Table 2. Assay of male accessory gland activity in *Aedes aegypti* by injection of females with serial dilutions of various gland preparations.

Dilution injected (1 μl per female)*	Females inseminated from treated preparation (%)†			
	With solution from males fed on apple		With solution from males fed on sugar	
	Unfrozen	Frozen and thawed ten times	Unfrozen	Frozen and thawed ten times
Full	0	0	0	0
1/2	0	0	0	0
1/4	0	0	0	10
1/8	0	60	0	50
1/16	0	55	15	70
1/32	35	100	50	95
1/64	45	90	90	85
1/128	90	95	85	100

* Stock solution from 250 male terminalia in 1 ml of saline. Injection of 1 μl gave a dose equivalent to one-fourth of a pair of male glands. † Twenty females injected for each dilution, placed with males 24 hours later, and examined for insemination 48 hours after injection.

then place females with 40 males; and (iv) in 48 hours after injection, dissect females and examine spermathecae for insemination. Control females injected with saline generally show 85 to 95 percent insemination after 24 hours with males.

Twofold serial dilutions were used to determine activity of stock solutions by titration (Table 2). Glands taken from males fed on slices of canned apple gave slightly more activity than those from males fed on dry sugar cubes. Activity of the stock solution is unaffected by freezing but is somewhat reduced by freezing and thawing ten times. The first column of results in Table 2 is typical of many other assays of stock solution. Each male apparently contains enough active principle to sterilize at least 64 females. The initial stock solution contained glands from 250 males in 1 ml of saline. Each female received 1 μl, a dose equivalent to one-half gland or one-fourth of a pair of glands. Thus, the gland material from one male was distributed among four females. The stock solution could be diluted 16 times and yet retain enough activity for complete prevention of insemination.

The stock solution of male accessory gland substance from *A. aegypti* is clear and colorless. Activity is retained indefinitely while the solution is frozen at −20°C and is reduced only slightly by repeated freezing and thawing. This activity was preserved for 12 days at 5°C and for 2 days at 25°C, but was then destroyed, probably by bacterial action; activity was destroyed by 5 minutes at 50°C but was not affected at 40°C. The active principle is nondialyzable and is readily precipitated with 50 percent ammonium sulfate. These data are consistent with the hypothesis that the substance is a protein or polypeptide.

The physiology of the female mosquito is changed markedly by mating and reception of seminal fluid. For *Aedes aegypti*, females receiving male accessory gland substance show increased oviposition (*13*) and modified biting behavior (*14*) as well as monogamy. One might consider the substance to be a pheromone (*15*), in view of the fact that it is secreted to the outside of one individual and releases a specific action in a second individual. This substance might be useful in insect control, provided that methods could be found to sterilize young females by spraying or by baits for feeding.

GEORGE B. CRAIG, JR.
Department of Biology, University of Notre Dame, Notre Dame, Indiana

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Life Cycle and Variation of Prototheca wickerhamii

Abstract. Prototheca wickerhamii *is a yeastlike organism that resembles the green alga* Chlorella. *Nuclear division in coordination with cytoplasmic cleavage gives rise to uninucleate cytoplasmic segments, each of which acquires a delicate cell wall and develops into an autospore. The autospores in this species are spherical; but in a variant that presumably arose as a result of spontaneous mutation, the cytoplasmic cleavage is irregular, and the resultant autospores are ovoid to bacillary. When these variant autospores grow, they swell and round up before the nuclear division begins, producing spherical cells like those seen in wild-type cultures. In view of the fact that species concept in the genus is based on size and shape of cells, the variation limits in these morphological characteristics have significant bearing on species classification.*

The genus *Prototheca* comprises a group of unicellular organisms whose phylogeny and taxonomic position are still a matter of debate and controversy. Krüger (*1*), who created the genus in 1894 and described it as a fungus, was the first to recognize its close resemblance to certain unicellular green algae which subsequently prompted Chodat (*2*) to describe it as an achloric strain of *Chlorella*. Experimental evidence for Chodat's view was presented by Butler (*3*) who obtained by irradiation white (lacking chlorophyll) mutants of *Chlorella pyrenoidosa* which, in his opinion, could be assigned to *Prototheca*. However, the white mutants of *Chlorella* are "autotrophic," in contrast to the thiamine-deficient species of *Prototheca* which are heterotrophic "like certain fungi and bacteria" (*4*).

Although the number of species included in the genus is relatively small, a precise species concept is not yet known, and the number of species is not definite. Based on morphological characteristics alone, five species have been recognized (*5*), each requiring thiamine for growth. A new species, *P. segbwema* (*6*), was isolated from a case of human protothecosis. I studied the development of a spontaneous variant of *P. wickerhamii* and its bearing on the species concept in the genus.

Prototheca wickerhamii was established by Tubaki and Soneda (*5*) to

accommodate a new isolate that can be recognized by the small size and spherical shape of its cells and autospores (Fig. 1a). When a stock culture of *P. wickerhamii*, grown on Sabouraud's dextrose agar and stored at cold temperature, was examined microscopically, small bacillary and ovoid cells were observed in high proportion among the spherical cells that typify the species. Assuming at first that the culture had been contaminated, I isolated single spherical cells with a de Fonbrune micromanipulator and transferred them separately to slants of cornmeal-dextrose agar. The cultures that arose from these single cells were of two types: one was typical of the species *P. wickerhamii*, and the other was distinguishable by its ovoid and bacillary autospores (Fig. 1b). Cultures from single bacillary cells were all of variant type. Therefore, the variant cells in the stock culture were apparently the result of spontaneous mutation. The variant cultures maintained their characteristics for several successive generations without reversion.

Their origin from single spherical cells is explained as follows. Autospores of the variant are bacillary to ovoid (Fig. 1, d and e), are 1 to 3.3 μ wide and 2.7 to 4.5 μ long, contain one or two refractile lipid droplets each, and are bounded by a thin cell wall. They are formed in a varying number that ranges from 8 to 16 within spherical mother cells whose size range (5.5 to 10 μ) is below that of the wild type. In the latter strain, both autospores and mother cells are spherical, their measurements being, respectively, 3.3 to 4 μ and 7.7 to 13 μ. Staining with aceto-orcein reveals that autospores of wild type and variant are uninucleate and are formed as a result of nuclear division accompanied by cell enlargement and followed by cytoplasmic cleavage. In most cells, coordination between nuclear division and cytoplasmic cleavage within the mother cell wall is such that each nuclear division is followed by a cytoplasmic division, and the resultant segments are therefore uninucleate (Fig. 1c). Occasionally, however, nuclear division may proceed to a certain extent before cytoplasmic segmentation begins; this gives rise to multinucleate cells or multinucleate cytoplasmic segments within a cell. Eventually, a delicate cell wall is formed around each uninucleate segment, which develops into an autospore. Upon rupture of the mother cell wall, the autospores are liberated; this marks the beginning of a new life cycle that passes through the same developmental stages outlined above. This life cycle is the same as that of *Chlorella*; the main difference between the autospore formation in *Prototheca* and the progressive cytoplasmic cleavage by which sporangiospores are formed in the Phycomycetes (*7*) is basically a matter of timing in the sequence of coordination between nuclear and cytoplasmic divisions.

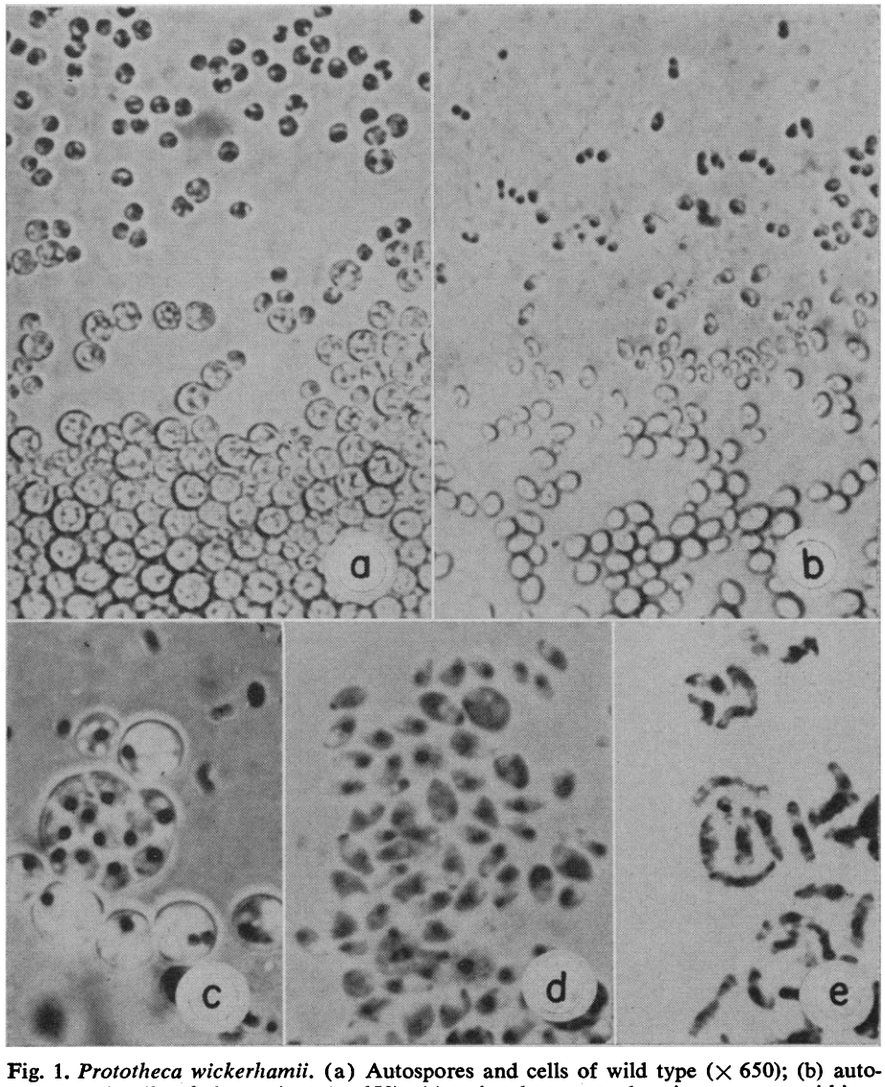

Fig. 1. *Prototheca wickerhamii*. (a) Autospores and cells of wild type (× 650); (b) autospores and cells of the variant (× 650); (c) uninucleate cytoplasmic segments within a mother cell sheath, and uninucleate and binucleate cells from a wild-type culture (× 1450); (d) uninucleate autospores and cells at various stages of development from the variant culture (× 1450); (e) bacillary uninucleate autospores of the variant (× 1450).

The life cycle of the variant differs from that of the wild type in two respects. (i) The liberated autospores of the variant swell and become round before nuclear division occurs (hence the origin of variant cultures from certain spherical cells); and (ii) cytoplasmic cleavage in the variant is irregular, even within the same mother cell wall, giving rise to autospores of variable shape. Physiologically, the wild type and variant are thiamine-deficient and capable of assimilating dextrose and galactose, but not of assimilating lactose, maltose, or sucrose. Both are sensitive to amphotericin B, which in a concentration of 0.2 μg per milliliter of Bacto Penassay broth inhibits their growth at 27°C for 72 hours.

The lack of a sexual cycle in *Prototheca* precludes determination of the genetic basis of the variant, but the variant presumably arose as a result of mutation that interferes with the

biosynthesis of an enzyme involved in regulating cytoplasmic cleavage.

In view of the great emphasis placed on shape and size of autospores in species classification, the variation limits of each morphological characteristic should be carefully determined before a species concept is formed. This variant would undoubtedly have been classified as a new species, if it had not arisen spontaneously in a stock culture of *P. wickerhamii*.

ARIF S. EL-ANI
Division of Laboratories and Research, New York State Department of Health, Albany 12201

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30 March 1967

Primordial Germ Cells in Blood Smears from Chick Embryos

Abstract. *The feasibility of studying avian primordial germ cells in blood smears has been demonstrated. Blood smears prepared from chick embryos of stages 13 to 15 (48 to 55 hours) contained primordial germ cells, which were revealed by the periodic acid-Schiff reaction. The presence of glycogen in the cytoplasm of the primordial germ cells facilitated their selective identification.*

Primordial germ cells (PGC's) have been consistently reported in the blood vessels of early chick embryos because the vascular system provides the chief, if not only, route for the migration of these cells from their place of origin, the extraembryonic germinal crescent, to the site of their ultimate disposition, the gonadal ridge (*1, 2*). Although the size and morphology of the PGC's are characteristic, cytochemical methods have had to be employed to demonstrate these cells, particularly in extragonadal areas. In contrast to mammalian PGC's, which possess sufficient quantities of histochemically demonstrable alkaline phosphatase activity to permit their selective identification (*3, 4*), chick PGC's cannot be distinguished by this technique, owing to the uniform distribution of enzymatic activity among all embryonic cells (*3*). Instead, the chick PGC's have been found to contain abundant cytoplasmic deposits of glycogen which greatly facilitate their recognition in tissue sections subjected to the periodic acid-Schiff (PAS) reaction. Using this technique, Meyer (*2*) observed that the maximum numbers of PGC's are present in the intraembryonic, vascular system during stages 13 (19 somites; 48 to 52 hours) to 15 (24 to 27 somites; 50 to 55 hours) (*5*). Therefore, blood smears prepared from embryos at these critical stages should provide an excellent means for further exploration of the PGC's provided, of course, that they can be reliably distinguished from the other blood elements. Such has not been the case with routine blood stains (*6, 7*).

Blood was removed from the dorsal aortas of chick embryos between stages 13 and 15 with the use of micropipettes according to the method outlined by Lucas and Jamroz (*6*). One half of the smears were dried and the other half were fixed in Gendre's fluid (for glycogen localization). Under the phase microscope, the PGC's were identified by their large size (related to that of the blood cells), their eccentric nuclei, and the presence of refractile vacuoles within their cytoplasm. This cellular appearance closely resembled that of living PGC's observed in squash preparations of whole embryos (Fig. 1). In preparations subjected to the PAS reaction, some of the cytoplasmic vacuoles became intensely magenta-colored; others remained unreactive (Fig. 2). We subsequently confirmed the PAS-positive material to be glycogen by subjecting control smears to malt diastase (*8*) prior to their treatment with periodic acid. The staining of unfixed, dry smears with oil red O (in propylene glycol) also revealed that the PAS-negative vacuoles were actually sites of lipid material (yolk). Dantschakoff (*9*) adopted this characteristic, among others, to identify the PGC's.

The distribution of the PGC's in the circulating blood of staged chick embryos is shown in Fig. 3. By stage 18 the presence of PGC's in blood smears was rare. A few blood cells displayed a slight diffuse reaction to the PAS technique, but they were easily distinguished from the PGC's by their small size.

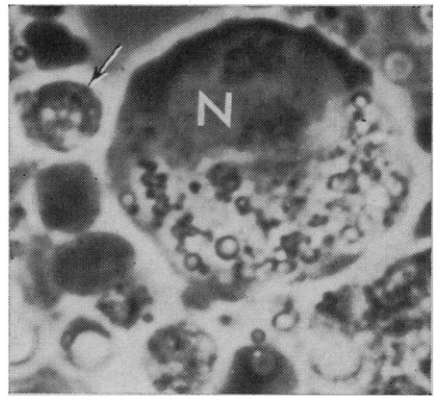

Fig. 1. Phase photomicrograph of living chick PGC in squash preparation of whole embryo, stage 15. Note the large size of the PGC in comparison to that of a blood cell (arrow). *N*, nucleus (× 2000). (Prepared in the laboratory of Dr. R. J. Blandau.)

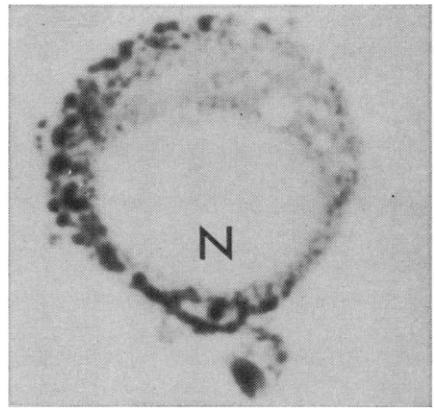

Fig. 2. Photomicrograph of PGC in blood smear at stage 15 stained by the PAS reaction. The dark staining bodies (magenta with the PAS reaction) are glycogen digestible with diastase. The unstained vacuoles represent lipid material. *N*, nucleus.

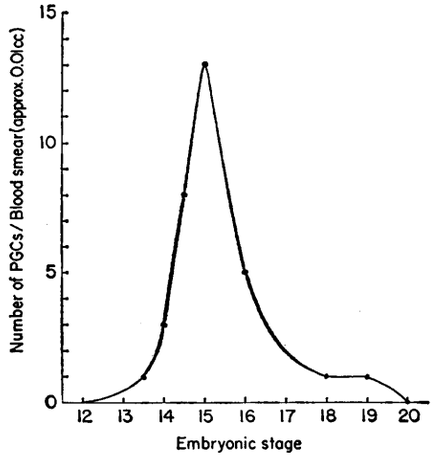

Fig. 3. Distribution of PGC's in the circulating blood of staged chick embryos based on the average of blood smears from five embryos at each stage.