STUDIES ON SIPUNCULID BLOOD: IMMUNOLOGIC PROPERTIES OF COELOMIC FLUID AND MORPHOLOGY OF "URN CELLS". (*)

by

Frederik B. Bang

and

Betsy G. Bang.

Department of Pathobiology, School of Hygiene and Public Health, The Johns Hopkins University, Baltimore, Maryland, The Marine Biological Laboratory, Woods Hole, Massachusetts, and the Station Biologique de Roscoff, Finistère, Brittany.

Résumé

Les réactions du sang de deux Sipunculides marins en réponse aux injections d'organismes étrangers sont décrites. Le liquide coelomique de *Phascolosoma (Goldfingia) gouldii* est stérile et peut détruire des concentrations massives de Bactéries. Le liquide coelomique de *Sipunculus nudus* a les mêmes propriétés et est aussi capable d'acquérir la possibilité d'immobiliser et de lyser de grandes concentrations d'un Protozoaire Cilié parasite. Cette propriété s'atténue après une semaine environ, mais il est possible de provoquer sa réapparition répétée. De nouvelles notions sont présentées sur la structure des urnes bicellulaires qui éliminent sélectivement les substances étrangères ou anormales du liquide coelomique.

I. INFECTION AND REACTION TO INJURY IN THE SIPUNCULIDS.

Sipunculus nudus is one of a large group of marine sipunculid worms. It was a favorite subject of study by Cantacuzène (1928), who kept several of them alive for some years and studied their reactions to injections of bacteria and other foreign substances into the coelomic cavity (Cantacuzène, 1922, 1). He emphasized the role of the urn cells of the coelomic fluid. Originally described by Cuénot, these remarkable muco-ciliated scavengers swim through the fluid and accumulate foreign matter and dispose of it; Cantacuzène demonstrated the cleansing role of these cells working with phagocytes in collecting bacteria and debris onto their mucous tails, then shedding

(*) These studies were sponsored by U. S. Public Health Service Grant E-135 and were carried out during the tenure of a John Simon Guggenheim Memorial Foundation Fellowship.

Cahiers de Biologie Marine
Tome III - 1962 - pp. 363-374.

them to form "brown bodies". He also believed that he evoked a slow immune response in *S. nudus* to repeated injection of a bacterium, a species of Vibrio (Cantacuzène, 1922, 2).

During several summers at the Marine Biological Laboratory, Woods Hole, working with *Phascolosoma* (*Goldfingia*) *gouldii,* and during one summer at the Station Biologique de Roscoff (1) working with *Sipunculus nudus,* we have confirmed the fact that the blood or coelomic fluid of these animals is free of bacteria, and that the fluid becomes clear following the injection of large numbers of bacteria. In *Sipunculus* we have found that a lysin which destroys invading ciliates can be experimentally evoked and can develop rapidly; and lastly have added observations to the record of the structure and action of the urns which scavenge debris in the coelomic fluid.

Material and methods.

Goldfingia gouldii were obtained by the Supply Department of the Marine Biological Laboratory from the ocean waters near Woods Hole, and were kept in running sea water in a minimum amount of sand. Since they survived poorly in the laboratory, most experiments were done within the first few days.

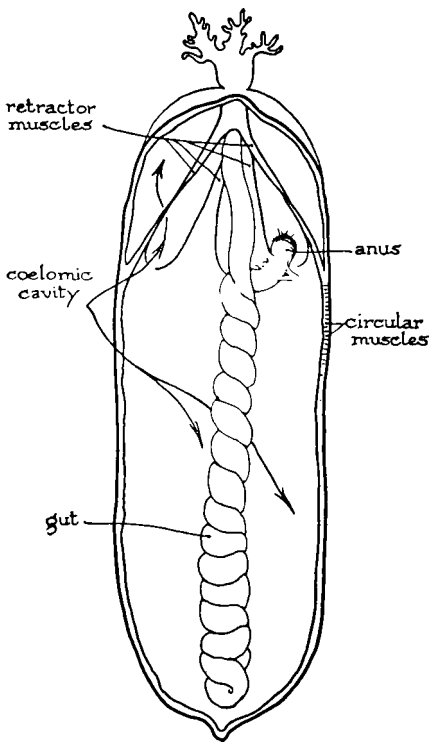


Diagram of *Sipunculus nudus,* omitting all details, to show great size of coelomic cavity.

Sipunculus nudus were obtained from Locquémeau and Crozon on the coast of Finistère in Brittany. Locquémeau was the source used by Cantacuzène; we collected at extremely low tide three times during the summer. The sipunculids were kept in several tanks with running sea water in sand six inches to one foot deep. Once the sand had been used for some weeks, the sipunculids survived very well; mortality of uninjected ones after the initial period was about 5 p. 100.

As pointed out by Cantacuzène, blood is readily drawn from the cavity through a puncture in the posterior portion of the worm, where the circular and longitudinal muscles join in a point (see diagram). When testing for anti-protozoal action, the blood was centrifuged for two minutes at 15,000 rpm and the supernatant tested immediately.

Marine bacteria: Vibrio sp. *limulus,* used in previous work with disease studies in limuli, crassostrea, and echinoderms, was used for much of the *in vitro* study at Woods Hole of the capacity of *Goldfingia* blood to destroy bacteria (Bang, 1955).

Cultures for marine bacteria were grown on Zobell's sea water agar (Zobell, 1946), and when the coelomic fluid of the worms was tested for sterility, the external surface was dried with a paper towel, swabbed

(1) We are indebted to Dr. Georges Teissier, Director of the Station Biologique de Roscoff, for the facilities of the Laboratory and for his continued encouragement.

with 80 p. 100 alcohol, and fluid was drawn with a sterile syringe and needle.

Protozoan: Anophrys sarcophaga was found by chance in an infected crab, *Carcinus maenas,* at Roscoff and was kept by continual passage every few days into new uninfected crabs. It was cultured on several occasions, but no attempt was made to maintain it in culture.

Results

Repeated tests of the blood of *Goldfingia gouldii* for bacteria, by culture on Zobell's sea water agar, showed that this fluid was free of bacteria, and no bacteria were found in repeated phase microscope examinations of the blood. This led to the question of the mechanisms by which bacteria were disposed of once they had gained entrance. Moderately cloudy suspensions of several unidentified marine bacteria, including *Vibrio* sp. *limulus* were injected into the coelomic fluid. In almost all cases these bacteria were either destroyed or disposed of, because cultures of the fluid some 24 hours later showed that the fluid was sterile. An occasional worm became sluggish, yielded a positive culture and smears, and died within one to two days after injection.

Blood removed from the worms was incubated at room temperature with varying concentrations of different bacteria (Bang and Krassner, 1958). In a number of individual tests such blood became sterile within six to twenty four hours. Consistent sterility was obtained within six to twenty-four hours when the combination of bacteria and blood was kept at 0°C. Various control preparations of these bacteria were kept alive for days at this temperature. At 0°C incubation, there was destructive activity by both serum and cells. Destruction of 1 million organisms was obtained with 0.2 cm^3 of whole blood within twenty-four hours.

Direct observations of the effect of the serum on the bacteria and of previous injections of the Goldfingia on the capacity of the serum to destroy the bacteria, would be of great interest in the light of the subsequent findings with *Sipunculus nudus.*

Response of Sipunculus nudus to the protozoan ciliate Anophrys

We studied the response of *Sipunculus nudus* to the marine protozoan, *Anophrys sarcophaga,* which is a natural parasite of the shore crab, *Maia.* This ciliate is known to reach a high density in the blood of the crab, and to eat its amebocytes (Poisson, 1930). When anophrys were added to cultures of blood or separated plasma of most normal sipunculids, the blood had no effect on the ciliates within a period of one to two hours' *in vitro* observations. Repeated bleedings showed no change in the effect of sipunculid blood on anophrys over a period of five weeks. However (in eight different experiments), in response to doses of 300 to 3000 or more parasites in .2 cm^3 of crab blood, whole worms regularly produced in 20 to

48 hours a heat labile substance which would lyse any parasites subsequently introduced into cultures of the blood of those worms (Charts 1 and 2). Within one to two minutes of mixture of plasma with the parasites the latter were immobilized, then lost their cilia, rounded up, and in the ensuing 10 to 15 minutes were completely

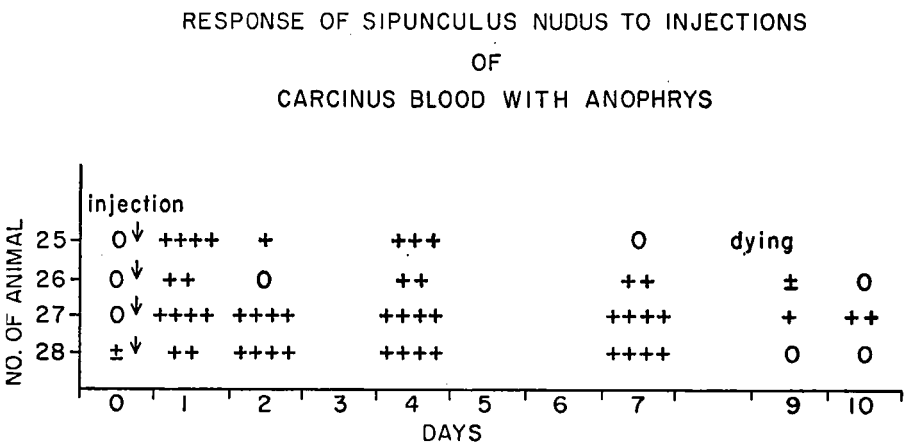


Chart 1

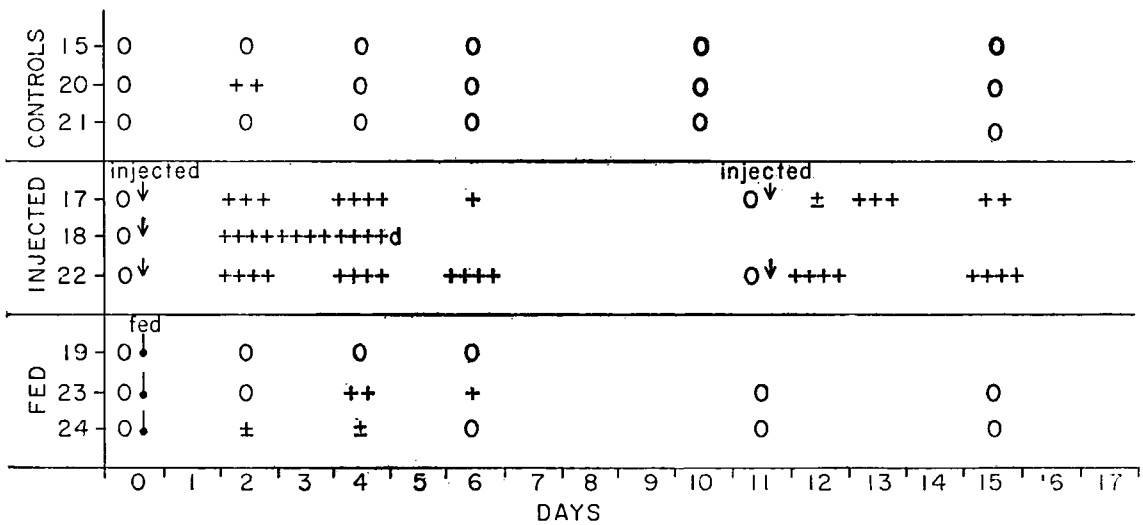


Chart 2

lysed so that only their outlines were recognizable (Plate 1, A and B).

The development of this capacity to destroy the ciliates was also provoked by the injection of normal crab blood or of bacterial suspensions (Chart 3), but only an irregular and weak reaction developed following the injection of normal unsterilized sea water (Chart 4). In view of the different kinds of stimuli used to provoke the reaction, it seems unlikely that the lysin is active only against *Anophrys* or even

against ciliates. No other protozoan species were tested. During the process of immunization—or changed reaction—no consistent change in the pH of the blood was observed (1), and the origin of the substance is not known. It may well be present in one of the numerous cell types of the coelomic fluid and may be released from the cell on stimulus. Known positive blood tested one to two hours after withdrawal often lost its formerly strong activity on standing

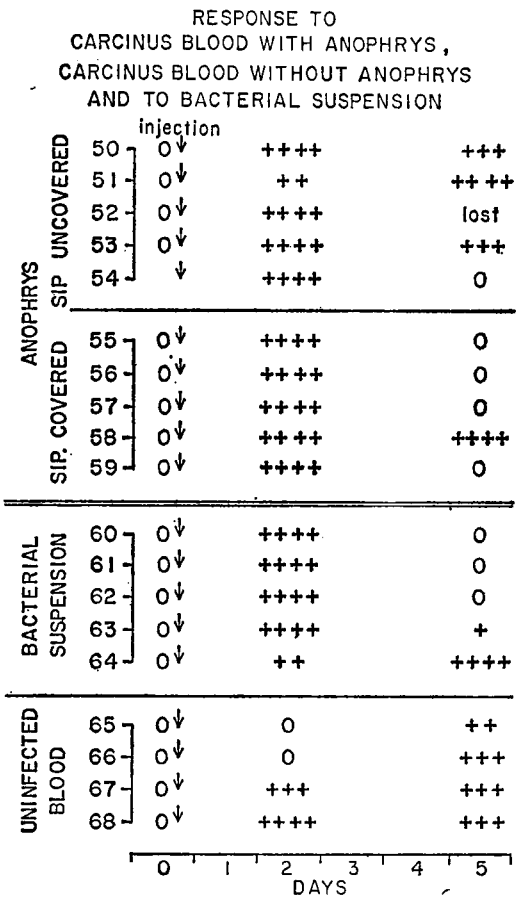


Chart 3

at room temperature. An effect of a positive serum was demonstrated at dilutions of one to 20 in sea water, but if tested under conditions of greater stability higher titers might be found. The stability of the substance under low oxygen tension should be studied. "Immunized" worms maintained a high activity in their plasma for five to eight days, and then apparently lost it. It reappeared again rapidly following reinjection of the animal (Chart 2). Repeated injection of

(1) Repeated tests with Merck pH papers.

four worms during a period of four to eight weeks failed to raise the titer or apparent strength of the activity (as measured by the rate and amount of destruction of the protozoan and by dilution titrations). No disease was provoked by these injections.

The sipunculids were kept in the laboratory for periods varying from a few days to several months. Of a grand total of 82 tested before injection, 10 had either slight or moderate lytic activity. These

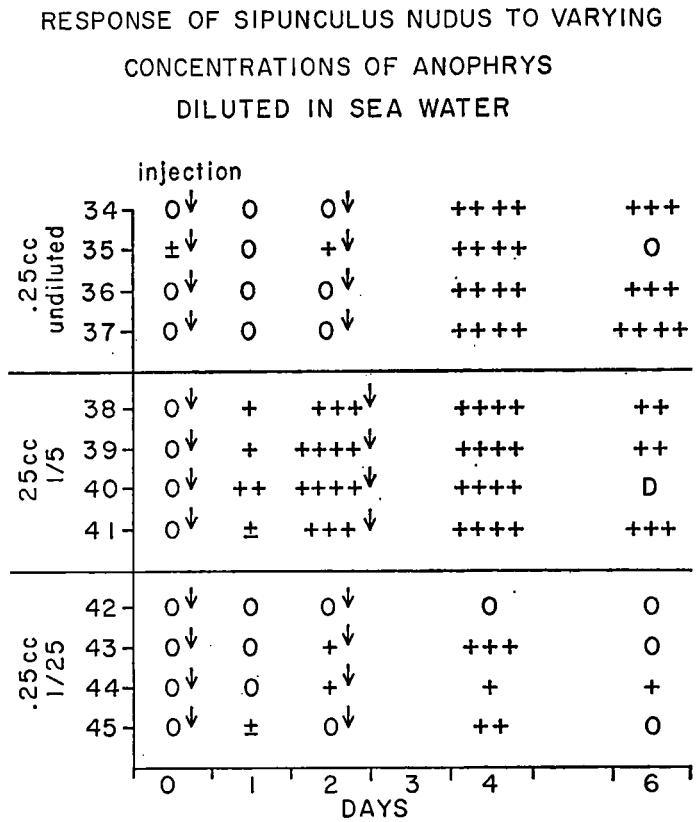
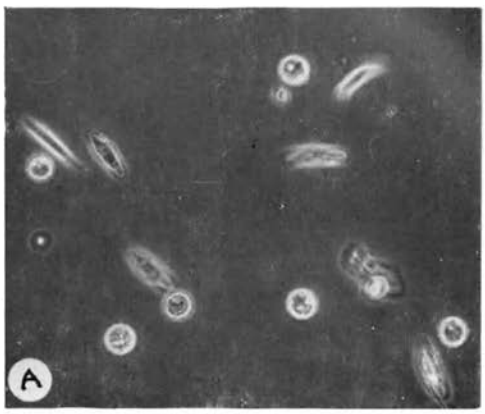


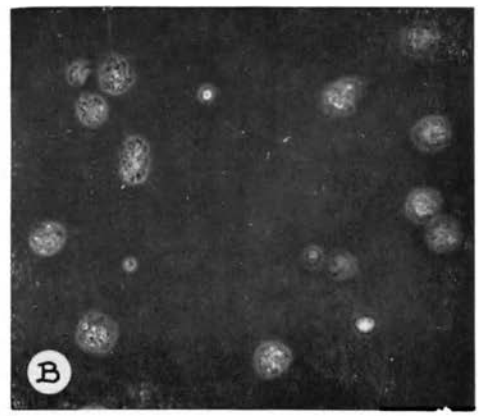
Chart 4

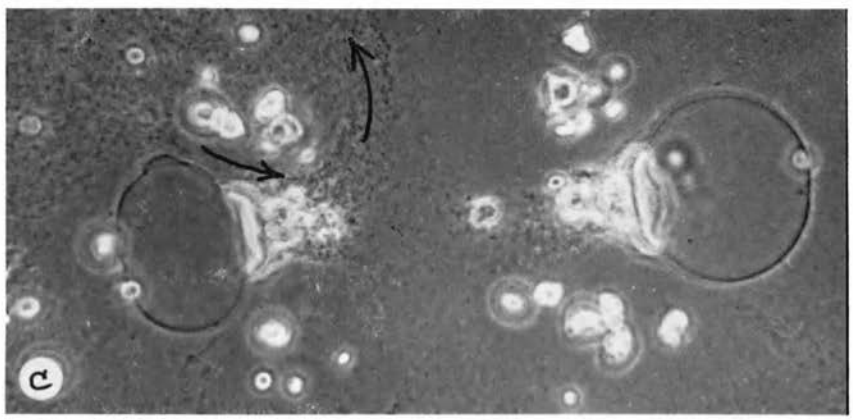
also lost their lysin when tested a few days later. Presumably these positive results in controls may be related to trauma during collecting, or to infection which has been recently overcome.

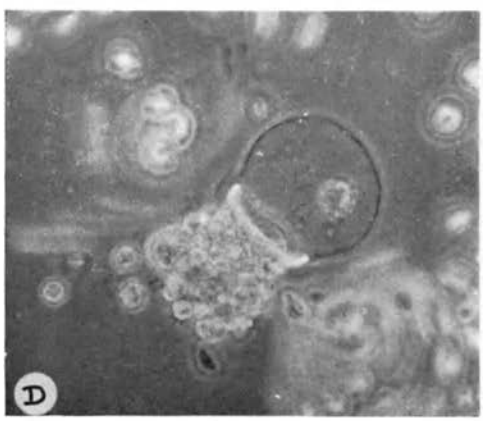
II. STRUCTURE OF URNS.

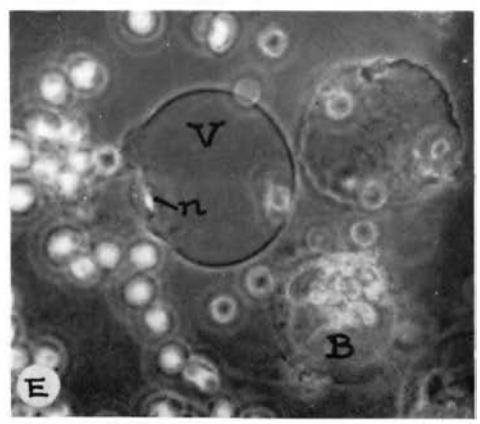
Although the "urn cells" in the coelomic fluid of *S. nudus* do not react with the ciliates while they are alive, they may collect dead anophrys; this is difficult to determine since lysed organisms are











F.B. Bang and B.G. Bang

PLATE I

Fig. A. Culture of sipunculid blood just after adding Carcinus blood ; about half the anophrys are still swimming and half have already rounded up, immobile.

Fig. B. Several minutes later, all anophrys are immobilized and lysis has begun.

Fig. C. Normal swimming urn on right with small tail and few bacteria (small dark dots) adherent ; urn on left swimming into heavy concentration of bacteria above. Arrows show ciliary current.

Fig. D. Urn tail with moderate load of amebocytes and debris ; blurred streaks are deflected red cells.

Fig. E. Separation of vesicular cell and base cell; enigmatic cell upper right. B=base, V=vesicle, n=nucleus.

not easy to identify. Urns have long been known to clean foreign particles and debris. Because of the urns' continuous motion, and because of phase refraction, the definitive structural details must await high resolution study. Cantacuzène's fresh and vivid descriptions of the general appearance and behavior of urns needs no restatement; the notes which follow describe only additional or disparate observations. Metalnikoff described the probable origin of the urns from the wall of the sipunculid body cavity (Metalnikoff, 1900). All of the pioneers, so to speak, who worked with urns described them as unicellular organisms.

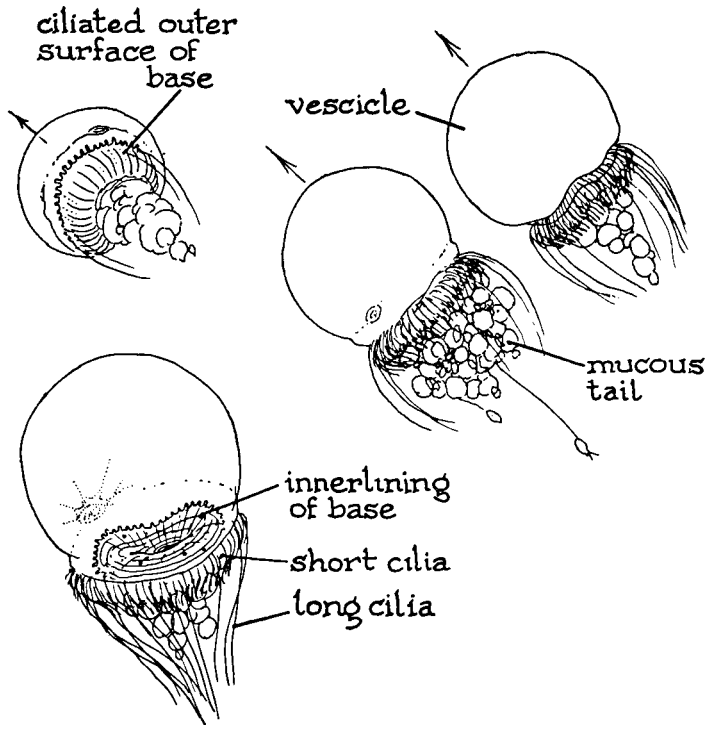


FIG. 1
Examples of swimming urns drawn while observed in phase microscope.

The "urn cell" (Plate I: C, D, E, and Fig. 1) is in fact two separate cells which are normally firmly attached, having considerable free play of motion at the attachment. The anterior cell looks rather like a transparent bubble with a flattened base and short neck (Fig. 2). The base and neck fit into the concavity of the saucer-shaped posterior cell which has a clear secretory central area (Fig. 4), and which is ciliated on its outer convexity (Fig. 3A). Cantacuzène calls the bubble-like cell the vesicular pole and the ciliated glandular cell the posterior pole. We observed urns in fresh cultures of sipunculid coelomic fluid and in cultures infected with marine bacteria and protozoans. There is some individual variation in sizes and shapes of urns in a given age of culture, and even more between those at

different culture ages or different physiological states. This is true of the bicellular urn as a whole and of each of its constituent cells.

As the urns dart among masses of erythrocytes in a drop of coelomic fluid, they may push through them or may run into an unyielding mass of them and get stuck. Rafts of urns may become

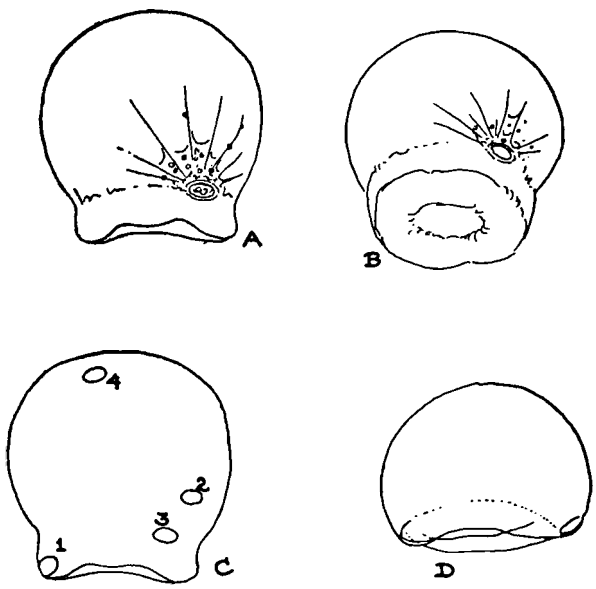


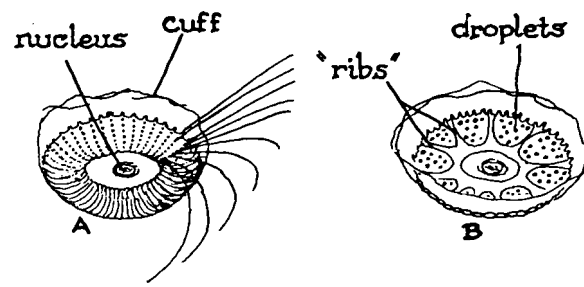
FIG. 2

Separated vesicles. C 1, 2, 3, 4 indicate relative frequency of position of nuclei.

stuck to one another by their mucous tails; individuals often float lazily for a few moments, so that there are opportunities to study them from all angles. There is always a wide halo of clear space around a swimming urn or a group of urns, for they keep flicking the red cells and other types of living blood cells away with their cilia

FIG. 3

Semi-diagrammatic drawings of separated bases, not shown in typical quadrifoil shape because of uncertain interpretation of non-motile (thus abnormal) base which loses cilia but retains nucleus, cuff, and refractile droplets. Ribs are actually of medium dark intensity.



(Fig. 5). Dead amebocytes, debris, and bacteria, however, are drawn into the ciliary current and swept summarily onto the mucous tail (Fig. 6).

The vesicular cell. After several weeks of observing, when "normal" and unusual urns could be discriminated with some confidence, it was noticed that there were occasional detached vesicles without bases and bases without vesicles in the fluid. The shape of the detached

vesicle was characteristic, and Figures 2A and 2B show its appearance in profile and semi-profile. In a few rare cases, the neck was partially contracted into the vesicle (Fig. 2D). In an occasional urn that had begun to decelerate, the outer membrane of a vesicle had partially slipped aside somewhat in the manner of a grape skin (Fig. 7), evidence of a least two membranes. The nucleus is nearly always at or near the junction of neck and vesicle, but is occasionally seen on other positions as diagrammed in Figure 2C.

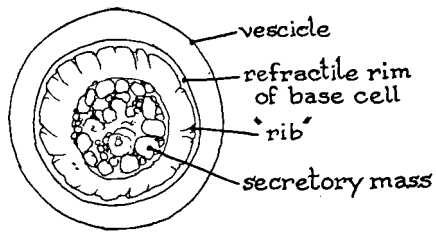


FIG. 4
Drawing of living urn head-on looking through vesicle into inside of base. From this angle the refractile rim is clearly seen, and the secretory tail is a luminous refractile mass.

In preparations of freshly drawn fluid, when neutral red is introduced under the coverslip the tails become brilliantly stained as the urns swim into the suspensions; as these swim into clear areas, the nucleus and paranuclear areas of the vesicle are seen to be brightly stained as well. This paranuclear area varies in size and clarity but is always distinguishable (Fig. 2A). The rounded base of the vesicular neck is not flat on the bottom, but has a softly molded quadrifoil shape and a slight central depression, as seen in Figure 2B.

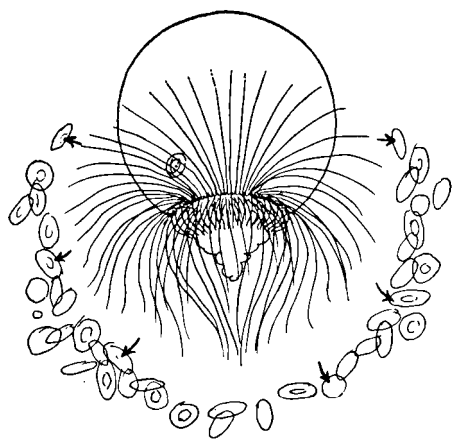


FIG. 5
Diagram of action of long cilia in deflecting red cells away from secretory mass.

The mucous-ciliated cell has a thin transparent cuff approximately the depth of the neck of the vesicular cell, and the two parts of the normal urn are apparently held together by some attractive force between this cuff and the bulge of the vesicle just proximal to the neck. The base of the vesicle fits into the concavity of the ciliated cell and is held firmly, but with a certain amount of free play, within the pliable cuff, so that if either the vesicle or the tail is stuck, the base may wobble and tug as though on a short tether.

This saucer-shaped cell has quite a complex structure, and is less standardized in its constituent parts than is the vesicle. It seems to be essentially a plaible decurved quadrifoil disc, the concave inner surface supporting the secretory mechanism, and the convex outer surface the cilia and their shafts. There are distinctly two rows of

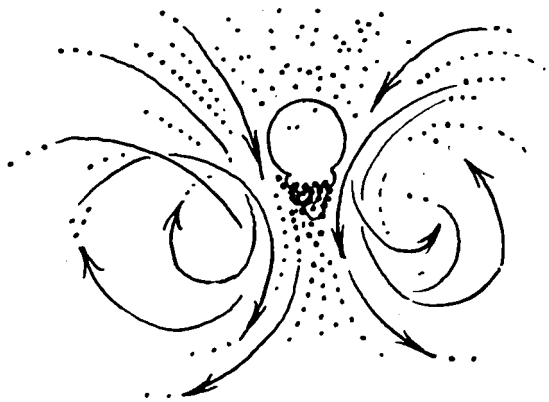


FIG. 6

Diagram of action of double ciliary current in attracting foreign particles; both dye particles and bacteria become involved in a whirpool and may get stuck either in the initial or an ensuing current or may escape to be caught on another urn. Drawn from life.

cilia, but whether they alternate or have separate zones of insertion could not be determined in the living urn, and they shed cilia while dying. The long row serves to fend off non-scavenged cells and to form an attractive current for material to be scavenged. Material is brought into the range of the short cilia which fling particles into the mucous mass (Fig. 7). Cilia serve also, of course, for propulsion. Refractile "ribs" can be seen inside the disc, varying in light intensity, numbers of ribs, and structure (single, branched; thick, thin) in individuals. The secretory mass may be negligible or may form a short fat or long thin tail (up to 4 times the vesicle length) to the whole urn (Plate I and Fig. 1). The nucleus lies in the secretory central portion, and is obscured by the mucous mass in the living cell; as may be seen in Figure 4. The nucleus of the base cell was most clearly demonstrated in urns stained by neutral red injected under the coverslip, then osmium fixed by the same route. As they died, the mucous mass and cilia shed, and the clear central area could clearly be seen, with the nucleus lying in the clear central area; and the insertion shafts of cilia showing faintly on the convexity (Fig. 3A). The ribs and secretory droplets showed in the concavity (Fig. 3B). The whole base cell must be quite plastic, for it was occasionally invaginated into the vesicle in pathological urns; and in healthy urns seen from above, fine orbicular striations as well as longitudinal ones were clearly seen, as indicated in Figure 1, lower, left. Bases have been drawn without

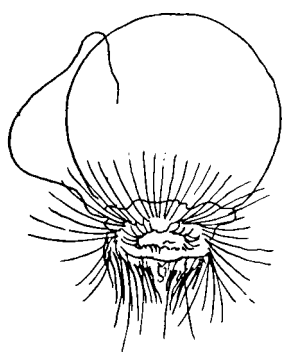


FIG. 7

Drawing of moribund urn, showing partial slipping of outer membrane, and appearance of decelerating cilia.

the quadrifoil shape because the contours are too variable to interpret dogmatically; each of the four "petals" in turn seems to be faintly bilobular, somewhat like a shamrock leaf.

Since separate vesicles and bases had been observed from time to time, we hoped to see the act of separation. Versene (EDTA) added to the cultures at .1 and .01 M solutions was lethal; .001 solutions had no apparent effect. Gradual changes, however, were effected with .0025 solutions: by 1/2 hour few urns were normal, the vesicles being either greatly distended, shrivelled, or distorted, yet some remained viable for over an hour. We watched many sets of such cultures, and at last saw two urns, stuck together by their tails, which were bumped sharply by an enigmatic cell; the vesicle was knocked off one urn, and the base remained stuck. The vesicle stayed close by, turning over and over in the ciliary current. A curious double urn was seen also in a normal, untreated culture: a single large vesicle and two complete bases.

To observe the action of and to try to localize the secretory areas of, the mucous of the base, 1 p. 100 suspensions of carmine and of India ink in sea water were injected into separate worms (1/2 cm^3 per worm). In urn cells seen in coelomic fluid twelve hours later, ink was concentrated on the tails and no particles of ink were free in the fluid; about 3/4 of the tails were ink-laden, the other 1/4 clear of ink. In the carmine specimens the dye particles were concentrated in the central part of the mucous tail. In both ink and carmine preparations occasional detached chunks of stained debris were seen floating in the fluid, presumably tails which had shed. The unstained minority of tails thus were probably ones which had shed and produced new secretions; or perhaps all the dye particles had already been picked up by others. India ink seemed to have a more harmful effect on the urns as a whole than carmine.

To try to get metachromatic staining of the secretory apparatus, filtered 1 p. 100 solutions of methylene blue in sea water were introduced into whole worms and onto slides of fresh fluid. The whole base stained intensely for the first 15-30 minutes in both cases; by 45 minutes it was evident that the cuff of the base was pale blue and the center of the tail intensely blue. The paranuclear area was brilliantly purpleblue at 30 minutes, deeper in intensity by 45, yet preparations seen after one hour showed no staining of this area. Our records do not mention nuclear staining with this dye. At two and three hours there was clear metachromatic staining of only the central part of the secretory tail, and of the many highly refractile droplets arranged in irregular rows inside the ribbed cuff of the base (Fig. 3B). Presumably the droplets are secretory, and in the living urn the continuous secretion forms the mucous tail which accumulates debris up to a certain loading point, then drops off; and gradually a new tail is secreted. We have seen thick accumulations of carmine, debris or ink several times the size of the vesicle. Whether the base cell is a sort of open sleeve, or whether the mucus is extruded onto the ciliated surface is not clear.

During the course of the studies of sipunculid coelomic fluid, it was found that urns could be kept alive and motile for as long as eleven days in culture tubes at room temperature. Different ages

varied quite a lot in appearance and behavior, and older ones lacked the secretory tail.

In effect, the information which may now be added to our knowledge of urns is that (1) the urn is composed of two cells which are separable and each of which contains a nucleus (2), the base cell has two distinct sets of cilia, a long and a short, (3) the secretory apparatus stains metachromatically, and (4) urns survive up to 11 days in culture, losing the secretory tails in the older cultures but remaining viable.

Summary

Properties of the blood of two marine sipunculid worms to respond to injections of foreign organisms have been described. *Phascolosoma (Goldfingia) gouldii* coelomic fluid was found to be sterile and to be capable of destroying massive concentrations of bacteria. *Sipunculus nudus* coelomic fluid had the same capacity, and was also capable of the induced ability to immobilize and lyse heavy concentrations of a protozoan parasitic ciliate. This ability became attenuated after about a week, but could be repeatedly re-invoked. New information on the structure of the bicellular urns, which selectively scavenge foreign or morbid matter from the coelomic fluid, has been presented.

Zusammenfassung

Die Eigenschaften des Blutes zweier mariner Sipunculiden in ihrer Reaktion auf Injektionen fremder Organismen werden beschrieben. Die Coelomflüssigkeit von *Phascolosoma (Goldfingia) gouldii* ist steril und ist fähig starke Konzentrationen von Bakterien zu vernichten. Die Coelomflüssigkeit von *Sipunculus nudus* hat die gleichen Eigenschaften und ist ausserdem fähig einen parasitischen Ciliaten selbst bei starker Bevölkerungsdichte zu immobilisieren und aufzulösen. Diese Fähigkeit wird schwächer nach ungefähr einer Woche, kann aber wiederholt in Erscheinung treten. Es werden neue Resultate dargestellt über die zweizelligen Urnen, welche fremde oder morbide Materie selektiv aus der Coelomflüssigkeit entnehmen.

REFERENCES

BANG, F.B., 1955. — A Bacterial Disease of *Limulus polyphemus*. *Bull. Johns Hopkins Hosp.* 98 - *pp.* 325-351.

BANG, F.B. and KRASSNER, S.M., 1958. — Antibacterial activity of *Phascolosoma gouldii* blood. *Biol. Bull.* 115 - *p.* 343.

CANTACUZÈNE, J., 1922 1. — Sur le sort ultérieur des urnes chez *Sipunculus nudus* au cours de l'infection et de l'immunisation. *C.R. Soc. Biol.* 87 - *pp.* 264-267.

CANTACUZÈNE, J., 1922 2. — Sur le sort ultérieur des urnes chez *Sipunculus nudus* au cours de l'infection et de l'immunisation. *C.R. Soc. Biol.* 87 - *pp.* 283-285.

CANTACUZÈNE, J., 1928. — Recherches sur les réactions d'immunité chez les Invertébrés. *Arch. Roum. Pathol. Exp. et Microb.* I - *pp.* 7-80.

CUÉNOT, L., 1902. — Organes agglutinants et organes cilio-phagocytaires. *Arch. Zool. Exp. Gén.* 10 (3) - *pp.* 79-97.

METALNIKOFF, S., 1900. — *Sipunculus nudus*. *Zeitschr. für Wiss. Zool.* 68, *pp.* 261-323.

POISSON, R., 1930. — Observations sur *Anophrys sarcophaga* (Cohn) et *A. maggii* (Cattaneo), Infusoire Holotriche marin, et sur son parasitisme chez certains Crustacés. *Bull. Biol. France-Belg.* 64 - *pp.* 288-331.

ZOBELL, C.E., 1946. — Marine Microbiology (A Monograph on Hydrobiology). *Chronica Botanica Co.*, Waltham, Mass.