with nitrate ion lend further support to this concept.

Morgan *et al.* (*13*) have reported that the permeability of isolated, perfused rat hearts to glucose is enhanced when cardiac output is increased by an increase in the filling pressure of the left atrium. It would be of interest to know whether or not such changes in cardiac output are associated with an altered balance of Ca^{2+} in the heart muscle cells.

JOHN O. HOLLOSZY
H. T. NARAHARA
Department of Biological Chemistry, Washington University School of Medicine, St. Louis, Missouri 63110

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17 November 1966

Genomic Exclusion: A Rapid Means for Inducing Homozygous Diploid Lines in Tetrahymena pyriformis, Syngen 1

Abstract. *Genomic exclusion is an abnormal form of conjugation occurring between cells with defective micronuclei and normal cells with diploid micronuclei. The progeny are heterocaryons; each cell has an old macronucleus but a new diploid micronucleus derived from one meiotic product of the normal mate. Such cells express genes found in the old macronucleus, are sexually mature, and can be specifically selected. When inbred, they give rise to lines genetically homozygous at all known loci.*

Few species of ciliated protozoa have been successfully employed in both biochemical and genetic research (*1*). *Tetrahymena pyriformis* is a favorite organism for biochemical experimentation (*2*), since it can be grown on defined medium (*3*) and propagated for many fissions without becoming senile (*4*). Rarely, however, has this experimentation been coupled to genetic analysis. This state of affairs is due to the use of asexual strains of *T. pyriformis*, which lack a micronucleus, and to a reluctance to use the known sexual strains because their maintenance and the techniques of crossing are relatively complex. A chief barrier to their use has been the lack of a rapid method for inducing homozygous diploid lines. Autogamy, a form of self-fertilization, does not take place in this organism; only conjugation, or cross-fertilization, occurs. Thus, genetic testing has necessitated making outcrosses followed by the tedious and time-consuming process of extracting homozygotes by inbreeding. Although genomic exclusion was revealed by the appearance of distorted genetic ratios in crosses of certain inbred strains of syngen 1 (*5*), only recently has the cytogenetic basis of this abnormal form of conjugation been understood (*6*). With proper manipulation, genomic exclusion can serve as a useful tool in the rapid genesis of pure homozygous diploid cell lines. The use of such lines should increase the scope of the biochemical experimentation that is possible with this organism.

For genomic exclusion to occur, one of the two parents must have a defective micronucleus (*7*). In my study a normal clone from the heterozygous AB strain was crossed to a defective clone C* from the inbred C strain. Stained preparations (*8*) of dividing cells showed a normal diploid micronucleus in AB cells (Fig. 1a), while C* cells were hypodiploid (Fig. 1b) or had no micronucleus at all (*9*). In the cross of AB and C*, meiosis was abortive in the C* conjugant (Fig. 1c), and genes were not transmitted from C* to the progeny (*5*).

The sequence of nuclear events that takes place during genomic exclusion was determined by cytological observation and by genetic tests of sample pairs obtained from timed matings of the AB and C* clones. Control crosses used two micronucleate clones from inbred strains A and B. Cultures grown in 1 percent proteose-peptone or in Cerophyl ryegrass inoculated with *Aerobacter aerogenes* were washed in Dryl's physiological salt solution and then mixed. Mating occurred after about 1½ hours. In some experiments, the formation of new pairs was stopped after a specific interval by the addition of nutrient. Samples of pairs were taken periodically for cytological examination (*8*), or single pairs were isolated in Cerophyl-*Aerobacter* medium, and genetic tests were conducted.

A difference in mating behavior was observed during genomic exclusion compared with normal conjugation, if mating was not stopped by the addition of nutrient (Fig. 2). The formation of newly paired cells was followed. In the normal cross (A × B) the frequency of new pairs fell to a constant value between 18 and 36 hours. In the AB × C* cross this frequency fell to 2.5 percent at 16 hours, rose to a second peak of 45 percent at 24 hours, and then fell to 4.2 percent at 36 hours. The timing of the second peak is significant in that it occurred after 12 to 16 hours, a time interval sufficient for completion of normal conjugation. This observation suggested that during genomic exclusion a second mating takes place upon completion of a first mating. The finding that both conjugants had diploid micronuclei in newly paired cells during the second peak (Fig. 1d) strengthened this hypothesis for reasons stated below. The formation of such pairs could be prevented if nutrient was added soon after the parental cultures were mated.

The two consecutive conjugations have been designated Round 1 and Round 2. Round 1 is abnormal. Meiosis is abortive in the C* conjugant. The AB conjugant contributes a single meiotic product, which divides mitotically and gives rise to sister haploid nuclei, one of which migrates to the C* conjugant. Diploidy is reestablished in both mates, probably by endoreduplication, although this point has yet to be documented (*6*). The

diploid nucleus in each mate then divides twice, giving rise to two new micronuclei and two new macronuclei; however, the new macronuclei are resorbed, and the old macronucleus becomes the functional somatic nucleus in the progeny.

Round 2 is normal. A new diploid nucleus is formed in each exconjugant from a product of meiosis contributed by each conjugant. This nucleus divides twice to generate two new micronuclei and two new macronuclei. The old macronucleus is resorbed, and the new macronuclei are functional in the progeny.

The above account of the behavior of the nuclei during Rounds 1 and 2 is based on the following major lines of evidence:

1) The micronuclear constitution of paired cells in Round 1 differed from that of Round 2 pairs. Round 1 pairs were made up of one diploid cell and one cell with a defective micronucleus, while Round 2 pairs contained two diploid cells.

2) The old macronucleus was retained after Round 1 but not after Round 2, since a cell type that has two macronuclei and one micronucleus, and is normally seen during conjugation before the first fission, was present after Round 2, but was absent after Round 1. Instead, cells with extra nuclei in various stages of resorption were observed. Some of these nuclei were capable of undergoing prophase of the first meiotic division when the cells remated (Fig. 1d). Furthermore, Round 1 exconjugants, like Round 2 exconjugants, had diploid micronuclei; yet Round 1 exconjugants were sexually mature, while Round 2 exconjugants were immature, or incapable of mating. Moreover, one exconjugant from each Round 1 pair had the phenotype of the AB strain and the other had the C* phenotype. This observation was made for genes at five different loci, and with all five loci the old macronuclear genes, not the new micronuclear genes, were being expressed. In contrast, both exconjugants from a Round 2 pair were phenotypically similar and expressed only genes that were derived from the AB parent.

3) The diploid nuclei of Round 1 exconjugants were derived from one meiotic product of the AB conjugant, while the nuclei of Round 2 exconjugants were formed from two meiotic products, one from each Round 1 conjugant. Strain AB is heterozygous for alleles for H serotype (H^A/H^D) distinct from those in C* (H^E/H^E). If the AB conjugant contributed only one meiotic product to Round 1 exconjugants, both exconjugants from the same Round 1 pair should be genetically identical and should have micronuclei that are either H^A/H^A or H^D/H^D. When remated, all the progeny within a cross should be phenotypically identical, but half the crosses should give rise to the Ha phenotype and half to the Hd phenotype. As a test of this prediction, 40 sets of Round 1 exconjugants were remated. Thirty-one of the crosses were viable. All progeny from a single cross were phenotypically identical. However, the crosses fell into two categories with regard to serotype: 13 crosses gave rise to Ha progeny and 18 to Hd progeny. Tests were extended to four other loci, and the results were those expected on the basis of the genotype of strain AB (*E-1*^{B}/*E-1*^{B}; *E-2*^{B}/*E-2*^{B}; *P-1*^{A}/*P-1*^{B}; *mt*^{A}/*mt*^{B}) (*10*). The progeny of 16 crosses were all E-1b, E-2b in phenotype, nine of which were P-1a and seven of which were P-1b. A 1:1:1:1 ratio was observed among the progeny for *H* and *P-1*. Of six crosses tested, two classes were observed for *mt*. Testcrosses of Round 2 progeny were made by selfing them or outcrossing them to inbred strains of known genotype, and the results of these tests confirmed their predicted genotypes. If Round 1 exconjugants were allowed to remate at random, three phenotypic classes (Ha, Had, and Hd) were observed among the progeny in an approximate 1:2:1 ratio. The appearance of the heterozygous class (H^A/H^D) indicates the derivation of the diploid nucleus of Round 2

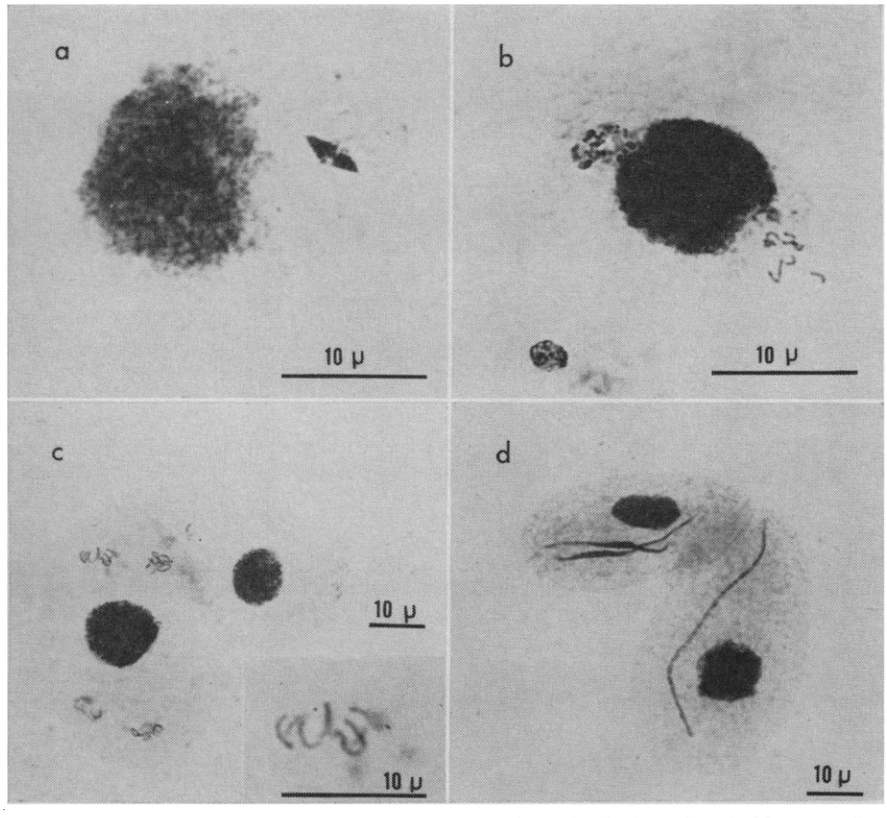

Fig. 1. (a) Micronucleus of cell from AB clone during mitosis (anaphase). Note spindle-shaped aggregate. Individual chromosomes are not resolved, as is normal for the micronucleus of ciliated protozoa. During meiosis AB cells can be shown to have the normal diploid ($2n$) number of chromosomes for *T. pyriformis* ($2n = 10$). Note insert in (c) where $n = 5$. (b) Mitotic chromosomes of micronucleus of C* cell at anaphase. The aggregate is not observed, and individual chromosomes are clearly resolved. These chromosomes are about one-fourth the size of normal meiotic chromosomes. Compare with those in insert (c). (c) AB mated with C*. C* conjugant is on right. Insert: enlargement of AB chromosomes. (d) AB × C*: Crescent stage, or prophase of first meiotic division, during Round 2. Each conjugant has one or more diploid crescents. As many as four diploid crescents have been observed in a few conjugants. In the pair shown, the conjugant on the left has two crescents.

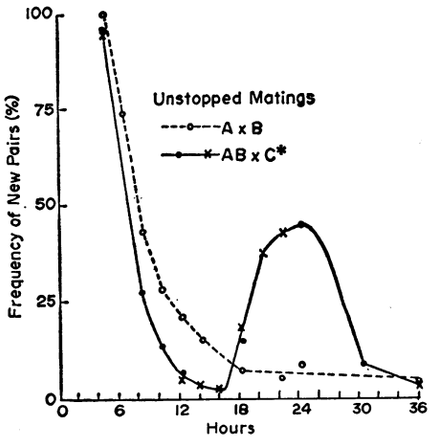

Fig. 2. Frequency of newly formed pairs over a 36-hour period in unstopped matings of AB and C* and a normal cross (A × B). The percentages of new pairs (from attachment through the crescent stage) from two experiments (●———●, x———x) are plotted from the AB × C* cross. For the normal cross these percentages (○----○) were derived from one experiment. Each point is based on observations of 100 pairs (○----○), 300 pairs (●———●), or 500 to 560 pairs (x———x).

exconjugants from two meiotic products contributed from both conjugants, each derived from a different Round 1 pair.

Genomic exclusion is unlike any of the standard forms of sexual reorganization in the ciliated protozoa, that is, autogamy, cytogamy, and normal conjugation (*11*). Since one meiotic product from a single mate is involved, and since homozygosity of both exconjugants is the result, it resembles autogamy more closely than it resembles the other sexual methods in its genetic consequences.

Genomic exclusion can be used to advantage as a rapid means for inducing homozygous diploid lines from heterozygotes. Round 1 exconjugants, which are heterocaryons, can be specifically selected if timed matings are effected between a micronucleate heterozygote and a clone such as C*, and if each of the resulting pairs is isolated into a separate container before the exconjugants come apart. When a pair is isolated in Cerophyl-*Aerobacter* medium and the two exconjugants are kept together, each exconjugant will replicate a population of cells. As the *Aerobacter* are exhausted, the two populations will mate and give rise to several thousand Round 2 pairs which are genotypically identical. Since some unmated cells are also present, sample pairs are removed. Each container will have a homozygous population of pairs, but different containers will have populations of pairs derived from different meiotic products. Screening for desired gene combinations is thus a simple matter of sampling a pair from each of these containers and examining the phenotypes of the cell lines that develop. Each line will be homozygous for a different combination of genes.

SALLY LYMAN ALLEN

Department of Zoology,
University of Michigan, Ann Arbor

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8. A sample containing several thousand paired or single *Tetrahymena*, or both, in Dryl's physiological salt solution [S. Dryl, *J. Protozool.* **6** (suppl.), 25 (1959)] was concentrated by centrifugation (500*g* for 3 minutes) into a soft pellet, and approximately 0.01-ml amounts of this pellet were diluted and spread on clean glass microscope slides with Nissenbaum's fixative [G. Nissenbaum, *Science* **118**, 31 (1953)]. The slides were stored in 70 percent ethanol before further processing, usually within 2 weeks. They were then further fixed in three parts of 95 percent ethanol to one part of glacial acetic acid for 15 to 20 minutes, hydrated, hydrolyzed in hot 1*N* HCl at 60°C for 15 minutes, and stained in Gomori's hematoxylin at 60°C for 10 minutes [Y. Melander and K. G. Wingstrand, *Stain Technol.* **28**, 217 (1953)]. Cytoplasmic staining was removed by 45 percent glacial acetic acid (23°C, 15 minutes). The preparation was covered, gently blotted, flattened over steam, and ringed with beeswax sealing compound (one part of paraffin to one part of gum mastic to one-fifth part of beeswax). The slides were stored, flat, at 4°C to prevent evaporation and to retard decomposition of the stain. These methods were based on those described earlier by C. Ray, Jr., *J. Protozool.* **3**, 88 (1956) and by C. Wells, *ibid.* **8**, 284 (1961).
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10. Esterase-1 and esterase-2 are specified by genes *E-1* and *E-2*. *P-1* controls an acid phosphatase. The esterases and phosphatases are typed by suitable staining methods after starch-gel electrophoresis. A spectrum of mating types is controlled by alleles at the *mt* locus. The *E-1* and *mt* loci are loosely linked; all others, including the *H* serotype locus, are unlinked [S. L. Allen, *Genetics* **49**, 617 (1964)].
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12. Supported by PHS research grant HD-01243. I thank Mrs. Barbara Morrison Licht and Mrs. Sharon L. Koch for technical assistance, Dr. Carolyn Wells for help in developing the cytological methods, and Dr. Eduardo Orias for fruitful discussions.

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Mongoose Throwing and Smashing Millipedes

Abstract. *African millipedes of the genus* Sphaerotherium *coil into tight spheres when disturbed. Their tough skeletal armor offers protection against some predators, but not against the African banded mongoose* Mungos mungo, *which smashes them by hurling them against rock.*

Millipedes of the order Glomerida (*1*) have the peculiar habit—shared with certain armadillos, pangolins, and some of the familiar isopod Crustacea known as "pillbugs" or "sowbugs"—of coiling into a tight sphere when disturbed (*2*). The behavior is usually assumed to be defensive, but this had never been tested with millipedes. We recently obtained from South Africa mature specimens of two large glomerids of the genus *Sphaerotherium* (Fig. 1), and offered these to several caged predators (*3*). In most cases the millipedes proved invulnerable, as expected. Nevertheless, they did fall prey to one particular enemy, which was singularly adapted to cope with them.

Both species have an unusually hard skeletal shell. The slightest provocation, even a mere tapping of the cage, causes them to coil. Coiling, plus the possession of armor, are the only noticeable means of protection. *Sphaerotherium* lacks the defensive glands found in some other glomerids (*4*) and

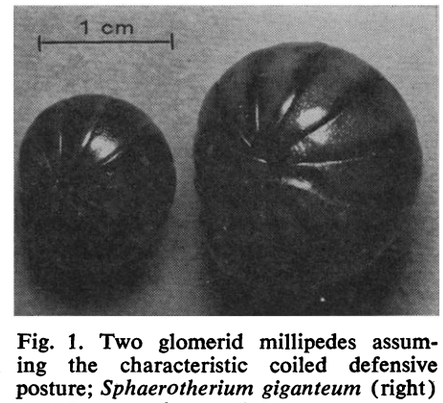

Fig. 1. Two glomerid millipedes assuming the characteristic coiled defensive posture; *Sphaerotherium giganteum* (right) and *S. punctulatum* (left).