"Abnormal" Schistosome Oviposition

Origin of Aberrant Shell Structures and Their Appearance in Human Tissues

Donald H. Kelley, MD and Franz von Lichtenberg, MD

A SUBSTANTIAL PROPORTION OF SCHISTOSOME EGGS normally contain nonviable or degenerated miracidia[1]; this has been interpreted as an example of the process of germ-cell blighting which is common to many reproductive cycles, particularly those in which egg production is rapid. Schistosome oviposition qualifies as an example of extremely rapid egg manufacture; a mean of 3500 eggs/day is produced by the most prolific human pathogen, *Schistosoma japonicum*.[2] This high-speed mechanism requires a relatively complex supply system, including separate ducts for transmission of germ cells (oocytes, originating in the ovary), and of yolk and shell material (vitellocytes, coming from the vitellaria). These ducts fuse just proximally to the chamber (ootype) where the egg components are assembled.

Defects in eggshell production, during the life of the parasite in untreated hosts, have received little attention. Interest in vitellogenesis has recently been kindled through studies of the newer antischistosomal compounds. Many of these compounds induce dramatic degenerative changes in the vitellaria, either as an early effect or as a final common endpoint preceded by alterations in other metabolic pathways.[3] Compounds such as TAC (tris *p*-aminophenyl carbonium salts),[4] niridazole,[4,5] SN 10,275, Wellcome 153C51, and Hoechst S-688 [6] have also been held to cause morphologic and functional changes in the vitellaria and their products. An early stage of this effect includes the presence of small, abortive egg-like structures in the ootype, well-illustrated in an article by Bueding.[4]

Changes similar to these had been described by Vogel and Minning [7] in *Schistosoma japonica* females that were regenerating after subcura-

From the Department of Pathology, Peter Bent Brigham Hospital, Harvard Medical School, Boston, Mass.

Supported by Grant AI-02631 from the United States-Japan Cooperative Medical Science Program, administered by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health, Department of Health, Education and Welfare, and by Contract DA-49-193-MD-2253 from the Armed Forces Epidemiological Board, Office of The Surgeon General.

Accepted for publication April 30, 1970.

Address for reprint requests: Dr. Franz von Lichtenberg, Department of Pathology, Peter Bent Brigham Hospital, 721 Huntington Ave, Boston, Mass 02115.

tive doses of tartar emetic. These authors gave the name *dwarf egg* to conglomerates of vitelline material found in the parasite uterus. Gonnert [8,9] used this term to designate an oocyte that had passed through the ootype receiving little if any shell covering. The latter author, in describing the effects of Miracil D on S *mansoni,* depicted the occurrence of spherical vitelline conglomerates in the livers of treated mice. An implicit assumption in such previous work would appear to be that eggshell formation is completely efficient in the untreated parasite.

In the course of earlier work on the pathogenesis of schistosome granulomas,[10-14] one of the authors (FL) had described [14] small pigmented particles in purified suspensions of eggs and of eggshells prepared by various methods. Although it is felt that these particles essentially correspond to the dwarf eggs of Vogel and Minning, use of the term *vitelline conglomerates* is proposed in order to avoid the confusion which has arisen regarding the older name.

The following work details our finding of these and other abnormal products of egg formation in normal female worms, and in experimentally infected host tissues. The origin of such products is traced through detailed examination of the female generative tract and its contents.

Materials and Methods

Female Worm Studies

Adult Swiss white mice of both sexes (average weight, 20 g) were sacrificed by cervical dislocation approximately 11 weeks after exposure (by tail immersion) to an infecting dose of cercariae of *Schistosoma mansoni* (Puerto Rican strain), S *haematobium* (Iranian strain), or S *japonicum* (Japanese strain). Whereas 12 mice were used for the study of S *mansoni,* 8 mice each were employed in the investigations of the other two species. The average worm yield/ mouse was roughly 10 females in all species.

After the mouse was sacrificed, an immediate abdominal evisceration was performed. Under a stereoscopic dissectiong microscope, worms were gently aspirated from the proximally transected portal vein using a Pasteur pipet. Additional worm pairs were carefully teased from mesenteric veins with fine teasing needles.

Separation of worm pairs was facilitated by immersion in Hank's solution containing 10^{-4} M carbachol.[4] Although this concentration caused enough muscle relaxation to allow worms *in copula* to be teased apart easily, this effect was rapidly reversed by immersing the worms in plain Hank's solution. Since Monteiro *et al,*[6] observed that artifactual oogenetic abnormalities (type not specified) can result when worms are allowed to stand at room temperature for a considerable length of time, the interval from evisceration to fixation was kept as short as possible.

Females that were to be examined in the fresh state were suspended in several drops of buffered Hank's solution on microscope slides, and coverslips gently applied.

Additional worms were fixed by successive immersion in Bouin's solution, fol-

lowed by increasing strengths of alcohol in Bouin's, then stained with Delafield's hematoxylin and mounted in glycerine.[15]

A final group of worms was fixed in osmium tetroxide s-collidine (Oscol), then dehydrated in cold absolute alcohol, impregnated with propylene oxide, and embedded in Epon 812. The plastic blocks were sectioned with glass knives on a Sorvall MT-1 ultramicrotome at 0.5 μ; the sections were stained with toluidine blue and mounted in Permount. Three females thus embedded were transversely sectioned in a serial manner from the caudalmost extension of the oviduct to the cephalic end of the ootype. All other worms were sectioned in a longitudinal orientation.

Host Tissue Studies

Formalin-fixed liver tissue from multimammate rats *(Mastomys coucha)*, obtained approximately 11 weeks after percutaneous infection with *S mansoni* cercariae, was stained by either the hematoxylin-eosin or acid-fast (Ziehl-Neilsen) technics.

Formalin-fixed human bladder and liver necropsy tissues were available from *S haematobium* and *S mansoni* infections, respectively. The sampling included tissues from untreated patients as well as from patients who had received a variety of antischistosomal drugs. Fresh-frozen, *S mansoni*-infected, white mouse liver tissue was obtained at necropsy evisceration for worm removal, as noted above. Five-micron-thick sections were produced on a cryostat, and alternate sections stained with hematoxylin and eosin; the remaining sections were left unstained for fluorescent microscopy.

Egg Suspensions

Saline suspensions of *S mansoni* eggs were obtained from KOH digests of murine liver according to the method of Coker and von Lichtenberg.[16]

Comparative measurements of abortive egg products were made from egg suspensions, whole worm mounts, and tissue sections using an American Optical ocular micrometer.

Whole worm preparations, fresh and unstained, were examined by bright-field phase-contrast microscopy using American Optical apparatus.

Autofluorescence of vitellocytes, eggs, and egg-like particles was examined using a Zeiss microscope equipped with an Osram 200 HBO burner, 0.8 mm dry darkfield condenser, exciter filters BG-12 (4 mm), UG-2 and B6-38, neutral filter 0.7 and barrier filter 50.

Results

General Observations of Female Reproductive Tract

Each female was examined for the presence of a sperm conduit corresponding to the Laurer's canal observed in other trematodes,[17] but this was not visible in our material.

By the plastic-embedding technic, spermatozoa were especially distinct as they lay radially aligned with their larger ends adherent to the oviduct luminal surface (Fig 1). Although most densely grouped in the proximal oviduct, in a slightly dilated initial segment which has been designated the seminal receptacle,[17] spermatozoa and their very long,

thread-like tails extended for a considerable distance distally toward the ootype. Whereas oocytes were found in the proximal oviduct in close proximity to spermatozoa (Fig 1), no penetration of oocytes by sperm cells was observed.

The vitellaria and vitelline duct contents showed green autofluorescence; only in the completed eggshell found in ootype and uterus was there the orange-yellow hue typical of shells within host tissues (Fig 2).

Evidence of Faulty Oogenesis

The existence of contractile tissue in the walls of oviduct and vitelline duct was indicated by propulsive motions of their contents in fresh preparations (often to and fro, similar to the observations of Monteiro *et al* in worms from hosts treated with Hoechst S-688, niridazole, or TAC pamoate).[6] Sphincters that prevented retrograde motion of cells at the junction of the two ducts were not observed, however, in contrast to the descriptions of Looss [18] and Gonnert.[19] Reflux of highly refractile vitellocytes into the oviduct was observed as it occurred on several occasions (Fig 3), illustrating the lack of competent sphincteric action at this junction.

The most frequently observed defect in egg formation was the passage of oocytes into the uterus. These cells could be identified by their nonfluorescent characteristics (Fig 4).

Excess vitelline material was occasionally seen lying external to an already formed eggshell in the ootype (Fig 5). A vitelline conglomerate corresponding to the dwarf egg of Vogel and Minning was rarely detected in the uterus of a mature female (Fig 6). Such particles have a highly refractile, amber-brown appearance, with a smooth or knobby outline. Their greatest diameters, as measured in worm uteri, tissue sections and egg suspensions (Fig 7), average approximately 27 μ.

The presence of these vitelline conglomerates could be screened for in frozen tissue sections by detection of their orange-yellow autofluorescence, a characteristic shared by the schistosome eggshell (Fig 8). These particles, when found in the tissues of *S mansoni*-infected mice, were also demonstrated to have the same acid-fast staining property as the eggshell of this species.[20] Schistosomal pigment aggregates and bile plugs were negative by both autofluorescent and Ziehl-Neilsen technics.

Vitelline conglomerates were found most frequently near the periphery of the schistosomal pseudotubercle in host tissues, and are demonstrated within liver parenchyma in the presence of natural human or experimental infections with *S mansoni* in animals (Fig 9–11) and

in the bladder wall of a human with S *haematobium* infection (Fig 12).

In the necropsy tissues of one Egyptian case of human mansonial schistosomiasis, in which hepatic changes had progressed to Symmer's pipestem fibrosis, these particles outnumbered the schistosome ova (Fig 9). This indicates the possibility that these particles are removed rather slowly from liver tissue, and suggests a possible means for confirming the etiologic relationships in those inactive, previously undiagnosed cases where the pattern of residual tissue damage is not considered pathognomonic in the absence of eggshells.

Discussion

Recently published reviews of schistosome physiology and chemotherapy [6,21,22] fail to associate aberrations in eggshell formation with the normal host-parasite relationship (with the exception of an illustration published by Senft [21] depicting excessive vitelline material surrounding the egg in the ootype). This phenomenon had been described by Looss in his classic paper.[18]

According to the current concepts of schistosome oviposition, approximately 40 vitelline cells, originating in the vitellaria and passing anteriorly along the vitelline duct, normally surround a single oocyte on its arrival within the ootype. At this point, the formation of a shell membrane around the group of cells ensues, an event thought by some to be mediated by the combined influences of the ootype epithelium and Mehlis' gland secretions.[19] Histochemical and extraction studies on schistosomes and other trematodes [23–25] suggest that phenolic compounds, contained within the vitellocyte cytoplasmic granules, are oxidized to quinones under the influence of a phenolase, resulting in tanning of structural proteins to a sclerotin-like shell substance, which is deposited in concert with lipoprotein membranes originating from Mehlis' gland. We have observed dark, granular (shell precursor-containing?) and light-staining (yolk lipid-containing?) inclusions in toluidine blue-stained schistosome vitellocytes, correlating well with the ultrastructural findings of Senft.[21] It would appear that the granular inclusions impart the green autofluorescent character to vitellocyte cytoplasm. The transition from green to orange-yellow autofluorescence after shell formation in the ootype would be consistent with a physicochemical change in the fluorescent species. This change in wavelength of fluorescence is coincident with a change in ordinary light microscopic appearance from colorless material in vitellocytes to yellowish shell substance as eggs traverse the ootype and uterus. Clegg and Smyth [23] have drawn a comparison between the latter change (in the color of

transmitted light) and the hardening and darkening of insect cuticles and egg cases, where it is known that the chemical process taking place is sclerotization—the stabilization of protein by quinone tanning.

Ovular bodies, very similar to the vitelline particles we describe, have been reported by Paraense [26] in experimental unisexual infections (where only female cercariae were allowed to invade the host). This author ascribed his observations wholly to the absence of fertilization by the male. Abortive eggs were apparently found with greater frequency than in our bisexually infected material; however, Paraense failed to detect an oocyte as a part of these bodies (paralleling our experience), which we feel is evidence in favor of primarily imperfect egg formation rather than developmental failure in an otherwise normal, but unfertilized egg. An increased frequency of this abnormality in unisexual female infections could perhaps be attributed to less specific effects of the male's presence.

Although aberrations in egg formation of the types described were present in our material from untreated hosts, they appear to be much more numerous in parasites from hosts receiving antischistosomal compounds.[4-6] This quantitative, rather than qualitative difference indicates the exaggeration of an already present tendency, which may be the occasionally dysynchronous motion of oocytes and vitelline cells into the ootype.

Whereas it could be contended that artifactual propulsion of these products occurs during the physical manipulations prior to examination of whole worms, this criticism applied to drug bioassays as well and fails to explain the presence of vitelline conglomerates in the tissue sections from untreated experimental hosts. The effects of the anticholinergic drug, carbachol, are similarly excluded by the findings in the host tissue.

One would not expect that vitelline conglomerates would play a major role in eliciting a cellular host response and subsequent tissue damage, judging not only from the paucity of inflammatory cells surrounding them in tissue sections, but also from the work of von Lichtenberg and Raslavicius,[14] which showed that purified eggshells were much less capable of engendering granuloma formation than intact eggs. From this and other [27,28] studies, it was concluded that the most readily demonstrable antigen was miracidial in origin, a secretion of the cephalic glands. On the other hand, Striebel [5] ascribes the plug-like accumulation of a "leukocyte granuloma" at the anterior end of the female schistosome to the rapid discharge of reproductive products during that stage when

the vitellaria and ovary are being depopulated by drug treatment. Striebel considers this rapid "antigenic release" to be correlated with the concurrent development of circulating schistosomal antigen.

It is presently impossible to assess the importance of other abortive egg products, such as "naked" oocytes, in this regard, as these have not yet been identified in host tissues.

Summary

Schistosome oviposition, a remarkably rapid and largely efficient process, occasionally results in aberrations of egg structure, notably conglomerates of vitelline (eggshell) material. That the deposition of these particles of apparently pure shell substance is usually unattended by a cellular host-response is additional evidence for assuming that the main antigenic stimulus is miracidial in origin. The passage of excess vitelline material into the ootype from the vitellaria has been documented, and appears to be a necessary condition before quinone-tanning and sclerotization of proteins and thereby the formation of characteristic, rounded refractile bodies. The detection of vitelline conglomerates in tissues, even under circumstances when eggs are difficult to find, suggests that an otherwise nondiagnostic histopathologic pattern of liver fibrosis might be related to a schistosomal etiology with the detection of these particles. Such studies would best include examination for autofluorescence.

References

1. Hernández-Morales, FH, Maldonado JF: The diagnosis of schistosomiasis mansoni by a rectal biopsy technique. Amer J Trop Med Hyg 26:811–820, 1946
2. Moore DV, Sandground JH: The relative egg-producing capacity of *Schistosoma mansoni* and *Schistosoma japonicum*. Amer J Trop Med 5:831–840, 1956
3. Bueding E, Fisher J: Biochemical effects of schistosomicides. Ann NY Acad Sci 160:536–543, 1969
4. Bueding E, Schiller EI, Bourgeois JG: Some physiological, biochemical and morphologic effects of tris (p-amino-phenyl) carbonium salts (TAC) on *Schistosoma mansoni*. Amer J Trop Med 16:500–515, 1967
5. Striebel HP: The effects of niridazole in experimental schistosomiasis. An NY Acad Sci 160:491–518, 1969
6. Monteiro W, Pellegrino J, Silva MLH: Some physiological and morphological changes in egg formation of *Schistosoma mansoni* following treatment with antischistosomal agents. Rev Brasil Pesq Med Biol 2:45–49, 1969
7. Vogel H, Minning W: Ueber die Einwirkung von Brechweinstein, Fuadin

und Emetin auf *Bilharzia japonica* und deren Eier im Kaninchenversuch. Acta Trop 4:21–56, 97–116, 1947

8. Gonnert R: Schistosomiasis-Studien II. Ueber die Eibildung bei *Schistosoma mansoni* und das Schicksal der Eier im Wirtsorganismus. Z Tropenmed Parasit 6:33–52, 1955
9. *Idem:* Schistosomiasis-Studien III. Ueber die Einwirkungen von Miracil D auf *Schistosoma mansoni* im Mäuseversuch und die Verteilung des Pigmentes in der Wirtsleber. Z Tropenmed Parasit 6:257–279, 1955
10. von Lichtenberg F: Host response to eggs of *Schistosoma mansoni.* I. Granuloma formation in the unsensitized laboratory mouse. Amer J Path 41:711–731, 1962
11. von Lichtenberg F, Sadun EH, Bruce JI: Host response to eggs of *Schistosoma mansoni.* III. The role of eggs in resistance. J Inf Dis 113:113–132, 1963
12. von Lichtenberg F: Studies on granuloma formation. III. Antigen sequestration and destruction in the schistosome pseudotubercle. Amer J Path 45: 75–94, 1964
13. Jaimes S, von Lichtenberg F: Host response to eggs of *Schistosoma mansoni.* IV. Fluorescent antibody titers in mice infected with normal cercariae, gamma-radiated cercariae and with purified eggs. Amer J Trop Med 14: 737–735, 1965
14. von Lichtenberg F, Raslavicius P: Host response to eggs of *Schistosoma mansoni.* V. Reactions to purified miracidia and egg shells and to viable and heat-killed whole eggs. Lab Invest 16:892–904, 1967
15. Mackie TT, Hunter GW III, Worth CB: A Manual of Tropical Medicine. Philadelphia, WB Saunders Co. 1945, p 665
16. Coker CM, von Lichtenberg F: A revised method for isolation of *Schistosoma mansoni* eggs for biological experimentation. Proc Soc Exp Biol Med 92: 780–782, 1956
17. El-Gindy MS: Post-cercarial development of *Schistosomatium douthitti* (Cort, 1914) Price 1931 in mice, with special reference to the genital system (Schistosomatidae-trematoda). J Morph 89:151–185, 1951
18. Looss A: Zur Anatomie und Histologie der *Bilharzia haematobia* (Cobbold). Arch Mikrosk Anat 46:1–108, 1895
19. Gonnert R: Schistosomiasis-Studien. I. Beiträge zu Anatomie und Histologie von S. *mansoni.* Z Tropenmed Parasit 6:18–33, 1955
20. von Lichtenberg F, Lindenberg M: An alcohol-acid-fast substance in eggs of *Schistosoma mansoni.* Amer J Trop Med 3:1066–1076, 1954
21. Senft AW: Considerations of schistosome physiology in the search for antibilharziasis drugs. Ann NY Acad Sci 160:571–592, 1969
22. Pellegrino J, Katz N: Laboratory evaluation of antischistosomal agents. Ann NY Acad Sci 160:429–460, 1969
23. Clegg JA, Smyth JD: Growth, development and culture methods—parasitic platyhelminths, Chemical Zoology. Vol 2. Edited by M Florkin, BT Scheer. New York, Academic Press, 1968, pp 395–466
24. Clegg JA, Morgan J: The lipid composition of the lipoprotein membranes on the egg-shell of *Fasciola hepatica.* Comp Biochem Physiol 18:573–588, 1966
25. Burton PR: A histochemical study of vitelline cells, egg capsules, and

Mehlis' gland in the frog lung-fluke, *Haematoloechus medioplexus*. J Exp Zool 154:247–258, 1963

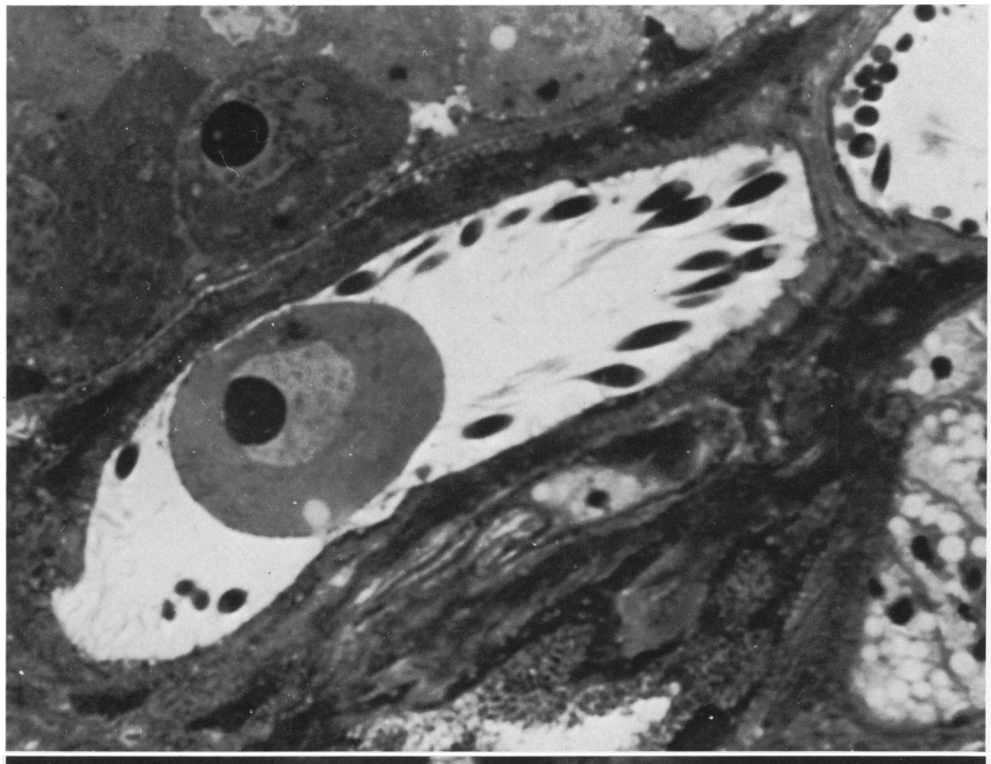
26. Paraense WL: Further observations on the sex of *Schistosoma mansoni* in the infestations produced by cercariae from a single snail. Mem Inst Cruz 47:547–556, 1949
27. von Lichtenberg F: The bilharzial pseudotubercle: a model of the immunopathology of granuloma formation, Immunologic Aspects of Parasitic Infections. Pan American Health Organization, Publ. No. 150, 1967
28. Smith HJ, von Lichtenberg F: The Hoeppli phenomenon in schistosomiasis. Amer J Path 50:993–1007, 1967

The technical collaboration of Mrs. Katherine Connolly and Mrs. Barbara Doughty is gratefully acknowledged. Parts of this project were greatly aided by the advice and critique of Jerome H. Smith, MD.

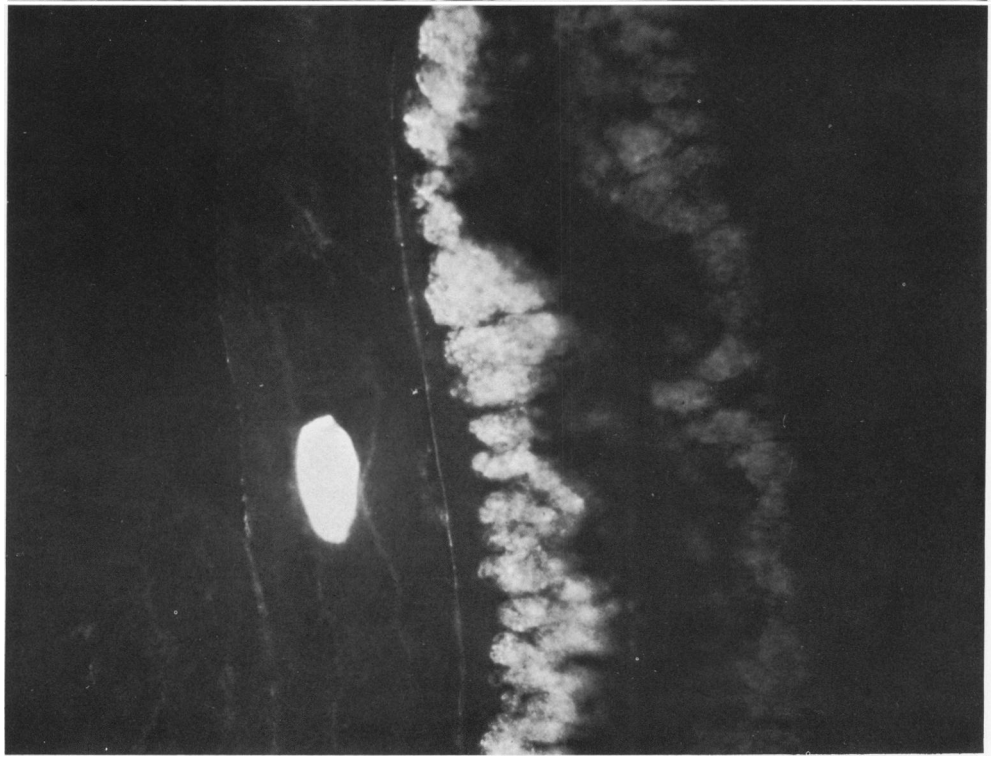
[Illustrations follow]

Fig 1. Oocyte within proximal oviduct of *S mansoni*. Spermatozoa are attached to ciliated oviduct wall. Ovary lies to upper left, vitelline duct to lower right. Toluidine blue stained, Epon-embedded. Approx × 1860.

Fig 2. This *S mansoni* female is doubled back upon herself in such a way that egg in uterus *(left)* and vitellaria *(right)* are juxtaposed. On examination of autofluorescence, formed eggshell is yellow-orange, whereas vitellaria have greenish hue. Unstained fresh whole worm preparation. Ultraviolet illumination. Approx × 112.



1

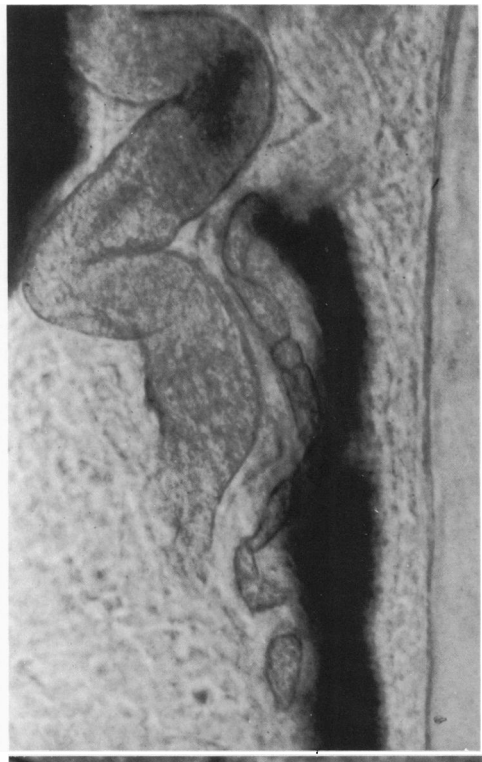


2

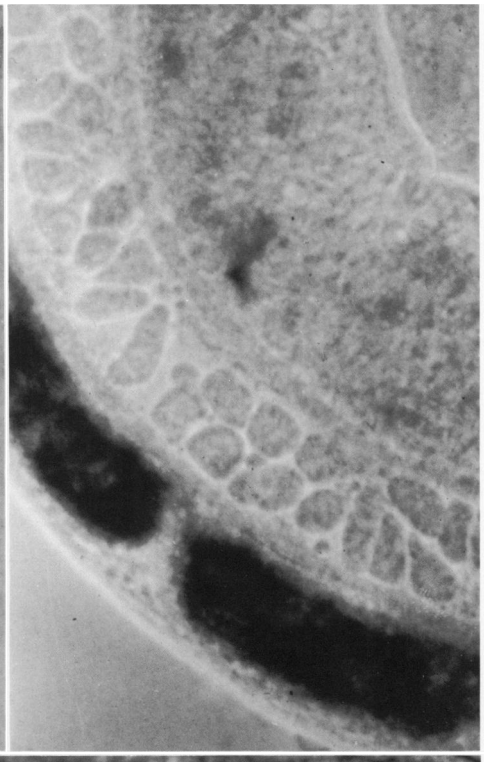
Fig 3. Refractile vitelline masses occupy smaller oviduct (*coiled structure on right*), as well as larger vitelline duct, after reflux of vitelline material into oviduct. Very dark shadows above plane of focus are portions of the blood-pigment-containing alimentary tract. Unstained *S mansoni* fresh whole worm preparation. Phase contrast. Approx × 400.

Fig 4. Nonrefractile germ cells (oocytes) within uterine canal of *S mansoni*. These cells did not exhibit autofluorescence. Unstained fresh whole worm preparation. Phase contrast. Approx × 300.

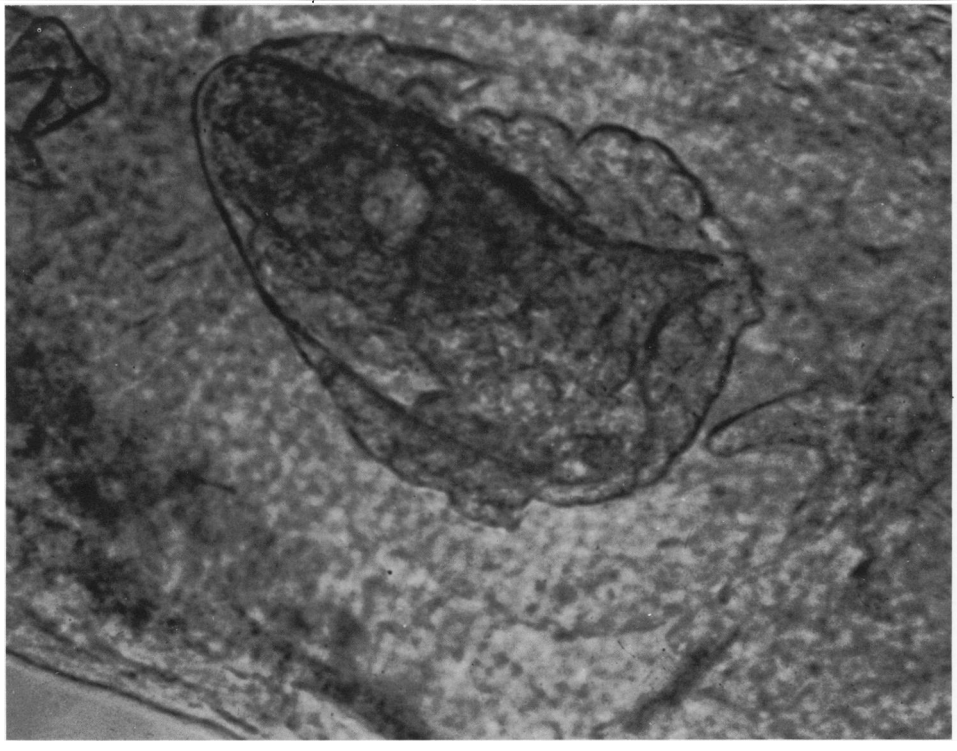
Fig 5. Excess vitelline material surrounds already formed eggshell in ootype (egg-assembling chamber) of *S mansoni*. Mehlis gland filaments can just be discerned to lower right. Unstained fresh whole worm preparation. Phase contrast. Approx 500 ×.



3



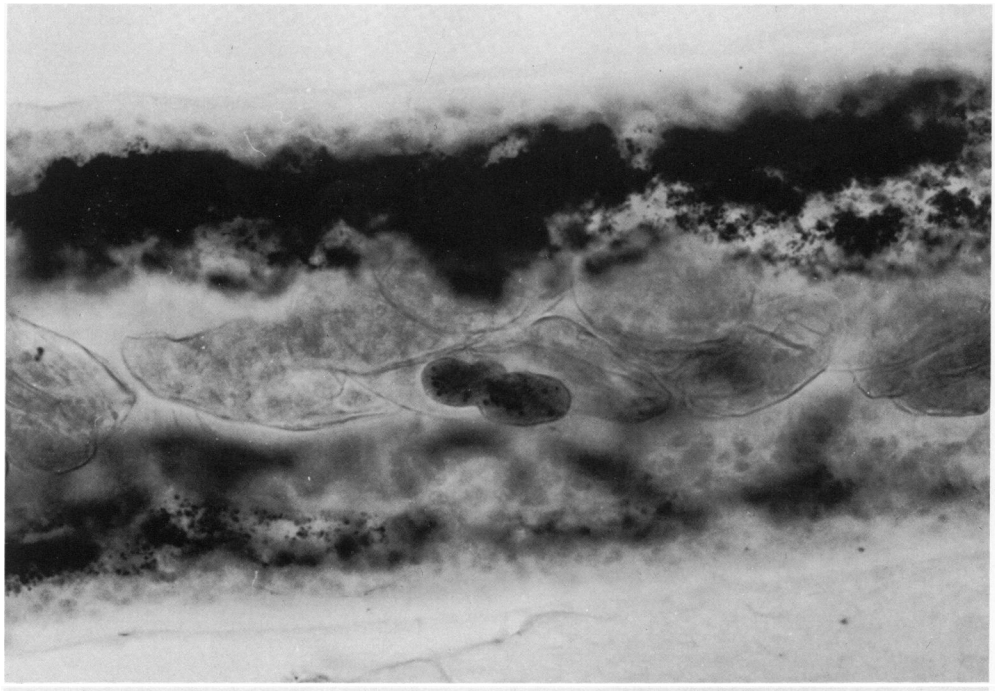
4



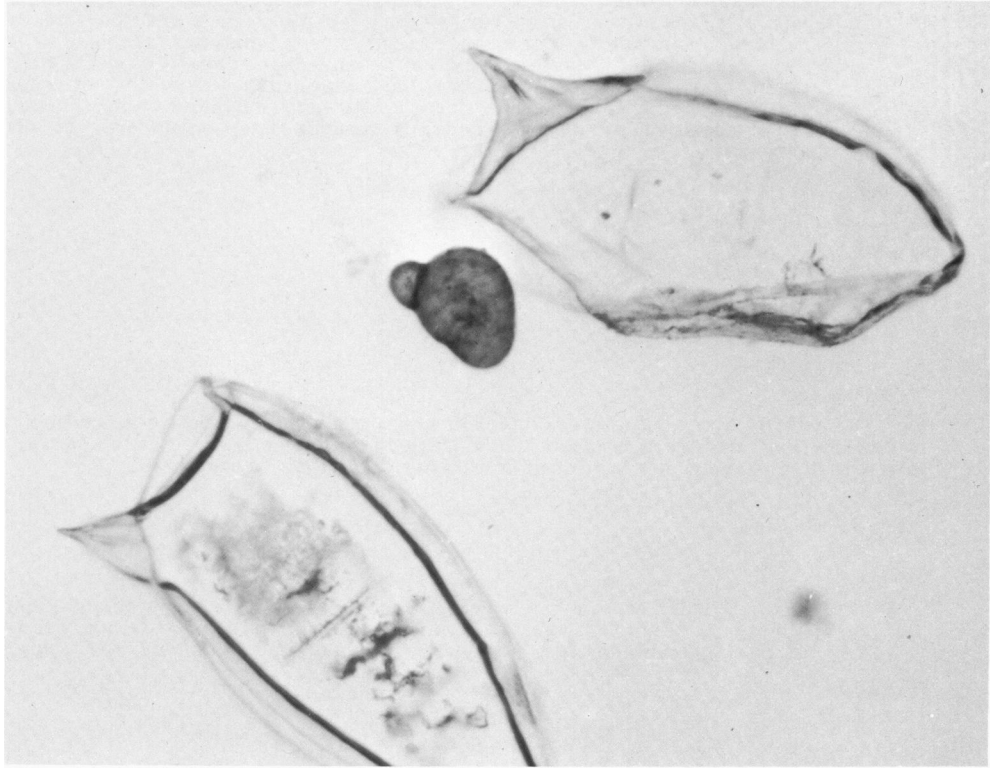
5

Fig 6. Rounded conglomerates of vitelline material along with numerous well-formed eggs in uterus of *S japonicum*. Internal granulation and relatively dark color of vitelline conglomerates can be easily appreciated here. Whole worm preparation. Delafield's hematoxylin. Approx × 465.

Fig 7. Egg suspension prepared by KOH digestion of *S mansoni*-infected white mouse livers. Note vitelline conglomerate between two eggshells of normal size. Unstained saline mount. Approx × 465.



6



7

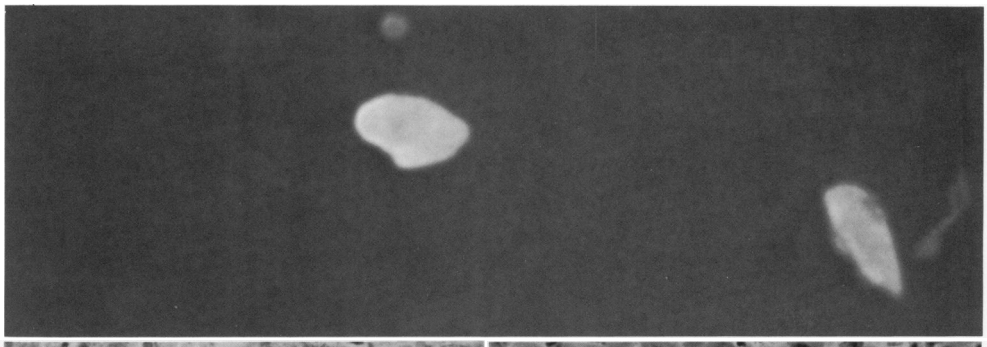
Fig 8. Autofluorescence of *S mansoni*-infected white mouse liver. Small round vitelline conglomerate (*upper left center*) exhibits same orange-yellow color as do shells of intact or partially absorbed eggs (*larger structures*). Unstained cryostat section. Ultraviolet illumination. Approx × 150.

Fig 9. Necropsy liver from human case of schistosomiasis mansoni (from Egypt). Eggshells were extremely scarce in this chronic, inactive infection. Much more numerous in liver were vitelline conglomerates such as one (*arrow*) pictured near fibrotic portal zone. H&E. Approx × 150.

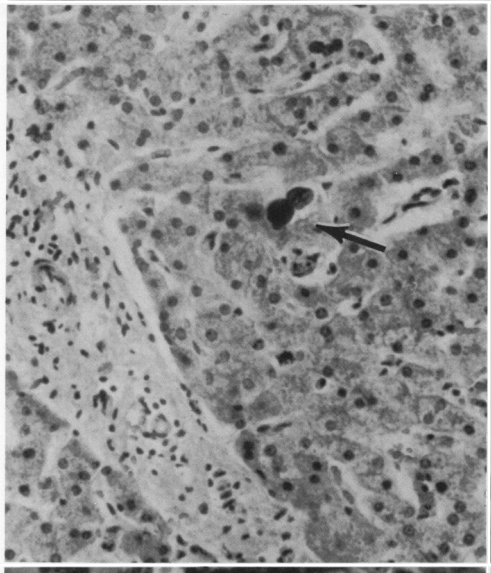
Fig 10. Experimental *S mansoni* infection in *Mastomys* liver. Both artifactually collapsed egg (*center*) and small vitelline conglomerate (*above at arrow*) are associated with little inflammatory response. In case of egg, this is probably due to recent arrival at this site, and little elaboration of miracidial antigens as yet. Often vitelline conglomerates are located at considerable radial distance from nearest egg, suggesting that their smaller sizes allow deeper centrifugal migration along sinusoids, often to extreme periphery of granulomatous reaction later engendered by egg. H&E. Approx × 150.

Fig 11. *Mastomys* liver after 11-week infection with *S mansoni*. Fully developed reaction to partially digested egg is evident (*below*). Granular vitelline conglomerate (*above*) is apparently only incidentally involved in this reaction. H&E. Approx × 465.

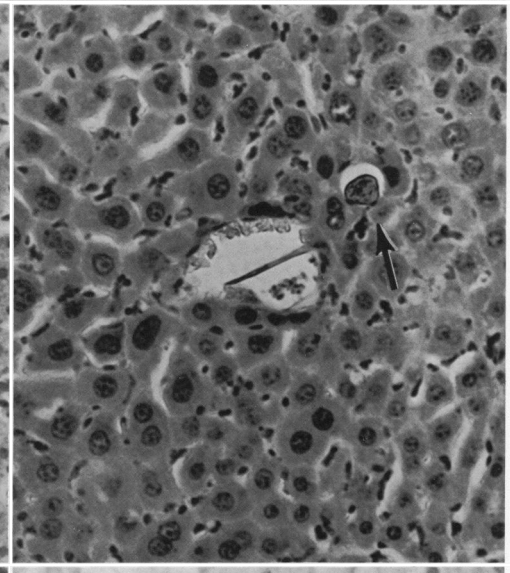
Fig 12. Bladder wall in human case of schistosomiasis haematobium (bilharziasis). In this field, well-formed eggs in various states of preservation and darker-colored vitelline conglomerates are present in approximately equal numbers. H&E. Approx × 150.



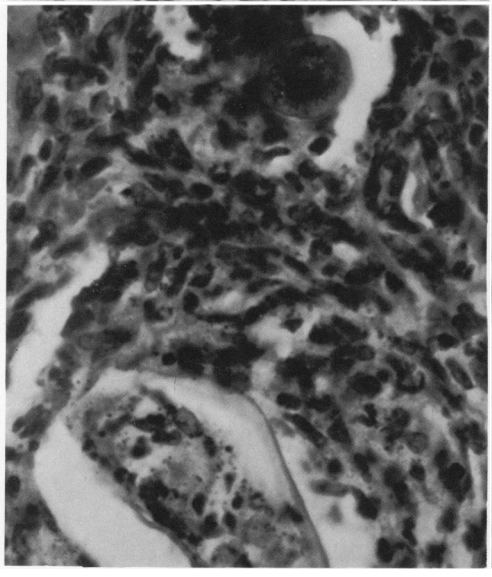
8



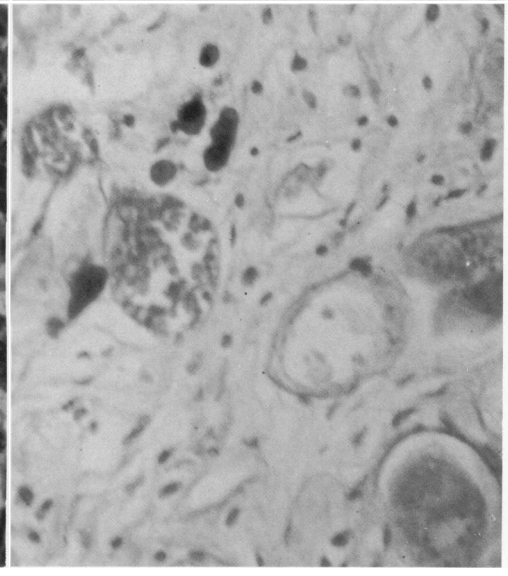
9



10



11



12

[*End of Article*]