[Jpn. J. Parasitol., Vol. 43, No. 2, 110–117, April, 1994]

Histochemical Nature of Egg-shell of some Trematodes

ABDULRAHMAN, M.N. KALANTAN[1)], MISBAHUL ARFIN[1)], MOHAMED S. AL-YOUSIF[1)] AND WAJIH A. NIZAMI[2)]

(Accepted for publication; February 16, 1994)

Abstract

The chemical nature and mode of stabilization of egg-shell of some trematodes belonging to different taxonomic groups and inhabiting various hosts and habitats have been investigated using histochemical and fluorescence techniques. Egg-shell in the monogenetic trematode *Heteromicrocotyle carangis* was stabilized by S-S linkages together with dityrosine while that in the digenetic trematodes *Pleurogenoides stromi* and *Dicrocoelium dendriticum* by quinone-tanning. Although the nature of proteins involved in the quinone-tanning was different in the two. Dityrosine and proteins having S-containing amino acids dominate the proteins in *D. dendriticum* while absent in *P. stromi*. The egg-shell in another digenetic trematode *Clinostomum complanatum* was stabilized by S-S linkages and dityrosine similar to that of monogenetic trematode, *H. carangis*, though an enzyme capable of oxidizing catechol was absent in the former but present in the latter species.

Key words: Chemical nature, egg-shell, trematodes

Introduction

Inspite of extensive histological and histochemical studies, much remains to be done on the mode of stabilization of egg-shell in a large number of helminths. Since many of the activities of helminths are directed towards egg-production and egg-shell formation, a process involving synthesis and stabilization of proteins is one of the major problems in developmental biology of parasites (Von Brand, 1979; Smyth and Halton, 1983; Wharton, 1983). Moreover, recent studies have revealed that egg/onchosphere protein could be used for production of monoclonal antibodies by using recombinant DNA technology which will be of immense use in the antigenetic characterization as well as by using sequence specific binding reagents for the pharmacological control of the parasite (Smithers, 1986; Cordingley, 1987).

In the present study, we investigated the chemical nature and possible mode of stabilization of egg-shell in a number of trematodes inhabiting different hosts and habitats and belonging to different taxonomic groups. As only few reports are available on the chemical nature of egg-shell in monogenea and some families of digenea such as Clinostomatidae and Dicrocoelidae, therefore, monogenetic trematode *Heteromicrocotyle carangis* from the gills of marine fish, and digenetic trematodes, *Pleurogenoides stromi*, *Clinostomum complanatum* and *Dicrocoelium dendriticum* from intestine of frog, oesophagus of chickens and liver of sheep, respectively were selected for the present study.

[1)]*Department of Zoology, College of Science, King Saud University, Riyadh, Saudi Arabia.* [2)]*Section of Parasitology, Dept. of Zoology, A.M.U. Aligarh, India.*

Materials and Methods

Specimens of *Heteromicrocotyle carangis* (Yamaguti, 1953) were collected from the gills of marine fish *Caranx sexfasciatus*, and *Pleurogenoides stromi* (Travassos, 1930) from the frog, *Rana ridibunda*. Adults of *Clinostomum complanatum* (Rudolphi, 1819) were obtained from the oesophagus of experimentally infected chick (*Gallus gallus domesticus*) while the specimens of *Dicrocoelium dendriticum* (Rudhphi, 1819) were collected from the liver of sheep slaughtered at the local abattoirs and brought to the laboratory in a vacuum flask containing Hank's saline at 37°C. The parasites were washed in saline and fixed in 10% buffered formalin/Bouin's fluid/70% ethanol/acetic acid-formol-alcohol (AFA) as per requirement. Serial

sections (7 μm thick) were cut and stained. The histochemical tests employed for the detection of various moieties were adopted from McManus and Mowry (1960), Pearse (1968) and Drury and Wallington (1976) unless otherwise stated and summarized in Table 1.

Fluorescence of the egg-shell protein was observed in VEB Carl Zeis Jenna NfPK research microscope equipped with high pressure mercury vapour lamp HBO 50. An excitation light filter U.V. filter type UG 1/3.5 (366nm wave length) was used. As barrier filters for fluorescence microscopy, filters GG9 and GG9/O g 1 (both in mounts) were fixed to the binocular tube (Andersen and Weis-Fogh, 1964; Ramalingam, 1973a).

Results

The results of histochemical tests performed to ascertain the chemical nature of egg-shell of *H. carangis, P. stromi, C. complanatum* and *D. dendriticum* are summarized in Table 1.

H. carangis:

The vitelline cells of *H. carangis* gave positive results for phenoloxidase, phenols and basic proteins while mature egg-shell was positive for basic proteins and phenoloxidase but negative for phenols and quinone. The egg-shell were refractory to Mallory's triple and Van Geison's stains and gave pinkish colour with Gomori's trichrome stain showing the absence of collagen.

The proteins having S-containing amino acids were present in the egg-shell as indicated by the positive reactions with DDD, ferric ferricyanide and performic acid alcian blue. Moreover, the intensity of reaction was increased on incubation in sodium thioglycollate prior to DDD and ferric ferricyanide, which showed the presence of S-S linkages. Another structural protein, elastin was also present in the egg-shell as shown by the positive reaction with aldehyde fuchsin and Verhoeff's stain.

Tyrosine was present in the egg-shell and vitelline cells while dityrosine was present in the mature egg-shell as indicated by the positive results with histochemical as well as fluorescence tests. The mature egg-shell showed blue fluorescence in U.V. light after treatment with ammonia vapours which was quenched by iodine indicating the presence of dityrosine.

P. stromi:

Phenoloxidase, phenols and basic proteins were present in vitelline cells and immature egg-shell while absent in mature egg-shell of *P. stromi* as indicated by results obtained with the specific tests. Though on prior treatment with sodium diethyldithiocarbamate and on heating at 80°C, the catechol reaction gave slightly positive reaction in mature egg-shell.

Other structural proteins like collagen, elastin, dityrosine and protein containing S-H and S-S linkages were absent in egg-shell of *P. stromi*. Tyrosine was found to be present in vitelline cells but absent in egg-shell.

C. complanatum:

The vitelline cells and egg-shell of *C. complanatum* gave negative results with tests for phenoloxidase, and phenols indicating the absence of phenols and phenoloxidase while gave positive results with bromophenol blue and malachite green methods showing the presence of basic proteins. Moreover the freshly laid eggs were colourless and gave positive results for basic proteins and even after prolonged incubation in catechol could not produced any brown colouration showing the absence of quinone in mature egg-shell.

Tyrosine and dityrosine were present in the egg-shell. The elastin was found to be absent in egg-shell but present in vitelline cells. Another structural protein, collagen was also absent in the egg-shell. Tests for proteins having S-containing amino acids were positive in egg-shell and vitelline cells. Moreover, the intensity of reaction was increased on incubation with sodium thioglycollate prior to DDD and ferric ferricyanide treatment showing the presence of proteins containing S-S and S-H groups in egg-shell and vitelline cells of *C. complanatum*.

D. dendriticum:

In *D. dendriticum,* the precursors of quinone-tanning (basic proteins, phenols and phenoloxidase) were present in vitelline cells and immature egg-shell while absent in mature egg-shell. Although slightly positive reaction was observed following

Table 1 Results of histochemical tests performed on egg-shell trematodes

Staining reaction for	Tests	*H. carangis*		*P. stromi*		*C. complanatum*		*D. dendriticum*		
		Vit. cells	Mature Egg-shell	Vit. cells	Mature Egg Shell	Vit. cells	Mature Egg-shell	Vit. cells	Egg-Shell Immature	Egg-Shell Mature
Phenoloxidase	Catechol method (Smyth, 1954)	++	++	++	++	–	–	+++	++	+
	Catechol after diethyl-dithiocarbamate treatment	–	–	–	+	–	–	–	–	+
Quinone	Catechol after heat treatment at 80°C (Hackman & Goldberg, 1967)	–	–	–	+	–	–	–	–	+
Phenols	Diazo test (Johri & Smyth, 1956)	++	++	++	++	–	–	++	+	–
	Ferric chloride (Lison, 1936 as cited by Smyth, 1954)	+/–	–	+/–	–	–	–	+/–	+/–	–
	Toluidine blue method (Ramalingam & Ravindranath, 1970)	+*	–	+*	–	–	–	+*	+*	–
Basic Proteins	Aqueous bromophenol blue	++	++	++	–	++	++	++	+	–
	Malachite green (Johri & Smyth, 1956)	++	++	++	–	++	++	++	+	–
Elastin	Aldehyde fuchsin stain	+	+	+	–	+	–	++	–	–
	Verhoeff's stain	+	+	+	–	+	–	++	–	–
Collagen	Van Geison's stain	–	–	+	–	+	–	+	–	–
	Gomori's trichrome stain	–	–	+	–	+	–	–	–	–
	Mallory's triple stain	–	–	+	–	+	–	++	–	–
Protein having S-containing amino acids	DDD Method	+	+	+/–	–	+	++	+	++	–
	DDD after sodium thioglycollate treatment	++	++	+/–	–	++	+++	+	++	–
	Ferric ferricyanide method	++	++	+	–	+	+	+	++	–
	Performic acid alcian blue	+	+	–	–	+	+	+	+	–
Tyrosine	Millon's test	+	+	+	–	++	+	+	+	–
Dityrosine	Methylene blue in glycerol and water (1:1) (Andersen & Weis-Fogh, 1964)	+	++	+	–	++	+	+	++	–
	Toluidine blue-light green in phosphate buffer of pH 7.2 (Andersen & Weis-Fogh, 1964)	+	++	+	–	++	+	++	++	–
	Fluorescence in u.v. light (at 366 nm wavelength (Andersen & Weis-Fogh, 1964)	–	yellow	–	–	Blue+	Blue+	Blue+	Blue+	Brown
	Fluorescence in u.v. light after ammonia vapours	–	Blue	–	–	++	++	++	++	Brown
	Fluorescence in u.v. light after treatment in iodine (Undenfriend, 1962)	–	–	–	–	–	–	–	–	Brown

+ Positive; ++ Intensely positive; – Negative; +/– Unsatisfactory colour produced * Produced green metachromasia

catechol method in the mature egg-shell.

The tyrosine and dityrosine were present in the vitelline cells and immature egg-shell. The tests for other structural proteins like elastin and collagen were negative in both immature and mature egg-shells and positive in vitelline cells. The tests for proteins having S-containing amino acids were positive in vitelline cells and immature egg-shell but negative in mature egg-shell showing the presence of protein rich in sulpher content in immature egg-shell and vitelline cells while its absence in mature egg-shell.

Discussion

H. carangis:

The positive results for basic proteins in the mature egg-shell of *H. carangis* provided the clue that the egg-shell might not be stabilized by quinone-tanning because tanned structures are refractory to the stains for basic proteins (Smyth and Clegg, 1959). The egg-shell and vitelline cells gave strongly positive reaction with 0.2% catechol which was controlled on treatment with diethyldithiocarbamate prior to catechol incubation, indicating the presence of an enzyme capable of oxidizing catechol. However, on heat treatment at 80°C following the method of Hackman and Goldberg (1967), the egg shell failed to produce any brown colouration even after prolonged incubation in catechol indicating the absence of quinone in egg-shell because it would have produced brown colour due to non enzymic oxidation of catechol if quinones were present (Mason, 1955; Dennell, 1958).

This enzyme may be phenoloxidase but not involved in the stabilization of egg-shell as in some cases it has been observed that phenoloxidase is present but not involved in the stabilization, rather may be associated with other physiological activities, since phenoloxidase is a group of enzymes and responsible for the oxidation of varied type of phenolic substrates. Threadgold and Read (1968) and Cheah and Prichard (1975) reported that some of the helminths produce hydrogen peroxide which is biologically toxic and this enzyme is involved in the detoxification mechanism.

Alternatively this enzyme may be peroxidase as suggested by Ramalingam (1973b) in *Pseudomicrocotyle* sp. and *Pricea multae*, which is involved in the bimerization of tyrosine.

Thus, the presence of basic proteins together with absence of quinone in the mature egg-shell of *H. carangis* suggested the absence of quinone-tanning, although its precursors were present in the vitelline cells. Similarly, all or some of the precursors of quinone tanning have been reported from the vitelline cells of a number of monogenetic trematodes: *Diclidophora merlangi, Gastrocotyle trachuri, Polystoma integerrimum, Rajonchocotyle batis, Protopolystoma xenopodis, D. luscae, Entobdella soleae, Calicotyle* sp., *P. multae, Protomicrocotyle* sp., *Polystomoides* sp., *Octomacrum lanceatum* and *Heteromicrocotyle indicus* (see Smyth and Halton, 1983; Kalantan and Arfin, 1984).

In view of the absence of quinone-tanning, various tests for other structural proteins were tried and collagen was found to be absent while dityrosine and proteins having S-containing amino acids were present in egg-shell. Therefore, it is concluded that the egg-shell of *H. carangis* is stabilized by S-S linkages together with dityrosine similar to the egg-shells of *P. multae, Pseudomicrocotyle* sp., and *H. indicus* (Ramalingam, 1973b; Kalantan and Arfin, 1984).

This study together with previous reports suggest that the egg-shell protein in all monogenetic trematode may be stabilized by formation of disulphide bondings and bimers of tyrosine. However, chemical nature of egg-shell should be investigated in the large number of monogenetic trematodes to make this generalization.

P. stromi:

The precursors of quinone-tanning were present in the vitelline cells as well as immature egg-shell. The light brown colour in the mature egg-shell with catechol on prior treatment with diethyldithiocarbamate and on heat treatment may be due to the presence of quinone, because as mentioned earlier catechol can be oxidized non-enzymatically in the presence of quinone to produce brown colourations (Mason, 1955; Dennell, 1958). Moreover, the phenols and basic proteins were absent in the mature egg-shell. The absence of phenol and basic proteins in the mature egg-shell was possibly due to the

formation of sclerotin (Pryor, 1940). Although mature egg-shell gave positive results with diazo test but this test is not very specific for phenols as it also gives colour with histidine, tryptophan, purines and pyrimidines (Smyth and Clegg, 1959). This diazo positive colour may be due to histidine thereby supporting the view of Lipke *et al.*, 1983 as cited by Cordingley (1987) that histidine could participate as nucleophilic part in the formation of cross-links.

Thus the presence of all precursors of quinone-tanning in the vitelline cells and quinone in mature egg-shell together with the absence of other structural proteins suggested that the egg-shell in *P. stromi* is stabilized by quinone-tanning. The precursors of quinone-tanning have also been demonstrated histochemically in the globules of vitelline cells of other trematodes belonging to the same family Lecithodendriidae: *Halipegus eccentricus* (Guilford, 1968), *Brandesia turgida* and *Pleurogenes claviger* (Gerzeli, 1968), *Ganeo tigrinum* and *Mehraorchis ranarum* (Rao, 1972), *P. tacapensis* (Kalantan and Arfin, 1985).

Therefore, the mode of stabilization of egg-shell in *P. stromi* is similar to that in *P. tacapensis* (Kalantan and Arfin, 1985) and many other trematodes where quinone-tanning is present, in which phenols are generally transformed into quinone with the action of phenoloxidase and this O-quinone condenses with free-NH_2 group of adjacent protein to give stable tanned proteins (Pryor, 1940). However, in some cases high molecular weight catechols are involved in quinone-tanning. These involve peptide or protein-bound 3,4 dihydroxyphenyl-L-alanine (DOPA) which is oxidized in the presence of catecholoxidase to give quinone-tanned structures (Waite and Rice-Ficht, 1987; Waite, 1990).

Eshete and LoVerde (1993) studied the characteristics of phenoloxidase of *Schistosoma mansoni* and demonstrated that egg-shell protein acts as a substrate for phenoloxidase which catalyzes the hydroxylation of free tyrosine to DOPA and oxidation of L-DOPA to dopa quinone and suggested that the enzymatic oxidation of DOPA residues is responsible for aggregation which can be interpreted as tyrosine-dependent aggregation of the protein that results in sclerotization.

C. complanatum:

The presence of basic protein in the mature egg-shell indicated the absence of quinone-tanned protein as tanned structures are refractory to the stains for basic proteins (Smyth and Clegg, 1959). The absence of quinone-tanning was further confirmed by negative results for phenoloxidase and phenols in vitelline cells and immature egg-shell and for quinone in mature egg-shell.

Therefore, the absence of quinone-tanned protein, elastin and collagen together with the presence of protein having S-containing amino acids suggested that the egg-shell of *C. complanatum* is stabilized by S-S and S-H linkages. Dityrosine which was found to be present in the egg-shell may provide further stability. The increase in the reaction intensity with DDD and ferric ferricyanide on prior treatment with sodium thioglycollate suggests that the egg-shell of *C. complanatum* contains large concentration of reducible disulphide bonds which is expected in keratin.

Although this keratin type of protein appears to be characteristic feature of the family paramphistomatidae as it has been reported in the egg-shell of many species of this family such as *Carmyerius spatiosus*, *Gastrodiscus secundus*, *Paramphistomum cervi* and *Diplodiscus mehrai* (Madhavi, 1966, 1968); *Megalodiscus temperatus* (Nollen, 1971); *Carmyerius synethes* (Eduardo, 1976); *Gastrothylax crumenifer* (Eduardo, 1976; Arfin and Nizami, 1986); *Diplodiscus amphichrus* (Kanwar and Agarwal, 1977) and *Gigantocotyle explanatum* (Arfin and Nizami, 1986). The only known exception is the *Fasciola hepatica* in which the egg-shell is also stabilized by S-S bondings together with dityrosine (Ramalingam, 1973a). The egg-shell in *C. complanatum* is stabilized by the dityrosine and S-S bonding similar to *F. hepatica*, thus adding another digenetic trematode species which does not belong to the family Paramphistomatidae where egg-shell contains S-S linkages.

D. dendriticum:

The absence of phenol and basic proteins in mature egg-shell of *D. dendriticum* was possibly due to the transformation of phenol and basic proteins to quinone-tanned proteins. The slightly positive reaction in mature egg-shell with catechol may

be due to the non enzymic oxidation of catechol in the presence of quinone (Mason, 1955; Dennell, 1958) indicating that quinone was present in the mature egg-shell.

Presence of all the three precursors of quinone-tanning in vitelline cells and immature egg-shell and quinone in the mature egg-shell together with the absence of other structural proteins like elastin, collagen, and keratin (protein having S-containing amino acids) in mature egg-shell of *D. dendriticum* suggested that the egg-shell protein is stabilized by the quinone-tanning although proteins having S-containing amino acids were present in vitelline cells and immature egg-shell. The process can be expected to occur as follows. The phenols are transformed into quinone with the action of phenoloxidase and this O-quinone condense with -SH or -NH_2 groups of adjacent protein to give stable tanned protein (Pryor, 1940). Alternatively, the tyrosine present may transform into quinone through one of the various modes, reviewed by Ramalingam (1973b) and this quinone may form tanned protein as also suggested by Waite and Rice-Ficht (1987) and Eshete and LoVerde (1993).

Quinone-tanned structures are distributed throughout the animal kingdom and serve critical supportive and protective functions in the life history of many organisms (Waite, 1990). Among helminths, in many trematodes the egg-shell has been reported to be stabilized by quinone-tanning no matter, what pathway is followed: e.g., *Fasciola indica* (Lal and Johri, 1967; Gupta and Puri, 1981); *Echinostoma revolutum, Echinoparyphium recurvatum* and *Glypthelmins* sp. (Fried and Stromberg, 1971); *Apatemon gracilis minor, Diplostomum spathaceum* and *Halostephanus luhei* (Erasmus, 1972); *Tremiorchis ranarum, G. tigrinum* and *M. ranarum* (Rao, 1972); *Isoparorchis hypselobagri* (Srivastava and Gupta, 1978); *Fasciolopsis buski* (Gupta and Puri, 1981); *F. gigantica* (Arfin and Nizami, 1986), *Helicometra pulchella* (Kalantan *et al.*, 1992).

From this study it can be concluded that the egg-shell protein in *H. carangis* is stabilized by S-S linkages together with dityrosine while in *P. stromi* by quinone-tanning. The chemical nature of egg-shell in *C. complanatum* is similar to that found in *H. carangis* as it is also stabilized by S-S linkages and dityrosine, however, in *H. carangis* an enzyme capable of oxidizing catechol is present while absent in *C. complanatum*. The egg-shell in *D. dendriticum* is stabilised by quinone-tanning similar to *P. stromi* but the nature of basic proteins involved is different. Dityrosine and proteins having S-containing amino acids dominate the basic proteins in *D. dendriticum* while absent in *P. stromi*. From the present study together with previous reports on the chemical nature of egg-shell it is clear that no generalization is possible as far as the chemical nature/mode of stabilization is concerned because even the two species of a genus and various species found in same habitat and host may have different mode of stabilization.

However, it has been observed that either S-S linkages or quinone tanning involving different types of proteins are major processes for the chemical stabilization of egg-shell in trematodes and the differentiating feature is the type of proteins involved in the stabilization. Therefore, it is suggested that the process of stabilization of egg-shell should be investigated in a number of parasites particularly by using recombinant DNA technology in order to work out the sequence in DNA molecule and their corresponding amino acids as recent studies have also revealed that egg/onchosphere proteins could be used for production of monoclonal antibodies (Smithers, 1986; Cordingley, 1987). Such studies will be of immense use in the antigenetic characterization and by using sequence specific binding reagents for the pharmacological control of the parasites.

References

1) Andersen, S. O. and Weis-Fogh, T. (1964): Resilin. A rubber-like protein in arthropod cuticle. Adv. Insect. Physiol., 2, 1–65.
2) Arfin, M. and Nizami, W. A. (1986): Chemical nature and mode of stabilization of egg-shell proteins of some digenetic trematodes. Helminthologia, 23, 157–164.
3) Cheah, K. S. and Prichard, R. K. (1975): The electron transport systems of *Fasciola hepatica* mitochondria. Int. J. Parasitol., 5, 183–186.
4) Cordingley, J. S. (1987): Trematode Egg-shells: Novel protein Biopolymers. Parasitol. Today, 3, 341–344.
5) Dennell, R. (1958): The amino acid metabolism of developing insect cuticle: The larval cuticle and puparium of *Calliphora vomitoria*. Pt. II. The non-specific hydroxylation of aromatic amino acids and the produc-

tion of polyphenols by the cuticle. Proc. R. Soc. Ser. B, 148, 280–285.

6) Drury, R. A. B. and Wallington, E. A. (1976): Carleton's Histological Technique. 4th ed. Oxford University Press, London.
7) Eduardo, S. L. (1976): Egg-shell formation in *Carmyerius synethes* (Fischoeder, 1901) and *Gastrothylax crumenifer* (Cerplin, 1847) Digenea: Gastrothylacidae. Philipp. J. Vet. Med., 15, 117–122.
8) Erasmus, D. A. (1972): The Biology of Trematodes, Edward Arnold, London.
9) Eshete, F. and LoVerde, T. (1993): Characteristics of phenol oxidase of *Schistosoma mansoni* and its functional implications in egg-shell synthesis. J. Parasitol., 79, 309–317.
10) Fried, B. and Stromberg, B. E. (1971): Egg-shell precursors in trematodes. Proc. Helminthol. Soc. Wash., 38, 262–264.
11) Gerzeli, G. (1968): Aspetti istochimici della formazione delgi in volucri ovulari in *Aspidogaster conchicola* Von Baer (Trematoda). Instituto Lombardo Scienze Lettere (Rend. Sc.), B102, 263–276.
12) Guilford, H. G. (1961): Gametogensis, egg capsule formation and early miracidial development in the digenetic trematode: *Helipegus eccentricus*. J. Parasitol., 47, 757–764.
13) Gupta, V. and Puri, M. (1981): Egg-shell formation in mammalian trematodes. Zool. Anz., 206, 252–256.
14) Hackman, R. H. and Goldberg, M. (1967): The O-diphenoloxidase of fly larvae. J. Insect. Physiol., 13, 531–544.
15) Johri, L. N. and Smyth, J. D. (1956): A histochemical approach to the study of helminth morphology. Parasitology, 46, 107–116.
16) Kalantan, A. M. N. and Arfin, M. (1984): Stabilization of egg-shell in *Heteromicrocotyle indicus* Ramalingam, 1960 (Monogenea: Microcotylidae). Proc. Saudi Biol. Soc., 7, 415–427.
17) Kalantan, A. M. N. and Arfin, M. (1985): Chemical nature and mode of stabilization of egg-shell in *Pleurogenoides tacapensis* Sonsino, 1894 (Digenea: Lecithodendriidae). J. Coll. Sci. King Saud Univ., 16, 109–118.
18) Kalantan, A. M. N., Arfin, M. and Alyousif, M. S. (1992): Chemical nature and mode of stabilization of egg-shell in *Helicometra pulchella* (Rudolphi, 1819) (Digenea: Opecoelidae). Acta Zool. (Stockh), 73, 153–156.
19) Kanwar, U. and Agarwal, M. (1977): Cytochemistry of the vitelline glands of the trematode *Diplodiscus amphichrus* (Tubangi, 1933) (Diplodiscidae). *Folia Parasitol.*, (Prague), 24, 123–127.
20) Lal, M. B. and Johri, G. N. (1967): The function of vitellocalycal glands in egg-shell formation in *Fasciola indica* Verma, 1953. J. Parasitol., 53, 989–993.
21) Madhavi, R. (1966): Egg-shell in Paramphistomatidae (Trematoda: Digenea). Experientia, 22, 93–94.
22) Madhavi, R. (1968): *Diplodiscus mehrai*: Chemical nature of egg-shell. Exp. Parasitol., 23, 392–397.
23) Mason, H. S. (1955): Comparative biochemistry of phenolase complex. In Advances in Enzymology, vol. 16, Nord, F. F. ed., New Interscience Publishers, New York, 105–184.
24) McManus, J. F. A. and Mowry, R. W. (1960): Staining Methods Histologic and Histochemical. Paul B. Hoeber, Inc., New York.
25) Nollen, P. M. (1971): Digenetic trematodes: Quinone-tanning system in egg-shells. Exp. Parasitol., 30, 64–72.
26) Pearse, A. G. E. (1968): Histochemistry: Theoretical and Applied. 3rd ed. vol. 1,: J. and A. Churchill Ltd., London.
27) Pryor, M. G. M. (1940): On the hardenings of the ootheca of *Blatta*. Proc. R. Soc. Lond. Ser. B, 128, 378–393.
28) Ramalingam, K. (1973a): The chemical nature of the egg-shell of liver fluke *Fasciola hepatica*. Int. J. Parasitol., 3, 67–75.
29) Ramalingam, K. (1973b): Chemical nature of egg-shell in helminths. II. Mode of stabilization of egg-shell in monogenetic trematodes. Exp. Parasitol., 34, 115–122.
30) Ramalingam, K. and Ravindranath, M. H. (1970): Histochemical significance of green metachromasia to toluidine blue. Histochemie, 24, 322–327.
31) Rao, L. N. (1972): Egg shell formation in three trematode parasites of *Rana tigrina, Varanus bengalensis* and *Chamaeleon zeylanicus*. Proc. Ind. Sci. Conqr. Assoc. Fifty ninth session, Section VII, 494.
32) Smithers, S. R. (1986): Vaccination against Schistosomes and other systemic helminths. In Parasitology Quo Vadit? edited by Howell, M. J. Pergamon Press, 31–42.
33) Smyth, J. D. (1954): A technique for the histochemical demonstration of polyphenoloxidase and its application to egg-shell formation in helminths and byssus formation in *Mytilus*. Q.J. Microsc. Sci., 95, 139–152.
34) Smyth, J. D. and Clegg, J. A. (1959): Egg-shell formation in trematodes and cestodes. Exp. Parasitol., 8, 286–323.
35) Smyth, J. D. and Halton, D. W. (1983): The physiology of trematodes. 2nd ed. Cambridge University Press, Cambridge.
36) Srivastava, M. and Gupta, S. P. (1978): Polyphenol-oxidase and scleroprotein/melanin pigments of *Isoparochis hypselobagri*. Z. Parasitenkd., 55, 55–58.
37) Threadgold, L. T. and Read, C. P. (1968): Electron-microscopy of *Fasciola hepatica*. V. Peroxidase localization. Exp. Parasitol., 23, 221–227.
38) Udenfriend, S. (1962): Fluorescence assay in biology and medicine. Academic Press, New York.
39) Von Brand, T. (1979): Biochemistry and Physiology of Endoparasites. Elsevier/North-Holland, Biomedical Press, Amsterdam.
40) Waite, J. H. (1990): The Phylogeny and chemical diversity of quinone-tanned glues and varnishes. Comp. Biochem. Physiol., 97B, 19–29.

41) Waite, J. H. and Rice-Ficht, A. C. (1987): Presclerotized egg-shell protein from the liver fluke *Fasciola hepatica*. Biochemistry, 26, 7819–7825.

42) Wharton, D. A. (1983): The production and functional morphology of helminth egg-shells. Parasitology, 86, 85–97.