# THE MUCOUS SECRETORY APPARATUS OF THE FREE URN CELL OF *SIPUNCULUS NUDUS*

by

**Betsy G. Bang** and **Frederik B. Bang**

**Department of Pathobiology, the Johns Hopkins University, School of Hygiene and Public Health, 615 North Wolfe Street, Baltimore, Maryland 21205**

and

Station Biologique, 29211 Roscoff.

## Résumé

Les appareils sécrétoires de deux systèmes sécréteurs distincts de la même cellule ont été identifiés dans des cellules muqueuses vivantes. Chaque type de sécrétion répond négativement au colorant vital spécifique de la forme adverse. L'une de ces sécrétions est formée lentement, entourée par une membrane et se colore de manière métachromatique sous l'action des colorants basiques d'aniline (groupe thiazine, oxazine et azine). L'autre sécrétion, induite par des stimulus spécifiques, consiste en un mucus circulant librement ; la forme violette du vert Janus, colorant azobasique, se concentre au lieu même de synthèse de ce système tandis que, suivant la nature du stimulus utilisé, le colorant se lie ensuite au mucus libéré, soit sous sa forme violette, soit sous sa forme bleu-verdâtre. Les cellules utilisées dans ces expériences sont les « cellules urnes libres », une population de cellules épithéliales muqueuses et ciliées qui baignent librement dans le liquide coelomique de l'Invertébré *Sipunculus nudus.* Ces cellules sont cultivées et éprouvées dans leur propre sérum. Sept liquides organiques d'origine humaine constituent des stimulus efficaces : ils induisent de manière reproductible la sécrétion des différents types morphologiques de mucus par les cellules urnes. Le vert Janus est également concentré et sécrété par les cellules des glandes muqueuses de fosses nasales de poussin, maintenues en culture d'organe.

## Introduction

Mechanisms which regulate the quality and quantity of mucous secretion are not well understood, and it is difficult to measure the mucin content of a given secretion. There is a population of discrete individual mucous cells 25-5(V in diameter in which these aspects of secretion can now be explored in vitro. The urn cells of the marine coelomate *Sipunculus nudus* are mucociliated epithelial cells which detach from the wall of the coelom and become free-swimming in the coelomic fluid. They are readily cultured in their own serum and their responses to a battery of stimuli can be directly observed in vitro in a light microscope. The hypersecretion, by urn cells, of mucus which consistently differs in rate, quality and quantity and which is produced in response to specific stimuli from autologous (J. Cantacuzène, 1928) and human (Bang and Bang, 1974) sources has been described previously.

CAHIERS DE BIOLOGIE MARINE
**Tome** XVII - **1976** - **pp. 423-432**

The present report describes the binding of the vital dye Janus green to mucous secretions which emerge as violet or green-stained products, depending on the stimulus, differentiates this mucus-secretory system from the membrane-enclosed secretory system of the cell in its unstimulated state, and summarizes the variety of specific stimuli tested to date which have consistently induced qualitatively different types of mucus in urn cells.

## MATERIALS AND METHODS

### The host animal.

*Sipunculus nudus*, phylum sipunculida, is a cosmopolitan marine invertebrate which can be dug from its burrows in the tidal sands in certain areas off the Breton coast of France during neap tides. The large worm-like adults are 10-15 centimeters long and the undivided coelom contains about 12 ml of richly cellular fluid (blood) (Bang and Bang, 1974). It has been recently suggested (Nichols, D., 1971) that the sipunculoids are ancestral to the deuterostome group from which the chordates were derived.

### The test cells.

Cultures of urn cells are prepared by withdrawing about 3 ml of whole blood by sterile procedures, placing it in a sterile 12 x 75 mm Falcon plastic snap-cap test tube and allowing the cells to settle at room temperature. The heavy blood cells precipitate while the buoyant mucociliated urn cells remain swimming in the supernatant serum from whence they can be withdrawn with a clean Pasteur pipet as needed. When effects of stimuli are tested, one drop of urn supernatant fluid is put into each of two wells of a double depression slide, and one drop of test stimulus is added to one of the wells, the other serving as control. When not in use, the test tube preparations are stored at 4°C.

### Stimuli.

*S. nudus* serum, human tears and nasal fluid, and invertebrate and human seminal fluids were used undiluted. Freshly drawn human and chicken sera and fresh human urine were diluted 1/10 in filtered boiled cooled seawater. Chick nasal fluids were obtained by washing with 1 ml of normal saline. Human saliva was washed three times in and resuspended in seawater, final dilution 1/2. Aliquots of millipore-filtered stools were tightly sealed and stored at 4°C until tested. Most fluids were tested both unheated and after heating to 85 °C for 5 minutes. *S. nudus* sera, human sera, and human nasal fluids were equally active after freezing at — 25 °C. Human urine was found to be extremely unstable and has not been standardized as a test stimulus.

**Vital stains.**

Methylene blue, brilliant cresyl blue and toluidine blue were used in 1/5000 solutions in filtered boiled seawater, neutral red in a 1/3000 solutions. Janus green (British Drug House Ltd.) or Janus green B (Allied Chemical Co.) were prepared as 1/500 solutions in seawater, filtered, and prepared as final 1/5000 or 1/10,000 solutions in filtered boiled cooled seawater.

## Background

Urn cells originate in the lining epithelium of the *S. nudus* coelomic cavity. Individual mucociliated cells become loosely attached to an underlying cell which then fills with fluid; the two-celled organelle then pinches off from the epithelial wall and becomes free-swimming in the fluid (blood, or serum). The vesicle cell fits loosely into the saucer-shaped mucociliated cell, somewhat like an acorn in its cup (Fig. 1 and Fig 3, inset). Like goblet cells, urns normally secrete mucus at a slow rate and, as they swim at random in the fluid foreign particles and cell debris, become stuck to the small tails of mucus. Autologous blood cells do not stick to this secretion. However, if pathogenic bacteria get into the coelom, the urn population secretes long streamers of free mucus in which the bacteria are trapped until the fluid is free of them. In nature, urns respond to a variety of foreign particles by secreting various amounts of excess mucus.

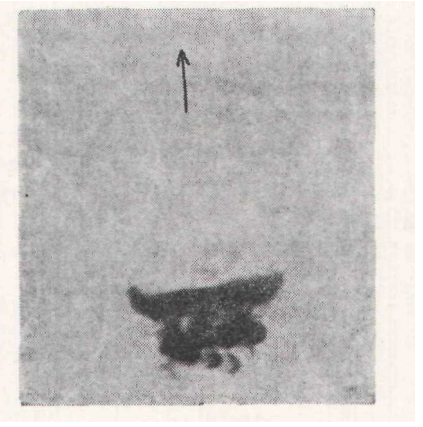


**FIG. 1**

Swimming normal, unstimulated, urn. Dead cells are adherent to the small secretory tail. Size approximately 20 μ, long diameter.

All stages of secretion, from the unstimulated state to the rapidly secretory, can be induced *in vitro* in preparations of urns in their own coelomic fluid (J. Cantacuzène, 1928). We had observed that serum from bacterially infected *S. nudus,* when it was heated to 85°C for 5 minutes, induced rapid secretion of great volumes of excess mucus, while heated sera of normal S. *nudus* and unheated sera of infected animals did not (Bang and Bang, 1972). Normal human serum made isotonic with seawater and heated to 85° C induced equal amounts of excess mucus but the secretion was quali-

tatively different; unheated human tears, urine and saliva each induced excess secretions of still different morphological types (Bang and Bang, 1974).

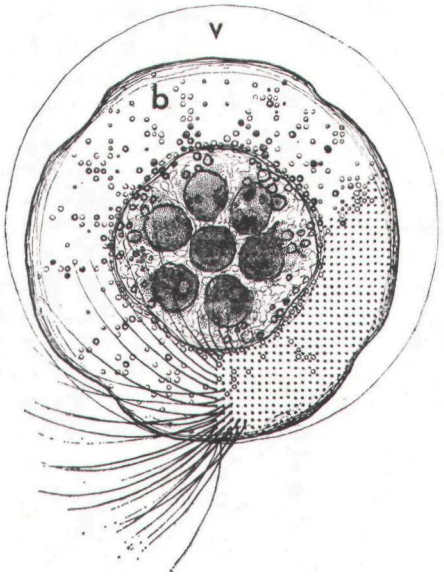



FIG. 2

Diagram of relationship of secretory granules, membranous sacs, and cilia. Tail-on view of base (b) and vesicle (v). Prepared from fixed urns, using combined data from low-power electron microscopy and alcian blue-PAS staining.

In attempting to characterize the secretions produced by urns in both the unstimulated and hypersecretory states, the cells were stained supravitally and after fixation, using standard staining methods. Fig. 2, 3 A and B show the way in which the secretory

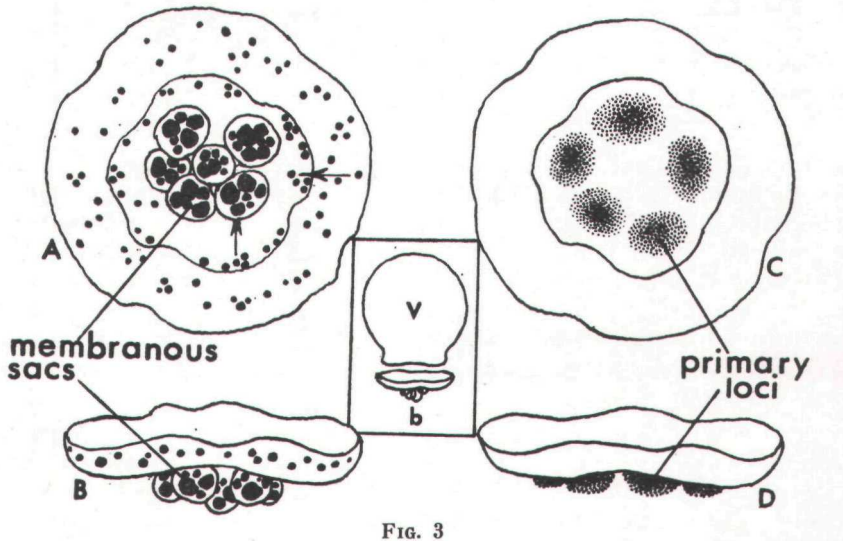



FIG. 3

Diagrams showing different loci of secretory apparatus of membrane-bound secretory system and free-flowing mucus system. Inset shows relationship between vesicle cell and base cell.

**A:** tail-on view of secretory droplets and membranous sacs; B: profile; C: secretory loci of free-flowing mucus system 5 minutes after adding Janus green; loci are rose-violet; D: profile.

droplets in the unstimulated urn stained in vitro with methylene and toluidine blue, brilliant cresyl blue and neutral red. The secretions were seen to form as droplets and to accumulate as granules in sac-like evaginations of the cell membrane. In fixed cells, the same secretions stained with alcian blue-PAS.

The free-flowing excess mucus produced by the stimulated cell, however, stained with none of these dyes. In the living cell, this colorless mucus could be seen to emerge peripherally to the secretory sacs. The latter were stretched distally as the newly emerging mucus surrounded them and moved distally. Figure 4 shows this distal stretching of the sacs by emerging mucus. During continued prolonged secretion the sacs were stretched beyond their tensile capacity and broke at the proximal end; the cell membrane closed over the break point and the sacs with their enclosed secretions gradually reformed. If a preparation of hypersecreting urns was sealed with a cover-glass on a ring of vaseline, the elongated sacs retracted within 15-20 minutes.

### Experimental findings

To date, twelve types of human, chicken and invertebrate fluids, most of them of secretory origin, have been found to stimulate excess secretion in urns. Responses to particular stimuli clearly have

**TABLE 1**

Substances tested to date which have induced secretion of excess mucus in urn cells

| Stimulus | **Relative** degree **of hyper-secretion** | Effective **when** unheated | Effective **when heated 85°C, 5 min.** | Effective **If** undiluted | **Effective If** diluted |
|---|---|---|---|---|---|
| Suspension of marine bacteria | + + + | + | 0 | | |
| Sera of bacterially infected S. *nudus* (1, 2) | + + + | 0 | + | | |
| Human serum (1, 2) | + + + | 0 | + | h | |
| Chicken serum | + + + | 0 | + | h | |
| Human tears | + + + | + | + | | |
| Human saliva | + + | + | + | | |
| Human nasal fluid | + + | + | + | | NT |
| Chicken nasal tissues | + + | + | NT | | NT |
| Human urine (2) | + + | + | 0 | | |
| Cholera stool | + to + + | + | + | | 0 |
| Human semen | + | + | NT | | NT |
| Invertebrate sperm (3) | + | + | NT | | NT |
| Janus green dye 1/5000 solution | + | + | NT | | |

Each result represents six or more separate tests, except in the case of invertebrate sperm.

(1) Withstands successive freeze-thawing (2); others not tested.

(2) Large (well over 50,000 daltons m.w.) molecules as tested by ultracentrifugation and ultrafiltration (2).

(3) Sperm of *S. nudus*, *Asterias*, *Notomastus*, *Ascidia* (2).

+ = response; 0 = no response; h = hypotonic, kills urns; NT = not tested.

degrees of specificity since the mucus produced by a given stimulus is consistently granular or homogenous; glassy or thready; thin or wide; elastic or brittle (Fig. 4). Table 1 summarizes some of the

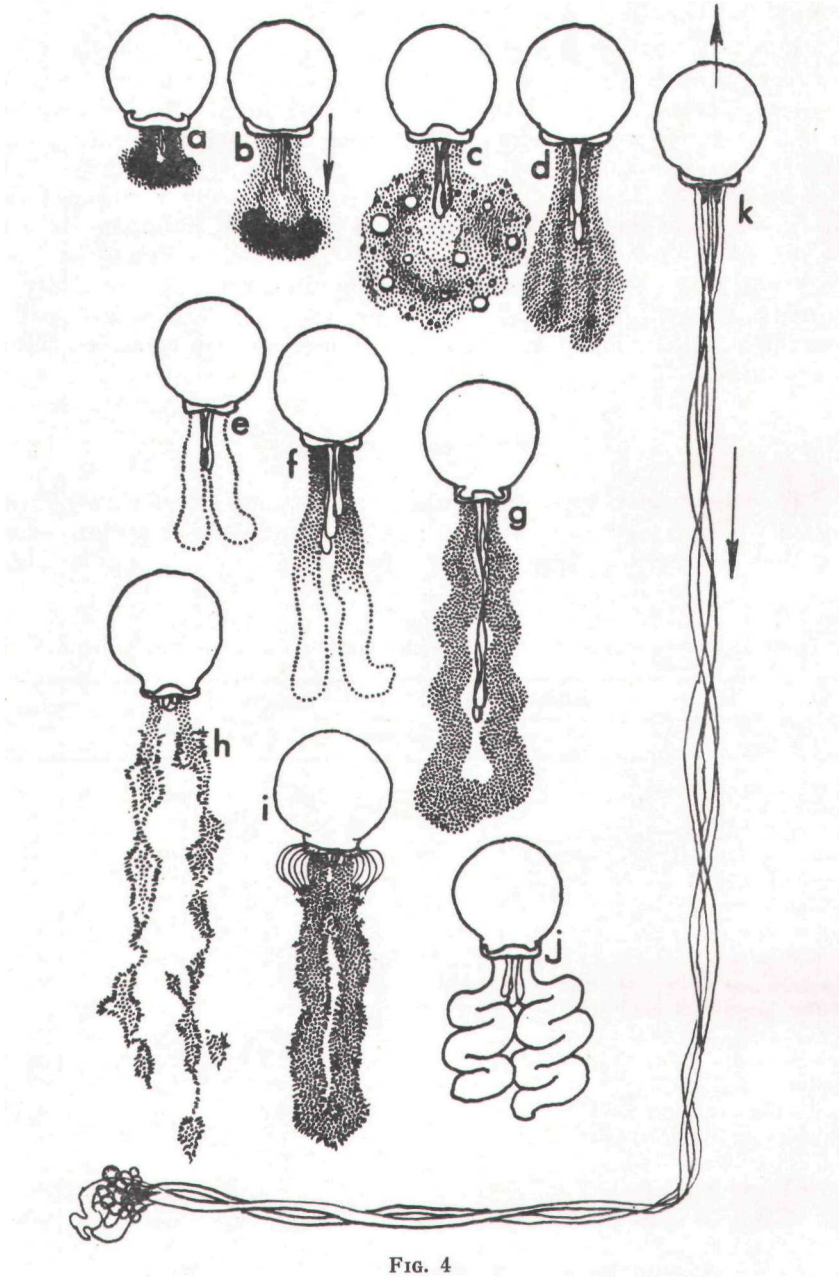



FIG. 4

Summary of urn cell secretions induced by different stimuli. Arrows indicate direction in which urn **is** swimming and direction in which mucus emerges. Time refers to time after stimulus was added to urns.

a: Janus green 1/5000, 10 minutes; violet colored mucus; b: the same cell 10 minutes after stimulation with human serum diluted 1/40 in seawater; green secretion has pushed violet secretion distally; c: undiluted human saliva, 20 mi-

known characteristics of the materials which have been found to induce degrees of excess secretions in cultures of living urns. Most bacteria and many kinds of foreign cellular and fluid materials simply accumulate on the small normal mucous tail and induce no excess secretion.

TABLE 2

Some properties of mucous secretions induced by particular stimuli and stained vitally with Janus green

| Stimulus | Secretion | | Stain intensity | Mucus quality |
|---|---|---|---|---|
| | Color | Rate | | |
| Janus green itself | violet | slow- | dense | viscous |
| Human saliva | violet | sudden, then moderate | moderate | fine, then bubbly |
| Human nasal fluid | violet | | moderate | clear, viscous |
| Chicken nasal fluid | violet | moderately rapid | moderate | clear, elastic |
| Marine bacteria | violet | rapid, slows with time | initially pale, changes to moderate | ragged, friable, changes to compact |
| Heated sera (1) of bacteremic S. *nudus* | bluegreen | quite rapid | moderate | granular |
| Heated normal human sera | bluegreen | very rapid | moderate | smooth, homogenous |
| Heated normal chicken sera | bluegreen | very rapid | moderate | smooth, homogenous |

(1) All sera were heated at 85°C for five minutes. Human and chicken sera were diluted 1:10 in seawater; *S. nudus* sera were undiluted.

Successful staining of the free-flowing mucous system was accidental. A drop of a 1/5000 seawater solution of Janus green was added to an urn cell preparation to identify the mitochondria of the secretory cell. Within a minute a series of bright rose-violet loci appeared in the periphery of the central part of the cell (Fig. 3 C and D, and Plate I,2). In the following ten minutes a modest amount of violet-stained secretion had emerged from each locus (Plate I,3). A known stimulus of excess secretion—human serum diluted 1/40 in seawater— was then added to the same preparation, and a newly emerging bluegreen secretion gradually pushed the ori-

nutes; pale violet; d: human nasal fluid, 15-20 minutes; violet; e: serum 1/40, 5-10 minutes; colorless; f: same preparation 10 miputes after adding Janus green; green proximally, colorless distally; g: twenty minutes after adding serum 1/40 and Janus green simultaneously; secretion is homogenous bluegreen; h: Janus green and bacterial suspension simultaneously; after 5-10 minutes, secretion is pale violet, fragmented, brittle; i: same preparation after 30 minutes; mucus has become compact or contracted, and is deeper violet; j: filtered acute human cholera stool after 30 minutes; secretion was slow, translucent and viscous (no dye); k: extremely rapid continuous secretion of type induced by serum 1/10; note remnants of membranous sacs in central part of tail proximally, and very distal bundle of broken-off sacs and adherent debris (no dye).

In each diagram, only two of the four to six streams of mucus are shown. Cilia are shown only in example « i ».

ginal violet secretion distalward (Plate I, 4). This was repeated several times, adding the stimulus at various intervals after the initial staining of the primary loci. In all cases the original violet loci were pushed distally by newly emergent bluegreen secretion.

To determine whether mucus which already been secreted would stain with Janus green, dye was not added to actively secreting cells until colorless tails had already been produced. The original mucus then remained colorless but the newly emerging secretion was bluegreen and had the greatest color intensity at the most proximal part of each stream (Fig. 4, e and f). When dye and serum were added simultaneously there was no violet prestaining of primary loci and the entire emergent secretion was clear homogenous bluegreen (Fig. 4,g).

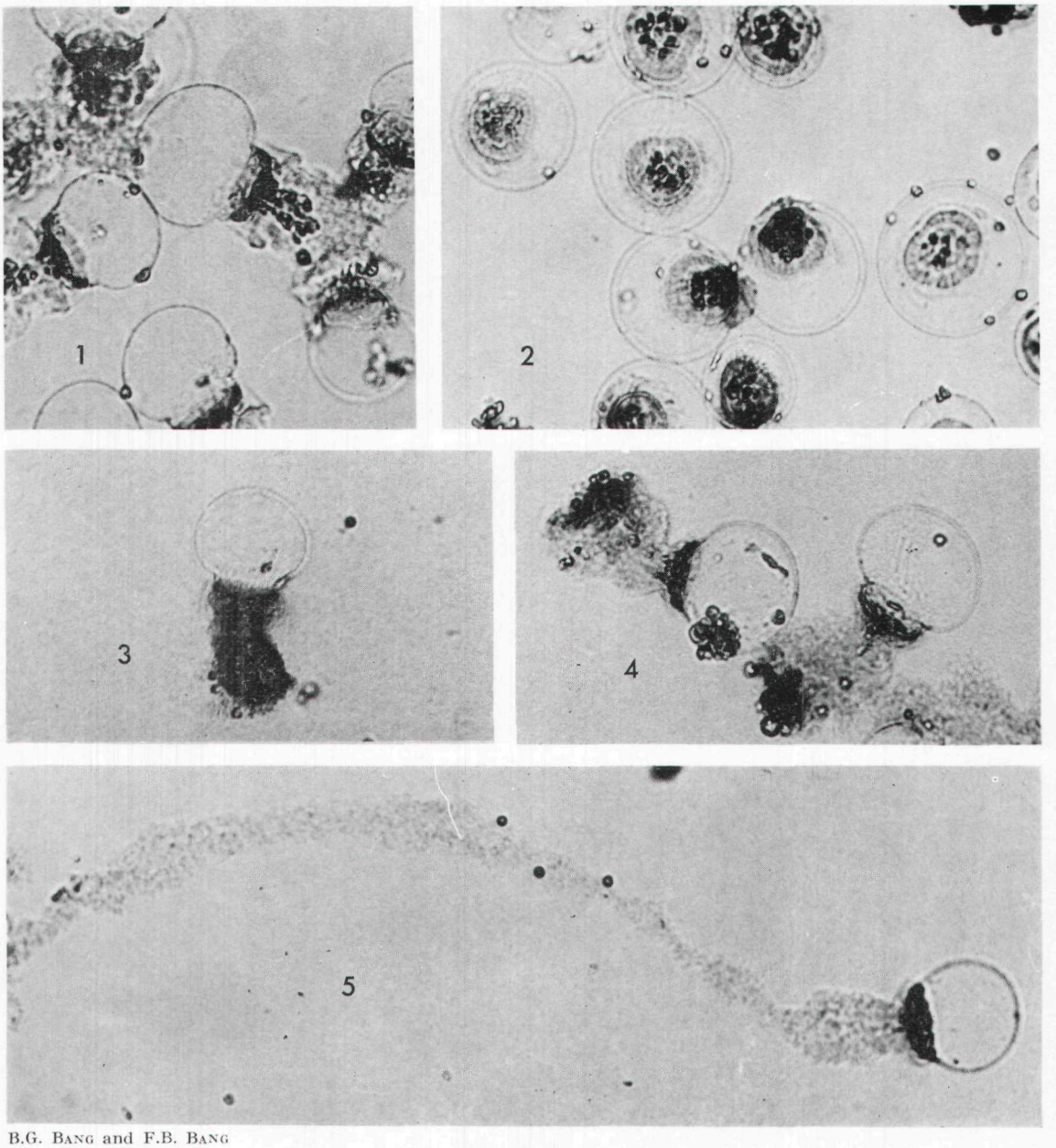
Not all of the known stimuli of excess secretion in urns have been tested for the Janus green effect. Those tested to date are summarized in Table 2 and some of the morphological and staining characteristics are summarized in Figure 4.

The membrane-bound secretory system and the mucous hypersecretory system were differentially stained in the same cell. Peripheral streams of bluegreen secretion were stimulated by adding serum 1/40 and Janus green simultaneously; then a drop of neutral red was added. The previously colorless granules in the membranous sacs rapidly took up the neutral red, while the peripheral bluegreen mucus was unaffected. Plate I,1 is a photograph of such a preparation. Thus, the membrane-enclosed granules consistently stained with metachromatic dyes but not with Janus green, while the free-flowing hypersecretory streams of mucus stained with Janus green but did not stain with metachromatic vital dyes. This indicates that two secretory systems coexist in the same cell.

The cell mitochondria do, incidentally, stain with Janus green. These small packages of enzymes are scattered throughout the muco-ciliated cell in both the ciliated and the non-ciliated areas. They disappear when the cell dies.

#### Secretion of Janus green by organ cultures of chick nasal turbinates

Preliminary experiments with fifteen pairs of chick nasal turbinates grown as organ cultures in synthetic media indicate that both the acinar glands and the goblet cells concentrate and secrete Janus green. Whole or longitudinally hemisected turbinates of chicks from 7 days to 4 1/2 weeks old were placed in 35 x 10 mm Falcon plastic culture dishes, covered with either Medium 199 or Modified Eagle's medium (Grand Island Biological Co.) containing a 1/100,000 dilution of Janus green, and incubated at 37°C in a moist incubator containing 95 percent oxygen and 5 percent carbon dioxide. While the culture methods need much further refining, it is clear that in both culture media the acini concentrate the dye within an hour, either as a rose-violet (less common) or a bluegreen stain. After overnight incubation the stained elements are found to have been liberally secreted into the medium, always as rich bluegreen "prints" of the secretory cells.




B.G. BANG and F.B. BANG

PLATE I

1. Profile view of living urns with moderate mucus secretion stimulated by human serum diluted 1/40 in seawater. Note stretching of sacs distalward. Sac secretion stained with neutral red.
2. Group of swimming urns 5 minutes after adding Janus green to preparation. Some are still unstained, others show beginning concentration of dye, a few are secreting the dye.
3. Urn cell 30 minutes after adding Janus green to preparation. Maximal size of tail secreted in response to the dye; purple form of dye is bound to the mucus.
4. Cells in duplicate preparation to which human serum 1/40 was added 10 minutes after Janus green. Bluegreen secretion has pushed primary violet locus distally.
5. Urn cell still secreting new mucus an hour after original stimulus (serum 1/10 in seawater).

## DISCUSSION

Obviously the urn cell has great flexibility in providing the amount and quality of mucus required for ridding the coelomic fluid of debris in vivo, whether noxious or benign. The hypersecretory mucous apparatus seems to be induced only when the membrane-bound secretory system is unable to cope with a sudden demand for rapid clearance of an invading substance. In mammals the mechanisms which regulate the variations in the quality and quantity of mucus in different physiological and disease states are not well understood. In urn cells the hypersecretory apparatus evidently remains quiescent until stimulated. Presumably the many types of mucus which are experimentally induced in vitro represent types produced in vivo in response to a wide spectrum of invasive agencies.

The membrane-bound secretory system of the unstimulated urn renders the cell remarkably sticky; sheep and human red cells stick with much greater avidity to the membranous sacs than to hypersecreted mucus (Bang and Bang, 1975). Since the secretory droplets are never seen to penetrate the cell membrane, it is assumed that enzymes or molecules are released from the granules by way of submicroscopic pores in the membrane, or that the membranes have some special surface property. These same characteristics could account for the distalward stretching of the sacs by contact affinity with the outpouring mucus.

The hypersecretory response raises several questions which are subject to investigation: for example, what kind of information turns this apparatus on, and what is the source of the energy which allows continuous prolonged rapid secretion by dozens of urns in one drop of serum?

The chemical structure of reduced forms of Janus green B (JG-B) has been considered theoretically by Lazarow and Cooperstein (1953) who have postulated that there may be several species of leuco JG-B, the reduced form of the dye. The first of these, JG-B-I, would be formed if the azo linkage of JG-B were reduced to a hydrazo group, and the color of this form might be red-violet or purple. It is known that Janus green sticks tenaciously to protein.

Thus, it is possible that the secretory apparatus of the urn cell reduces the bluegreen dye solution to the postulated violet colored leuco JG-B-I, and subsequently secretes it either in the same form or in a reoxidized bluegreen form, depending on the nature of the stimulus which induces the secretion.

We have no knowledge of the components of urn cell mucus nor of the affinity of Janus green for specific components of mucus. The fact that it stains urn mucus only if it is metabolized by the secretory apparatus may account for the lack of previous reports

on this facet of the dye. Analogs and biochemically related stains have not yet been tested.

We believe that the living urn cell, maintained in its own physiological fluid, is a system in which mucous metabolism, mucus regulatory mechanisms, and mucus structure can be profitably explored. It is the only model that we know of in which (i) production of viscous or cobweb-fine, elastic or brittle, grainy or glassy mucus can be induced at will; (ii) the secretory apparatus concentrates and metabolizes two forms of a vital dye (presumably a reduced and an oxidized form (Lazarow and Coperstein, 1953) ; and (iii) the metabolites in the serum in which prolonged secretion takes place can be monitored.

**Acknowledgements**

We thank the Director, Dr. J. Bergerard, the Administrator, Mr. Jean-Claude Lorgeré, and the field and administrative personnel of the Station Biologique for their help and for generous use of facilities.

**Summary**

The secretory apparatus of two distinct secretory systems in the same cell has been identified in living mucociliated cells. Each type of secretion fails to stain with the supravital dye which stains the other. One secretion is slowly produced, is membrane bound, and stains metachromatically with basic aniline dyes in thiazin, oxazin and azin groups. The other secretion is a free-flowing mucus which is induced by specific stimuli; the violet from of the basic azo dye Janus green is concentrated at the site of synthetis of this system, and the dye is then bound to the emerging mucus in either its violet or its bluegreen form, depending on the stimulus. The test cells are "free urn cells", a population of mucociliated epithelial cells in the coelomic fluid of the invertebrate *Sipunculus nudus*. They are cultured and tested in their own serum. Stimuli which consistently induce secretion of different morphological types of mucus by urn cells include seven human body fluids. Janus green is also concentrated and secreted by mucous gland cells in organ cultures of chick nasal turbinates.

## REFERENCES

BANG, B.G. and BANG, F.B., 1974. — *Lancet*, ii, p. 1292.

BANG, B.G. and BANG, F.B., 1972. — *Am. J. Pathol.*, 68, p. 407.

BANG, B.G. and BANG, F.B., 1975. — *Nature*, 253, p. 634.

CANTACUZÈNE, J., 1928. — *Arch. roum. Pathol. exp. Microb.*, 1, 7.

LAZAROW, A. and COOPERSTEIN, S.J., 1953. — *Exp. Cell Res.*, 5, p. 59.

NICHOLS, D., 1971. — The invertebrate panorama, p. 377, Weidenfeld and Nicolson, ed., London.