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RELATIVE ROLE OF VITELLINE CELLS AND MEHLIS' GLAND IN THE FORMATION OF EGG-SHELL IN TREMATODES *

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ABSTRACT

The tanning precursors of the egg-shell of trematodes are contributed by vitelline cells. These precursors are localized in the shell globules, in a masked condition. The phenol exists as "Dopa" and is masked by sulphated acid mucopolysaccharide, and remains in an unoxidizable state. The enzyme exists as prophenolase and is substrate specific, oxidizing only deaminated and decarboxylated phenol; this constitutes a 'built-in' inhibition system preventing premature tanning *in situ*. As the vitelline cells carrying these precursors reach the oötype surrounded by Mehlis' gland, this 'built-in' inhibition system is released, leading to a sequence of events resulting in sclerotization of the egg shell. The releasor mechanism of this 'built-in' inhibition system lies in the Mehlis' gland secretion. The phospholipid present in the secretion of Mehlis' gland seems to trigger the above mentioned processes and govern the mechanism of tanning. It is also suggested that Mehlis' gland secretion may contribute to the formation of ground substance of the egg-shell.

RESUMEN

Los precursores de la induración de la cáscara del huevo de los tremátodos son proporcionados por las células vitelinas. Dichos precursores están localizados en los glóbulos de la cáscara de un modo enmascarado. El fenol existe en forma de "Dopa" y está enmascarado por un mucopolisacárido de ácido sulfatado, y permanece en estado inoxidable. La enzima existe como profenolasa y es específica para el substrato; oxida sólo a los fenoles desaminados y descarboxilados y constituye un sistema inhibitorio preparado ahí ("built-in") que evita el endurecimiento prematuro *in situ*. Cuando las células vitelinas portadoras de esos precursores llegan al ootipo rodeado por la glándula de Mehlis, este sistema inhibitorio es liberado y conduce a una serie de resultados que se manifiestan en la esclerosis de la cáscara del huevo. El mecanismo liberador de este sistema inhibitorio radica en la secreción de la glándula de Mehlis. El fosfolípido presente en dicha secreción parece desatar los procesos arriba mencionados y regir el mecanismo de la induración. Se sugiere también que la secreción de la glándula de Mehlis pueda contribuir a la formación de la substancia fundamental de la cáscara del huevo.

Although it is realized that vitelline cells and Mehlis' gland are involved in the formation of egg shell in trematodes, knowledge of their relative roles is still incomplete. Egg-shell in a number of trematodes is known to be hardened by

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phenolic tanning (Smyth and Clegg, 1959 and Clegg and Smyth, 1968 for a review). It is known that phenolic tanning involves a ground substance comprising predominantly protein, a phenolic precursor and an enzyme system (Gilmour, 1961). In this connection, it is also known that vitelline cells of trematodes cotribute to the precursors of sclerotization (Vialli, 1933; and Stephenson, 1947). A direct evidence in support of the involvement of vitelline cells in sclerotization process of egg-shell in trematodes is provided in the isotopic and autoradiographic studies of Burton (1963) and Nollen (1968). Mehlis' gland is also reported to be involved in the formation of egg-shell (Dawes, 1940; Yosufzai, 1953; and Ebrahimzadeh, 1966) besides other functions, that have been attributed to it (see Dawes, 1940; Yosufzai, 1953; Smyth and Clegg, 1959; Burton, 1963; and Löser, 1965b for a review). Direct evidences regarding the role of Mehlis' gland in the shell formation are required. The present study is an elucidation of the relative role of vitelline cells and Mehlis' gland in some aspects of the formation of egg-shell in trematodes.

Vitelline cells

In trematodes, the cytoplasm of immature vitelline cells is homogeneous and strongly basophilic (Kohlmann, 1961, and Ebrahimzadeh, 1966). In this respect, immature cells of the monogenetic trematode *Pricea multae* showed similarity and stained blue in Mallory. On the other hand, the cytoplasm of mature vitelline cells was filled with globules and stained red in Mallory. The globules in the cytoplasm of mature vitelline cells have been reported to contain phenolic precursors. In *Fasciola hepatica*, Vialli (1933) and Stephenson (1947) reported the presence of *o*-dihydroxyphenol in the globules. However, Smyth and Clegg (1959) are of the opinion that they contain a phenolic protein. In *Pricea multae* a detailed investigation on the cytochemistry of the globules of mature vitelline cells was carried out. It was observed that the shell globules were positive to Millon's, chromaffin and ferric chloride tests and showed a yellow-green colour to Morner's and Liebermann's tests, similar to that exhibited by synthetic 3-4 dihydroxyphenylalanine ("Dopa") (Ramalingam and Ravindranath, 1970 and Ramalingam, 1917a). Presence of "Dopa" in globules has been shown to give an unusual colour reaction to periodic acid-Schiff's reagent (PAS) and its control (Ramalingam, 1971a). It has also been shown that this phenol exists in a masked state. This conclusion is based on its giving a more intense reaction to Fontanna's ammoniacal silver nitrate subsequent to methylation. It is known that a treatment in hot HCl-methanol (Methylation) will liberate phenol from acid mucopolysaccharide (Monne, 1959). It was shown recently that this complex gives green colour to Toluidine blue (Ramalingam and Ravindranath, 1970). Further, it has been shown that the acid mucopolysaccharide is of sulphated type on the basis of its stainability to Alcian blue at low pH (Ramalingam, 1970, 1971a). This association thus forms a built-in inhibition system preventing the phenol from oxidation and the consequent tanning of the proteins *in situ* as well as being detoxicated. Further, the observations made on vitelline cells that are in the proximal uterus are of interest. These cells on their passage through the oötype have come under the influence of Mehlis' gland secretion, release their shell precursors but are not enclosed in the egg-shell and remain as remnants in the uterus anterior to the ootype. These cells showed an intense reac-

tion to Alcian blue at pH 2.8 and to Hale's colloidal iron, possibly thereby suggesting the release of acid mucopolysaccharide from the phenol.

Vitelline cells are also known to synthesize phenolase. (See Smyth and Clegg, 1959 and Clegg and Smyth, 1968 for a review). In *Pricea multae*, phenolase enzyme was localized only in the mature vitelline cells, using cytochemical techniques. The enzyme has been reported to exist as a latent, inactive prophenolase and is activated by Mehlis' gland secretion (Ramalingam, 1970) and it has been recently characterized as substrate-specific. It was observed to oxidize only deaminated and decarboxylated di- and polyphenols such as catechol, hydroquinone and pyrogallol. The enzyme did not act on L-tyrosine, DL-3,4 dihydroxyphenylalanine ("Dopa") or protocatechuic acid as revealed from studies on incubation experiments in the above substrates separately (Ramalingam, 1971 b). In these respects the enzyme strongly recalls the enzyme phenolase of unhardened cuticles of the cockroach *Periplaneta americana* and larval cuticle of the fly *Calliphora vomitoria* (Dennell, 1958). This substrate-specific proenzyme was noted to be activated by detergents like sodium oleate (Ramalingam, 1970) and by other artificial activators like alcohol, formalin and sodium carbonate (Ramalingam, 1971b). The enzyme's thus remaining in a proenzyme state while in the vitelline cells in the vitellaria, and its inability to react with "Dopa" owing to its substrate-specificity, contribute further in preventing the precursors from premature tanning *in situ*. The results of this study have been summarized in the Tables I and II.

TABLE I

RESULTS OBTAINED ON MATURE VITELLINE CELLS OF FRESH, UNFIXED SPECIMENS OF THE MONOGENETIC TREMATODE *PRICEA MULTAE* ON INCUBATION IN 0.1% SUBSTRATE WITHOUT AND WITH TREATMENT IN 0.2% SODIUM OLEATE

Incubation in substrate	*Results*
1. L-Tyrosine	−
" after treatment in sodium oleate	−
2. D-L-3, 4 dihydroxyphenylanaline	−
" after treatment in sodium oleate	−
3. Protocatechuic acid	−
" after treatment in sodium oleate	−
4. Catechol	−
" after treatment in sodium oleate	+
5. Hydroquinone	−
" after treatment in sodium oleate	+
6. Pyrogallol	−
" after treatment in sodium oleate	+

−: negative means absence of activity of the enzyme on the substrate by absence of colour development by vitelline cells.

+: positive means activity of the enzyme on the substrate provided by formation of colour development by vitelline cells synthesizing the enzyme.

TABLE II

RESULTS OF HISTOCHEMICAL TESTS IN SHELL GLOBULES OF MATURE VITELLINE CELL OF TREMATODES

	Fasciola hepatica Vialli, 1933	*Fasciola hepatica* Stephenson, 1947	*Diclidophora merlangi*, Rennison 1953	*Fasciola hepatica* Smyth & Clegg, 1959	*Polystoma integerrimum*, Kohlmann, 1961	*Rajanchocotyle batis* Rigby & Marx, 1962	*Haematoloechus medioplexus*, Burton, 1963	*Parastrigea mexicanus* Coil, 1969	*Pricea multae*
Millon's	0	+	+	+		++	++		+
Chromaffin	+	+	+	+	+	++	++		+
Ammonium molybdate	+	+	+	+	+				+
Sodium iodate	+	+	+	+					+
Diazo	+		0	+	+		++		0
Fast redB						++	++		+
Ferric chloride	+	+		+	+	++	++		+
Ferric chloride/sodium carbonate									+
Ferric chloride/ammonium hydroxide		+							+
Morner's									yellow
Liebermann's									green yellow green
Fontanna's ammonical silver nitrate	+	+	+	+	+	++	++		+
—do— after methylation									++
Periodic acid-Schiff						−		−	Reddish brown
PAS without Schiff									brown
PAS without periodic acid									−
PAS after acetylation									Reddish brown
PAS after diastase									+
PAS after trypsin									+
PAS after pyridine									+
PAS after chloroform									+
PAS after deamination									+
PAS Hale's colloidaliron									
Alcian blue at PH 4.0									
Alcian blue at pH 2.8								−	
Toluidine blue at PH 7.0								green	green
Toluidine blue at pH 4.0									green
Toluidine blue at pH 2.8									green

0 : means not done ?: means not certain −: means no reaction +: reacts to the tests ++: reacts intensely to the tests

The precursors by remaining in a non-reactive state in the vitelline cells constitute a "built-in inhibition" system and prevent the precursors from tanning *in situ*. The nature of the association of phenol while in the vitelline cells, and how it is prevented from reacting with the enzyme *in situ*, have hitherto defied helminthologists (Smyth and Clegg, 1959; Hanumantha Rao, 1960; Burton, 1963, 1967; Clegg, 1965; Wilson, 1967; Coil, 1969; Threadgold and Irwin 1970).

It has already been shown that the enzyme acting on catechol exists in an inactive state or as a proenzyme or latent enzyme. This inactive enzyme has been observed to become active under the influence of Mehlis' gland secretion in the oötype. This is based on vitelline cell contents giving a positive reaction by developing a colour on incubation in catechol after their passage through the oötype. It has also been shown that the precursor of tanning phenol exists as "Dopa" in the vitelline cells but the enzyme present in them is specific to catechol. Hence it is inferred that the precursor of tanning phenol "Dopa" has undergone chemical conversion to tanning phenol, catechol by undergoing deamination and decarboxylation before being acted upon by the activated enzyme. Obviously, Mehlis' gland that surrounds the oötype may have an influence over these changes. In this context the statement of Smyth and Clegg (1959) "that the location of Mehlis' gland plays some part in the formation of the shell" is pertinent.

Mehlis' gland

Mehlis' gland is composed of a cluster of unicellular glands around the oötype, opening into it by fine ducts Dawes, (1940). A "number of authors have identified two types of cells in the gland, based on their affinity to acid and basic dyes (Ujiie, 1936; Williams, 1960; Gönnert, 1955, 1962; Kohlmann, 1961; Löser, 1965; Ebrahimzadeh, 1966; and Threadgold and Irwin, 1970). However, other workers have found only one type of cells (Dawes, 1940; Stephenson, 1947; Rennison, 1953 (cited in Smyth and Clegg, 1959) and Yosufzai, 1953). Gönnert (1962) termed the cells "mukösen", to refer to the large and numerous peripheral cells having affinity for aniline blue, and "serösen", to refer to the smaller and fewer cells close to the oögenotop with affinity to acid fuchsin and eosin. In *Price* a only one type of cell could be observed, showing an affinity for aniline blue of Mallory.

Stephenson (1947) suggested that an understanding of the functions of Mehlis' gland depends on determining the exact chemical nature of the secretion. Investigations carried out in this line are still inadequate. The results obtained on Mehlis' gland of various trematodes are presented in Table III.

A perusal of the table on PAS-positive material, though fragmentary, yet provides some clue in regard to the probable nature. A fact emerging from these studies is the presence of a lipid owing to a positive reaction to acid haematin, Sudan Black B, Sudan IV and Nile blue sulphate, and its being abolished subsequent to pyridine and chloroform-methanol extraction (Rao, 1959; Clegg, 1965). A detailed chemical analysis of Mehlis' gland of *Fasciola hepatica*, carried out by Clegg and Morgan (1966), not only confirms the presence of lipid but also sheds light on its exact nature. The results of the above mentioned studies reveal the presence of phospholipids. Phospholipids are known to contain both saturated and unsaturated fatty acids (Snider and Bloor, 1933, cited in Lovern, 1957). Presence of phospholipids in the secretion of Mehlis' gland seems to be of great significance to the events that take

TABLE III

RESULTS OF HISTOCHEMICAL TESTS OF MEHLIS' GLAND OF TRAMATODES

	Fasciola hepatica			*Artyfechinostomum mehrai* Hanumantha Rao, 1963	*Ogmocotyle indica* Coil, 1966	*Parastrigea mexicanus*, Coil, 1969	*Haematoloechus medioplexus* Burton 1963	*Polystoma integerrimum* Kohlmann 1961	*Rajanchocotyle batis* Rigby & Marx, 1962
	Hanumantha Rao, 1959b	Clegg 1965	Threadgold and Irwin, 1970						
PAS	+	+	+	+	+	+	++	+	+
PAS/analyse or diastase		+		+		+	++		
PAS/pyridine	−			−					
PAS/chloroform methanol		−							
Sudan IV		−							
Sudan Blach B	+	+		+					
Sudan Black B/pyridine	−								
Sudan Black B/chrloroform methanol		−							
Nile blue	*								

−: means no reaction to the tests

+: means reaction to the tests

place in the oötype, although no significance has been attributed to it so far (*vide infra*).

Yet another feature arising out of previous studies is with regard to PAS-positive substances. These PAS-substances in the Mehlis' gland have been observed to be resistant to amylase, diastase, pyridine and chloroform extraction. Electrophoretic studies on the PAS-positive material of Mehlis' gland of *Fasciola hepatica* by Clegg (1965) would suggest that it may be heterogeneous. In this connection Kohlmann (1961) reported that PAS-positive material in the mukösen cells has an iso-electric point ranging from 6.75 to 6.99, suggestive of a neutral mucopolysaccharide. The findings of Stephenson (1947) and Yosufzai (1953) are of interest. They have reported that the contents of peripheral cells, on reaching the medullary region, become refractile to staining and are hyaline in nature. These observations strongly recall the PAS-positive material in the formative stages of cuticle of arthropods (Taylor and Richard, 1965) and radula of molluscans (Runnham, 1962). The above authors likewise observed the PAS-positive material becoming refractile to staining. They attributed this to the synthesis of long chains of chitin. The above findings also suggest the possibility of chitin being synthesized by mukösen cells. In this context it is interesting to note the report of Lillie (1954) that the egg shell of schistosomes and *Capillaria hepatica* are chitinous in nature. Preliminary observation on the egg-shell of the monogenetic trematode *Pricea multae* with the iodine sulphuric acid test of Campbell (1929) showed the presence of a light violet colour. However, further studies in this direction are necessary and are under way.

Relative role of vitelline cells and Mehlis' gland

Vitelline cells' contributing the precursors of phenolic tanning of egg-shell is well known (see Smyth and Clegg, 1959 and Clegg and Smyth, 1968 for a review). However, the enzyme that exists as a proenzyme and phenol existing in a masked condition in the vitelline cells of *Pricea* (Ramalingam, 1970; Ramalingam and Ravindranath, 1970; and Ramalingam, 1071a) are of interest. The fact that the enzyme becomes reactive to substrate after coming under the influence of Mehlis' gland secretion (Ramalingam, 1970) and the substrate being freed from its association with acid mucopolysaccharide, as evidenced by intense reaction of acid mucopolysacchasiде in the vitelline cells remaining in the uterus after passing through the oötype, suggests that the secretion of Mehlis' gland, in addition to activating the enzyme, also brings about the liberation of phenol from acid mucopolysaccharide. The changes thus initiated lead to the process of sclerotization, as evidenced by completed egg shell leaving the oötype. A further note-worthy feature pertaining to the nature of the phenol in the oötype is that it is not only unmasked but also deaminated and decarboxylated, so that the activated enzyme can act on it, as evidenced by its substrate specificity to deaminated and decarboxylated phenols. As activation of the enzyme, unmasking of the phenol and chemical degradation of the phenolic precursor viz. 'Dopa' to tanning phenol, catechol, take place in the oötype surrounded by Mehlis' gland, it is reasonable to suspect that Mehlis' gland secretion in some way brings about these changes. In this context, it is of interest to recall the statement of Smyth and Clegg (1959) that "although the precursors of quinone tanning system are derived from shell globules, it is possible that Mehlis' gland

secretes a fluid which affects the tanning process in some way."

Therefore, the chemical nature of the Mehlis' gland secretion may provide a clue to the above changes. Results from histochemical and analytical studies on Mehlis' gland show the presence of 1. mucoprotein, 2. glycoprotein and 3. phospholipid. The secretion of mucoprotein into the oötype by the gland may suggest a function similar to that proposed by Kouri and Nauss (1938) in facilitating the passage of the egg by serving as a lubricant. Although the significance of glycoprotein is not clear, it is suggested, as proposed earlier, that it may be involved in the formation of the ground substance of the egg shell material, as envisaged by Dawes (1940), Yosufzai (1953) and Ebrahimzadeh (1966). However, this suggestion requires further attention.

The two types of chemical substances mentioned above do not provide a clue regarding their function related to (1) activation of the enzyme (2) unmasking of the phenol and (3) degradation of the phenolic precursor to tanning phenol. Hence attention is directed to phospholipid that is also reported to occur in the Mehlis' gland secretion of *Fasciola hepatica* (Hanumantha Rao, 1959, 1960; Clegg, 1965; and Clegg and Morgan, 1966). It seems to shed some light on these aspects. It is suggested that activation of prophenolase in helminths may be brought about by phospholipids that occur in Mehlis' gland secretion. In support of this, studies on the chemical nature of natural activators of prophenolase in insects are pertinent. In insects, where the enzyme is activated by artificial activators such as sodium oleate, the natural activator is known to be lipoidal in nature (Bodine and Allen, 1938; Lewis and Lewis, 1963; Hackman and Goldberg, 1967; and Heynman and Vercauteren, 1968). As the prophenolase of trematodes is also known to be activated by artificial activators like sodium oleate (Ramalingam, 1970), it is suggested that phospholipid present in Mehlis' gland secretion may bring about the activation. This suggestion seems to be further strengthened by the fact that phospholipids are known to function as activators of latent mitochondrial ATPase (Racker, 1965), and phenolases in insects are known to be stored in mitochondria (Karlson *et al.*, 1964). In the light of the above findings, it is not unreasonable to assume that the phospholipid secretion of Mehlis' gland is activating the phenolase synthesized by vitelline cells.

Unsaturated fatty acids are known to influence as surface acting agents (Lovern, 1957). This property is significant in the light of the suggestion of Löser (1965) and Ebrahimzadeh (1966) that the secretion of Mehlis' gland brings about softening and confluence of shell globules leading to their coalescence. In addition, unsaturated fatty acids are known to bring about solublization of lipoproteins (Lovern, 1957). This is significant in the context of the suggestion of Ujiie (1936), Dawes (1940), and Hanumantha Rao (1959b), who have stated that Mehlis' gland secretion functions in the release of shell precursors. Daws (1946) noted that "the secretion of the shell glands may perhaps by virtue of contained electrolytes induce vitelline cells to extrude their secretion at a given instant, as they undoubtedly do." This solublization of the lipoprotein of the membrane of vitelline cells may bring about under its influence the liberation of phenol from acid mucopolysaccharide, as it is known that injury causes the unmasking of phenol from acid mucopolysaccharide (Monne, 1959).

Unsaturated fatty acids are known to be involved in bringing about an oxidation, or in acting as oxygen carriers, owing to the presence of easily oxidi-

zable double bonds (Lovern, 1957). It is known that oxidation of phenol by phenolase is accelerated in the presence of lipids (Hurst, 1945; Pryor, 1955; and Lewis and Lewis, 1963). Phospholipid secretion of Mehlis' gland may likewise accelerate the process of tanning, precursors of which are derived from vitelline cells.

The role of various physiological funtions referred to above and attributed to phospholipids is possibly one of involving reactions which not only complement each other but also reinforce the reactions set forth earlier, all contributing to the single aim of successfully completing most efficiently and effectively the whole series of events within the brief time available to the organism from the release of shell precursors in the oötype to the formation and release of the completed egg capsule.

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