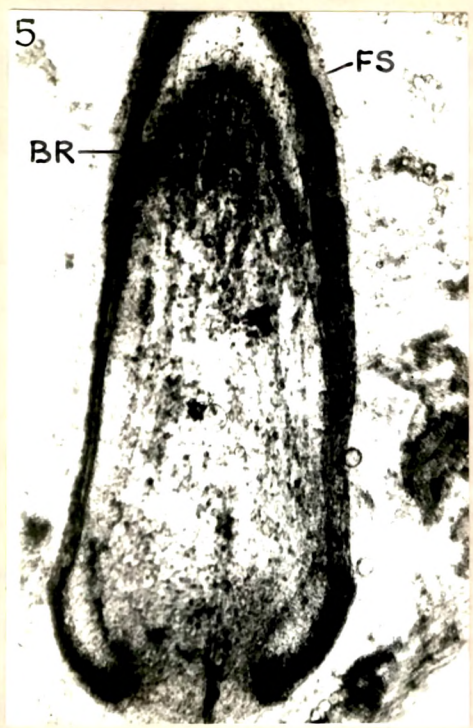


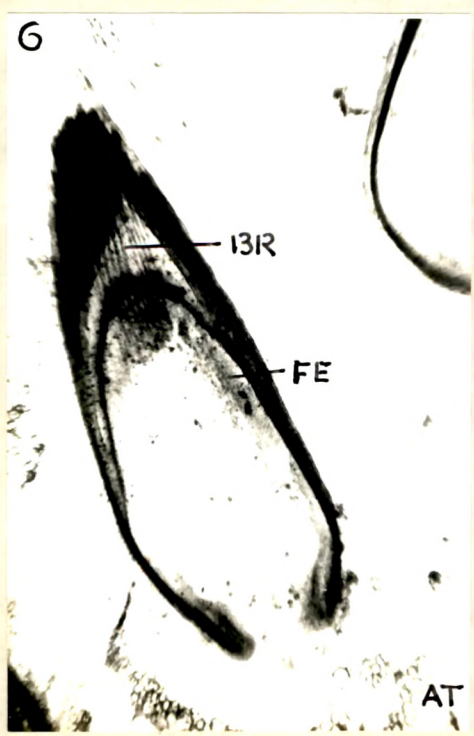


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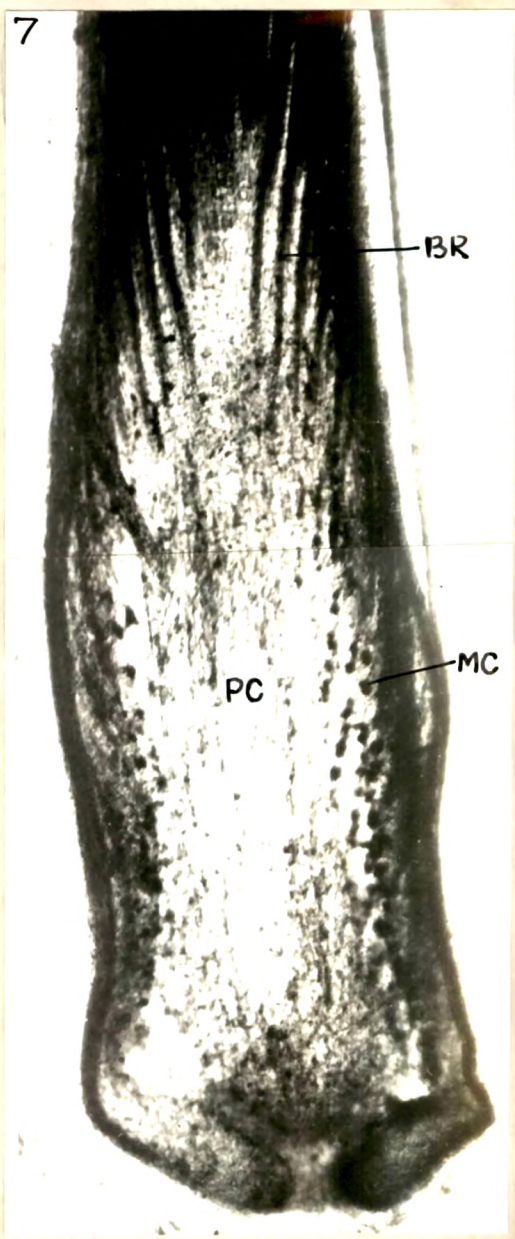


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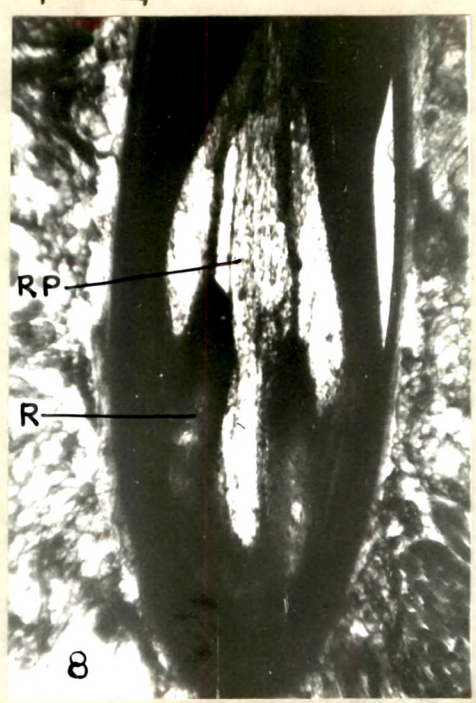


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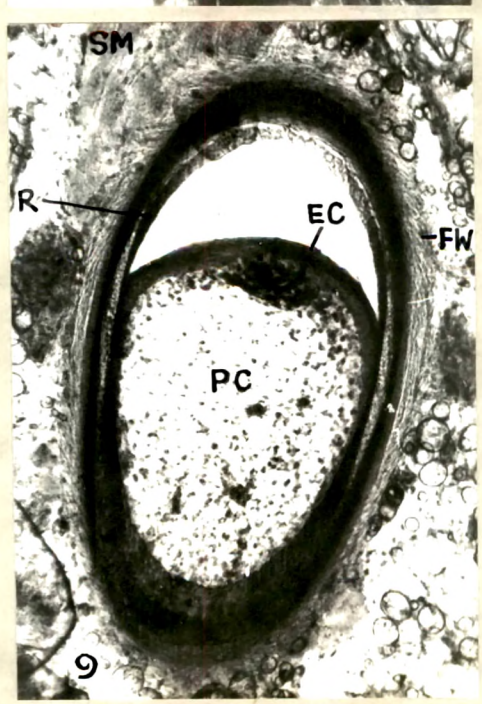


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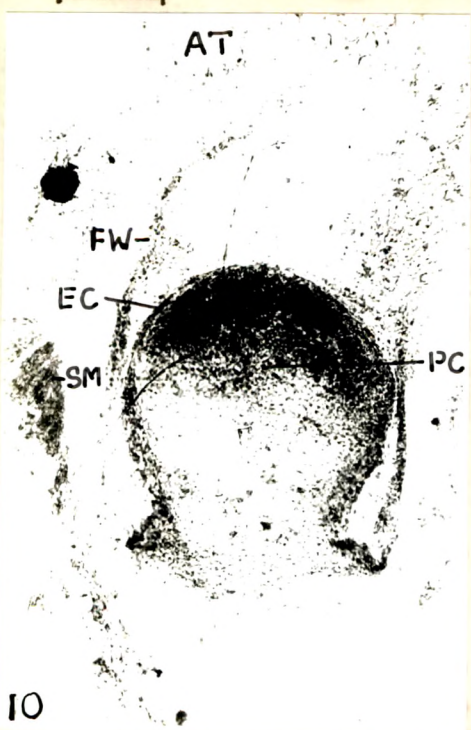


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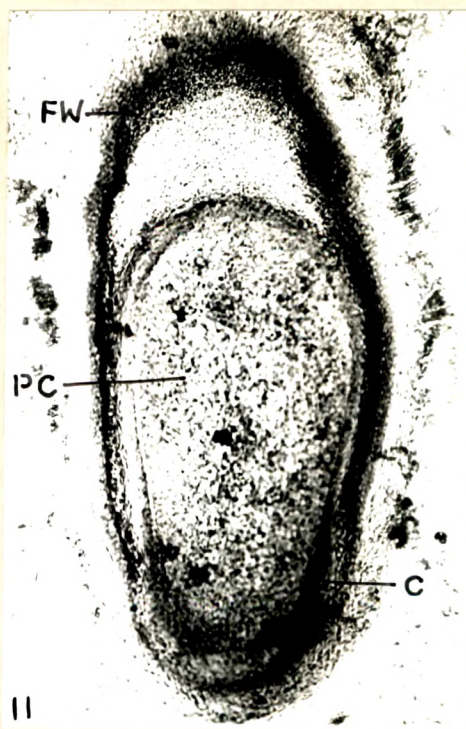


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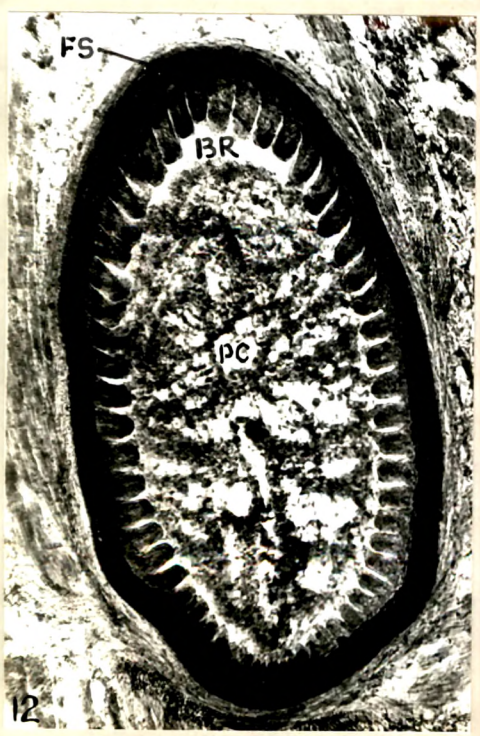


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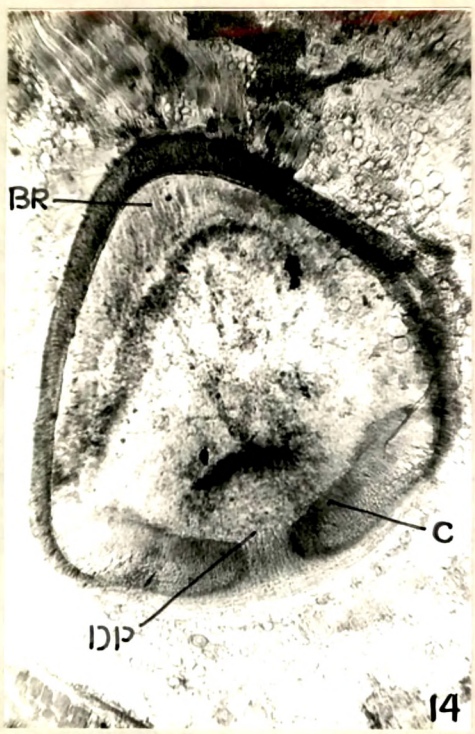


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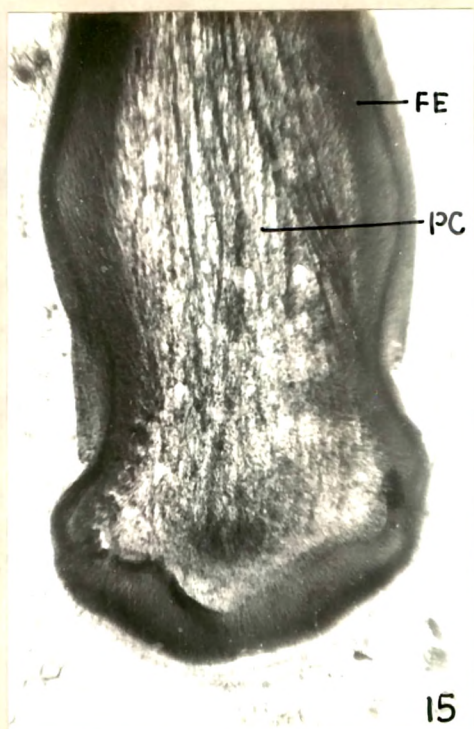


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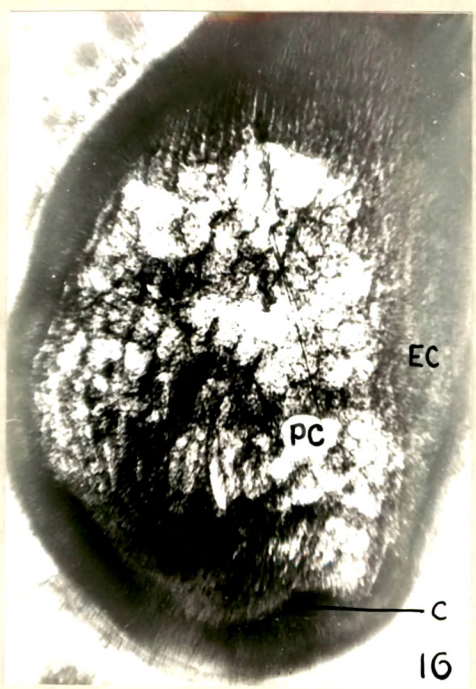


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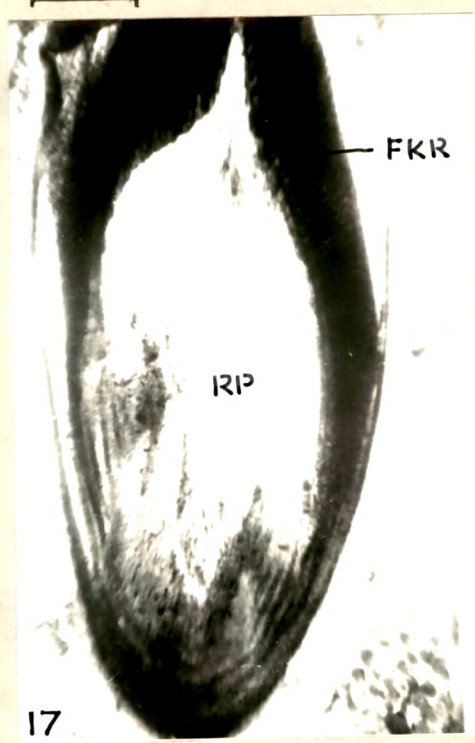


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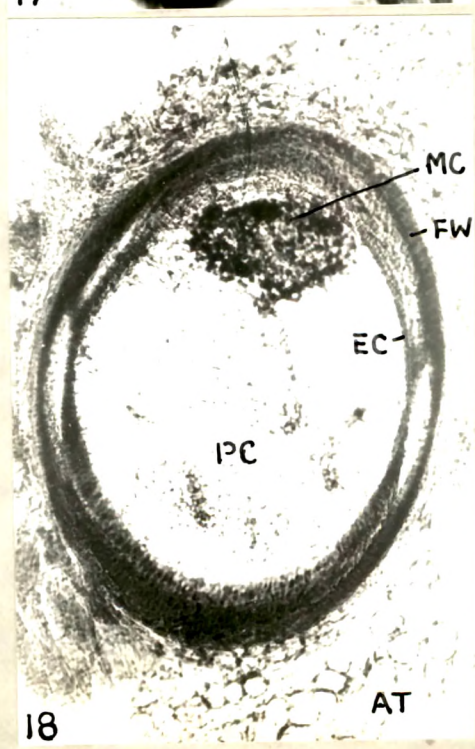


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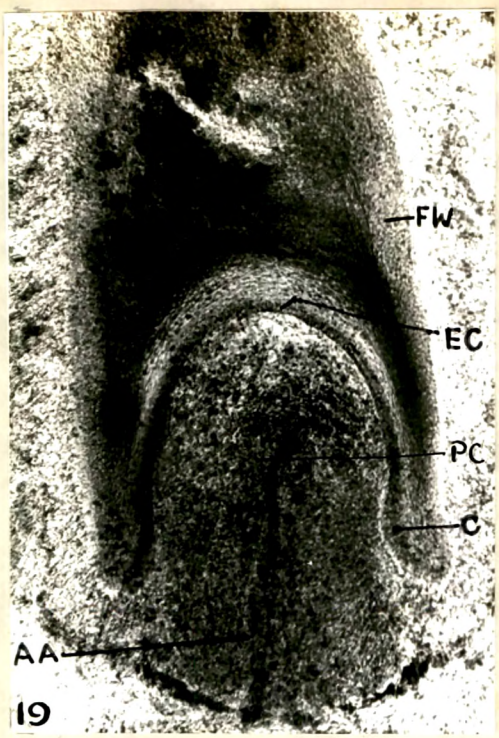


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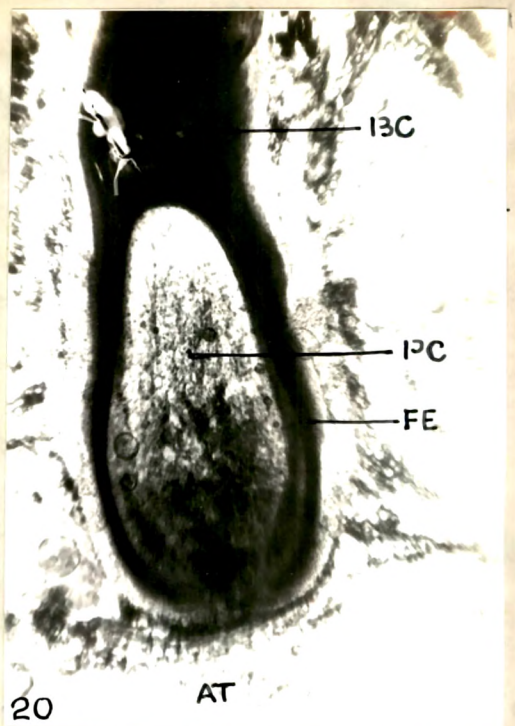


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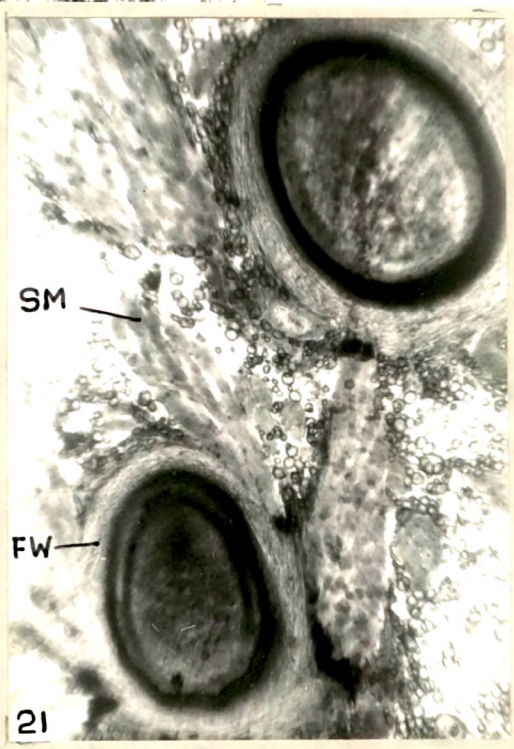


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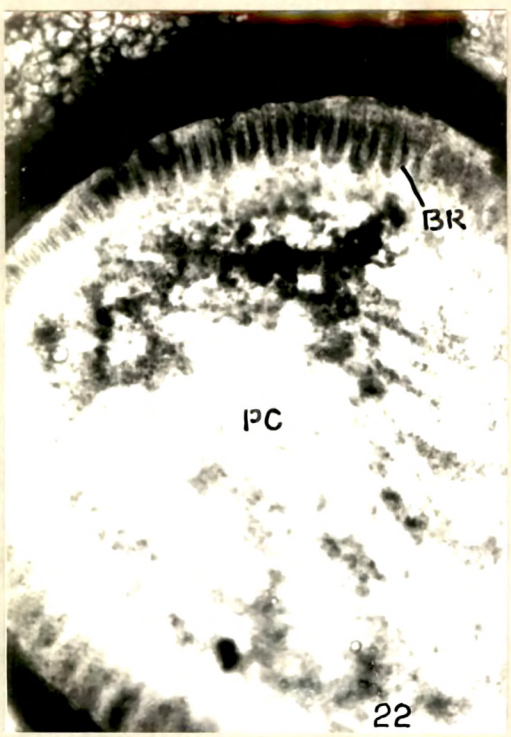


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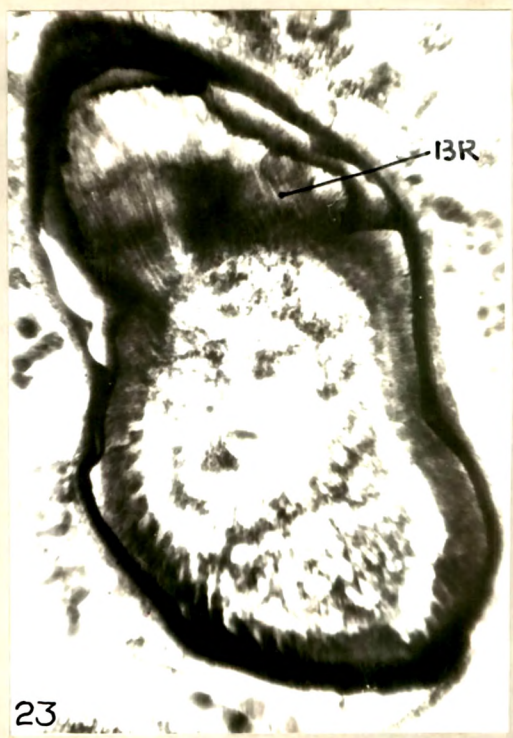


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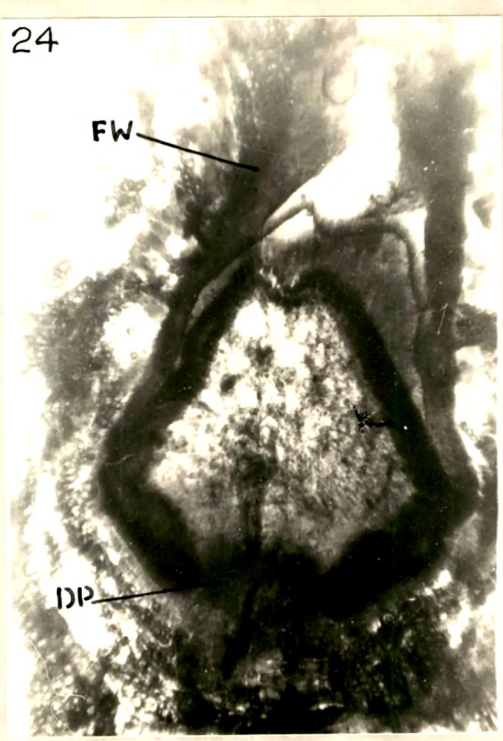


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Induced Development: (Figs. 10–18)

Fifteen hours after plucking the adult feather a considerable increase in G6PDH concentration in the germ was observed. This increase was all the more evident in the epidermal cells and the pulp cells subjacent to them. Epidermis of the neighbouring non-feather regions and the fibrocytes, smooth muscles and adipose tissue of the epidermis also registered an increased enzyme activity which was about two fold of that observed in the corresponding tissues of the normal adult pigeons. This gradual increase culminated in a peak enzyme response in the, by now, developing germ on the 2nd day and continued to remain at such a peak uptill the 7th day. However, the enzyme response in all the other component tissues of the skin fell to a level found in the corresponding tissues of the normal adult skin by about the 10th day of the induced development. This declined G6PDH activity was, however, short lived and soon increased to reach a second peak level by the 14th day and that lasted upto the 20th day after which, a gradual decline resulted in the enzyme activity reaching by the 35th day of inducing feather development (by which time the feather had fully developed) a condition almost similar to that observed in the resting germ and other components of the normal adult skin.

Regeneration: (Figs.19-24)

Twentyfour hours after plucking the inducedly developing feather a slight decline in the enzyme reactivity was noticed in the injured regions. Nevertheless, smooth muscles and fibrocytes of the dermis registered relatively high enzyme concentration. Epidermis of the non-feather regions and the adipose tissue were also appreciably enzyme reactive. By the 3rd day, the pulp cells and the epithelial cells of the collar region of the feathers which have healed their wound, showed a considerable increase in G6PDH activity, reaching a level almost double to that found in them on the first day. However, no noticeable change was observed in the ~~pulpa~~ papilla but a reduced enzyme reactivity in the other components of the skin, including the epidermis of the non-feather regions was observed at this stage. The increased enzyme concentration in the pulp and epithelial cells reached the first high peak level of activity by the 5th day. However, the epidermis in the non-feather regions and dermal components retained the same low level of the enzyme activity noticed in the previous stage. Among the epithelial cells of the developing feather, those of the collar region and the barb ridges showed

maximum G6PDH activity. This elevated level of enzyme activity continued to remain so uptill the 10th day of regeneration, only to show a drop in the epithelial cells and the pulp cells in that order, by about the 12th day. An overall decline in the enzyme reactivity was also noticed in the various components of skin at this stage. This decline was followed by a gradual increase in the enzyme activity in all the component tissues to reach ultimately the second peak level in the pulp and epithelial cells by the 20th day. Thereafter, a gradual decrease of enzyme concentration reached a level characteristic of the resting germ and other components of the normal skin by the 25th day and remained so with the completion of the process of feather regeneration.

## DISCUSSION

At the very outset, it may be noted that a considerably high level of G6PDH activity was clearly discernible in the various components of the skin soon after hatching. It is well known that the operation of HMP shunt during early stages of development of various vertebrate tissues is indicated by the presence of this enzyme in the tissues concerned. The period after hatching is marked by a dynamic

state of development and at the epidermal region, the development of the first generation of definitive feathers denotes the beginning of the maturation processes of the integument. This state of development marked by a high rate of cellular proliferation and differentiation can well be translated in terms of structural as well as physiological demands calling for increased energy yield and production of important cofactors respectively. These demands appear to be satisfactorily fulfilled by the observed first high peak level activity of G6PDH in the developing feathers (post-natal) which in all probability is an indication of an active operation of the HMP shunt pathway yielding ribose sugars much needed for the synthesis of nucleosides, nucleotides and nucleic acids. Apart from this, during this hectic phase of cellular proliferation and differentiation, the $NADPH_2$ generated by the activity of the shunt pathway could also be useful in the synthesis of nucleic acids and lipids. Significance of HMP shunt in the processes associated with cellular proliferation has been stressed by Burt and Wenger (1961); Newburgh *et al.* (1962) and Moore and Hurlburt (1962). During the initial phases of development of the definitive feathers, the active proliferation of epithelial cells would require an increased

turnover of nucleic acids and hence the participation of an active HMP shunt pathway for the supply of ribose sugars and $NADPH_2$ is understandable. Brachet (1950) had suggested that one of the important pathways of DNA synthesis is from RNA, and Cohen (1961) found that $NADPH_2$ is required for the reduction of ribose to deoxyribose. Reichard (1958, 1960 & 1961) demonstrated the existence of an $NADPH_2$ dependant mechanism for the direct conversion of ribonucleotides to deoxyribonucleotides, in chick embryos. Moore and Hurlburt (1962) also found a similar pattern of deoxyribonucleotide synthesis in rat ascites tumour cells and suggested that this mechanism is associated with rapid cell proliferation. All these factors when taken together, lend credulance to the assumption of an active association between G6PDH and cellular proliferation in developing systems. However, in the feather development, proliferation is accompanied by differentiative activities and clear cut signs of differentiation in the form of barb ridge formation could be evidenced as early as the 5th day after hatching, even before the emergence of the definitive feathers from the follicles. A general concept about metabolic regulation is that the HMP shunt activity decreases during periods of differentiation. This has been reported by Newburgh et al.

(1960 & 1962) in chick nervous system and by Burt (1965) in developing chick spinal cord. This pattern is known to hold good for retinal cells also (Moog, 1965). Whereas it might be said to be true in all the above mentioned cases, where differentiative activities are initiated only after cellular proliferation is complete, in those cases where proliferation and differentiation occur simultaneously, Balinsky (1960) has opined the possibility that differentiation could be initiated by a differential partitioning of the nutrients. The developing feather might be included in this category as the epithelial cells which are differentiated (as barb ridges) and getting pushed further and further away from the pulp separated from it by the underlying epithelial cells could be expected to have a nutritional supply lesser than those close to the pulp and which have just begun to differentiate. At the same time, the proliferative and differentiative activities require the cofactors produced by the HMP shunt activity and hence, the presently observed G6PDH activity during such a phase where growth and differentiation occur side by side, is self-explanatory. Further evidence for correlating an active shunt pathway with cellular differentiation could be obtained from the works of Burt and Wenger (1961) who suggested that increased activity of this

pathway is needed to meet the demands for $NADPH_2$ and RNA pentose essential for synthesis of new proteins for the differentiating cells. Moreover, as the differentiation of the feather (keratinization) is shown to require RNA (Hamilton, 1965) an involvement of the shunt pathway in the differentiative activities of the developing feather, however indirect through RNA synthesis, cannot be overlooked. A close correspondence between enhanced G6PDH activity and periods of predominant RNA synthesis has been reported by Backstrom (1959) during the development of the sea urchin. Another instance of a high activity of the enzyme corresponding to organogenesis and differentiation could be had from the works of Broyles and Strittmatter (1973) who observed an increase in its activity in various organs of the frog after the tadpole stage. Apart from this, the production of $NADPH_2$ which is an essential cofactor for lipogenesis could also be considered to be of importance, as lipids are found to increase in concentration in the pulp after hatching (Chapter 5). Lipids, apart from being metabolites of considerable significance, are also known to act as precursor substance for keratin (Bell and Thathachari, 1963). It is interesting in this light to consider the possibility that during feather differentiation lipids might be forming the

framework around which the more highly ordered forms of keratin are assembled, as envisaged by Bell and Thathachari (1963). Taking into consideration all the facts mentioned above, the peak histochemical reactivity found for the enzyme in the developing definitive feathers undergoing growth and differentiation simultaneously, thus appears understandable.

However, it is interesting at this stage to consider the slight decline in activity of G6PDH observable by the 10th day after hatching, in the pulp of the developing feather and other components of the skin. During this period characterised by active growth of the feathers as a whole, it could be assumed that the growth processes, for a while, assume priority over differentiative activities, thereby increasing the energy demands of the developing feather which could be most effectively met with by the operation of other energy yielding metabolic routes. A high incidence of aldolase together with peak LDH activity noticed at this stage (Chapters 3 & 4) are rather suggestive of an increased rate of anaerobic glycolysis known to be a significant source of energy in vertebrate tissues. Such a shift in the metabolic pattern in favour of anaerobic glycolysis would entail an increased channeling of carbohydrate

intermediaries through the EMP pathway, thus rendering a declined rate of operation and significant participation of the HMP shunt. The high LDH activity could be looked upon as a part of the metabolic regulatory mechanisms, whereby, the conversion of pyruvate to lactate, regenerating enough NAD would ensure the continued optimal activity of the EMP pathway. However, the epithelial cells of the collar region and the barb ridges, which are in a state of rapid proliferation appear still to depend upon the shunt pathway as could be inferred by the observation of a high incidence of G6PDH in them, even at a stage when the enzyme concentration declined in other cellular components of the developing feather.

The second peak activity of G6PDH reached by the 13th day after hatching which ended the short lived phase of the declined HMP shunt activity and which lasted upto the 20th day corresponded to an active phase of differentiation (keratinization) and growth. A high aldolase activity (chapter 3) and the presently observed peak G6PDH activity tend to indicate that both glycolysis and HMP pathways are operating on a well balanced basis providing energy ~~for~~ and cofactors respectively for the processes: proliferation, differentiation and growth. Such a balanced activity of these two important metabolic

routes would very much be in accordance with the mode of development of the feather during the later half (from 15th to 25th day) when both growth and differentiative processes are almost equally important.

The gradual decline in G6PDH reactivity observed in the regressing pulp and the epithelial cells surrounding it, from the 25th day onwards, till the 30th day, of a level characteristic of the normal resting germ is probably indicative of the completion of the process of development of the first generation of definitive feathers.

During the course of induced development of the feather, the enzyme reactivity followed a somewhat similar pattern of fluctuations. The peak reactivity of G6PDH prior to the emergence of feather outside the follicle is similar to that noticed during the initial phases of normal development of the feather. The second peak response for the enzyme noted during periods of differentiation also coincided with a similar level of enzyme activity during the corresponding stages of normal development.

During regeneration, however, three peak periods of enzyme activity were observed unlike in the other two modes of development mentioned above. The initial increase

reaching a peak level by the 2nd day of regeneration lasting uptill the 5th day, could well be for aiding in the proliferative activities of the epidermal cells during the process of wound healing and subsequent regenerative growth of the healed feather (blastema). The slight decline in G6PDH activity on the 7th day of regeneration, corresponded with an increased phase of aldolase activity (chapter 3) and could well be envisaged as indicative of the predominance of glycolytic activity over that of the HMP shunt, thus satisfying the increased energy demands of this active growth phase.

The second peak response for the enzyme registered by the regenerating feather on the 10th day was shortlived and thus could be considered as providing the necessary cofactors to be built up as reserves for the next phase of development. The decline shown thereafter and the enzyme activity which remained low uptill the 15th day could be correlated with the growth phase of the feather, whence most of the substrates are probably utilised via the EMP pathway for the production of energy. The third peak period corresponded well with a phase of growth and differentiation between 15th and 20th days.

G6PDH is already known to be a site for the control of the shunt pathway (Mckerns, 1966; Anstall & Trujillo, 1967; Criss & Mckerns, 1968). The presently observed fluctuations in the activity of G6PDH noted during the different phases of feather development, when considered in the light of the known potential importance of the shunt pathway in organogenesis and the fact that G6PDH is a controlling site of this pathway clearly indicate that this enzyme is involved in the metabolic regulations underlying the process of definitive feather development, diverting the carbohydrate intermediaries toward the shunt pathway when the demands for cofactors are increased, and stepping down its activity when energy demands take priority, thus helping in channeling most of the substrates towards the EMP pathway.