not explain this paradoxical effect, but we should mention that Bloom *et al.* also failed to detect a relation between chromosomally abnormal survivors in Hiroshima and Nagasaki and their estimated doses of radiation (*8*). Chromosome aberrations did not correlate with age or sex, nor could any relation be established between the occurrence of thyroid lesions and chromosome abnormalities.

We cannot account for the unusually high incidence of acentric fragments in the unexposed and their relative deficit in the exposed people. Radiation from ingested radionuclides is not likely to be a factor, since all Marshall Islanders have been exposed to low levels of residual radionuclides such as $Cs^{137}$, $Zn^{65}$, and $Sr^{90}$ since their return to Rongelap in 1957, and since body burdens of these elements were about the same in both groups. Although viruses and some chemicals, in addition to ionizing radiations, are known to produce breaks in human chromosomes in vivo and in vitro (*13*), the kind and distribution of the fragments seen in the cells of the unexposed group do not suggest this origin. If these agents were implicated, it is not clear why they failed to produce a similar effect in the exposed people who have lived on the same island under identical environmental conditions since 1957.

Similar chromosome aberrations have been reported in the Japanese fishermen exposed to radiation from the same fallout (*7*); the incidence of aberrations, excluding aneuploid cells, was 2.1 percent and thus identical with our finding; most were two-break aberrations. The incidence of acentric fragments in a control population of Japanese studied by the same authors was 0.11 percent —20 times less than our finding among the eight unexposed Marshall Islanders. In a controlled cytogenetic study of sampled survivors of the atomic bombings of Hiroshima and Nagasaki, Bloom *et al.* have found exchange-type chromosome aberrations in 33 persons —35 percent of 94 survivors examined 20 years after exposure (*8*); the incidence of aberrations was 0.6 percent in the exposed group—less than half our finding among the Marshall Islanders. Bloom *et al.* found only a single acentric fragment in 8847 cells from the 94 controls, an incidence of 0.01 percent; for the 33 aberration-positive individuals, the dose ranged from 237 to 891 rads, and for none of the entire group was it less than 200 rads. All persons examined were 30 years of age or younger at the time of the bombings.

The results of our study demonstrate that a small but significant number of chromosome aberrations persists in blood lymphocytes of some Marshall Islanders 10 years after exposure to fallout radiation. The conclusion that at least some of these aberrations were caused by the radiation, and not by other factors, rests on the finding that exchange-type aberrations were found only in the exposed people and not among the controls.

The biologic significance of persistent chromosome aberrations in blood lymphocytes of hematologically normal persons many years after exposure to ionizing radiation is not known. In particular, any role that aberrations of this type may play in the pathogenesis of radiation-induced leukemia can only be surmised at present. It is problematical and indeed doubtful whether chromosome aberrations in lymphocytes can serve to indicate abnormalities in other tissues, except perhaps inferentially. Indeed, of the ten individuals who have developed thyroid lesions since our examinations were made, only three show double-break aberrations.

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## Chlamydomonas reinhardi: Heterozygous Diploid Strains

Abstract. *Zygotes of the unicellular green alga* Chlamydomonas reinhardi *occasionally divide mitotically to give rise to stable diploid vegetative strains. As well as by their mode of origin, these strains are distinguished from haploids by cell and nuclear size, DNA content per nucleus, and chromosome number. Diploid strains heterozygous for a variety of mutant genes are phenotypically wild type and mating-type minus. Thus these mutant genes are recessive to their wild-type alleles, and the mating-type-minus is dominant over the mating-type-plus allele.*

The life cycle of the unicellular green alga *Chlamydomonas reinhardi*, as typically described, involves the fusion of two haploid gametes to form a diploid zygote which then develops into a thick-walled resting cell (mature zygote). Mature zygotes undergo meiosis and germinate to produce four or eight haploid cells that are capable of vegetative growth.

Recently it has been observed that newly formed zygotes may follow an alternate course of development (*1*): rather than differentiating into mature zygotes, some may divide mitotically to give rise to diploid cells that remain vegetative. I now describe a selective method for obtaining diploid strains, and some of the characteristics that distinguish them from haploid strains.

My method for recovering diploid strains is based upon the inability of mutant auxotrophic haploid cells to grow under conditions that allow prototrophic cells to proliferate rapidly. These conditions are met by plating a mating mixture of two different auxotrophs on a minimal agar medium lacking the required growth factors.

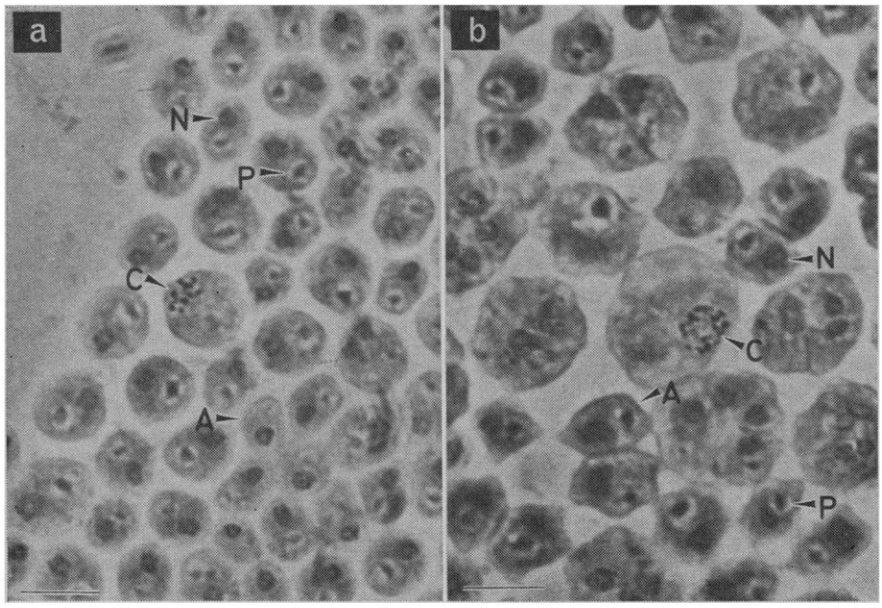


Fig. 1. Haploid (a) and diploid (b) vegetative cells of *C. reinhardi* stained with azure A (*4*), illustrating differences in chromosome number (*C*), nuclear size (*N*), and cell size (*A*); *P* indicates the pyrenoid. Scale, 10 $\mu$.

On the assumption that the mutant genes conferring auxotrophy are recessive to their wild-type alleles, mitotic division of heterozygous zygotes should lead to the appearance of diploid prototrophs. However, under these conditions haploid prototrophs might also derive from reverse mutation or from recombination during meiosis in germinating mature zygotes. By applying the method to be described, one can recover diploid prototrophic strains and distinguish them from haploid prototrophs (*2*).

The haploid auxotrophic strains initially used were arginine-1 (*arg-1*) and arginine-2 (*arg-2*); since these two are closely linked (*3*), they were chosen to minimize the appearance of prototrophs resulting from gene recombination. Gametes of both strains were combined, plated on minimal agar medium, and continuously illuminated at 26°C. After incubation for 30 to 36 hours, the plates were examined microscopically (×60) and found to contain, as expected, both thick-walled, mature zygotes and unmated cells; also present were small prototrophic colonies composed of 10 to 20 cells. Five days later each small colony had developed into a large, macroscopically visible colony. In addition, microscopic examination revealed the presence of germinated zygospores and new small prototrophic colonies.

Since the new small prototrophic colonies were identical in position with the mature zygotes observed earlier, they were assumed to be wild-type recombinants. As previously observed (*3*), the frequency of these recombinants was approximately 12 percent of the total number of germinating mature zygotes.

The large colonies were taken to be diploid prototrophs; they were not recombinants, since they appeared before zygote germination. It is improbable that they were revertants arising from either of the parental strains, since no wild-type colonies were detected when $10^8$ cells of each parental strain were plated separately. Moreover, at different mating efficiencies, the frequency of the large colonies was shown to be a function of the number of mature zygotes but not of the parental input. It was estimated that approximately 4 percent of the zygotes formed in the mating mixture failed to differentiate into mature zygotes, but divided mitotically to give rise to vegetative diploid colonies.

Several lines of evidence suggest that the nuclei in the cells from the large prototrophic colonies are diploid:

1) *Mode of origin.* The fusion of pairs of gametes to form zygotes, and the subsequent division of these zygotes to form vegetative colonies, were observed microscopically.

2) *Chromosome number.* The haploid number of the cells of the parental strains is between 10 and 12 (*4*). At least 20 chromosomes have been counted from cells of the diploid strains. Haploid and diploid mitotic figures are compared in Fig. 1; while the absolute chromosome number cannot be determined from this material, it is apparent that diploid nuclei contain approximately twice as many chromosomes as do nuclei from the haploid strains.

3) *DNA content.* The amount of DNA per nucleus in diploid strains is approximately twice that found in haploids (*5*).

4) *Cell and nuclear size.* Cells of diploid strains in the log phase of growth (liquid culture) are larger than cells from haploid strains grown under identical conditions. This size difference is most obvious at the "A-cell" stage (*6*) of the vegetative growth cycle; differences in nuclear size also are evident at this stage (Fig. 1).

5) *Behavior in crosses.* Mature zygotes resulting from diploid × haploid crosses give rise to tetrads of four or eight cells; however, most of the tetrads are "incomplete," since more than 80 percent of the cells fail to give rise to vegetative colonies. Incomplete tetrads are extremely rare in haploid × haploid crosses, but they are characteristic of all crosses of diploid × haploid strains. These results lead to the suggestion that death of the cells is a consequence of gross chromosomal imbalance; nuclei must be either haploid or diploid (or nearly so) in order to divide and survive. The results support the interpretation that the strains are really diploids and not disomic for certain chromosomes.

6) *Heterozygosity.* When diploid strains derived from the mating *arg-1* × *arg-2* were crossed to a haploid wild-type strain, most of the viable meiotic products were phenotypically wild type. However, the fact that both *arg-1* and *arg-2* mutant colonies were recovered indicates that the diploid parent was heterozygous for both mutant genes. Use of multiple mutants as parents produced diploid strains that were heterozygous for six loci situated in four different linkage groups. These diploids were not only heterozygous for characters used to select prototrophs, but also heterozygous for the nonselective mutant traits paralyzed flagella (*pf-16*) and streptomycin resistance (*sr*) (these two mutants are not linked to the selective markers). All six mutants were recovered when the diploid was crossed to a haploid wild-type strain. All mu-

tant genes so far introduced into heterozygous diploid cells have been shown to be recessive to their wild-type alleles. In addition, the two alternative mating-type alleles *mt*− and *mt*+ have been tested for dominance. All the diploid cultures so far examined have been mating-type minus, indicating that *mt*− is dominant over *mt*+. To confirm this supposition a diploid heterozygote was selected from the mating *nic-7 mt*+ *ac-29*+ × *nic-7*+ *mt*− *ac-29* (*nic-7*, *mt*, and *ac-29* are very closely linked). From a cross of this heterozygote with the haploid wild-type strain, both *nic-7 mt*+ *ac-29*+ and *nic-7*+ *mt*− *ac-29* progeny were recovered. These results show that nonrecombinant derivatives of both parental chromosomes are present in the diploid, and support the original observation that *mt*− is dominant in cells heterozygous for mating type.

7) *Stability*. All diploid strains recovered so far have remained diploid in successive subcultures longer than 12 months. The fact that haploid cells have not been detected indicates that the cells of vegetative diploid strains do not undergo meiosis. However, mitotic recombination probably occurs, although rarely, since cells homozygous for one or more markers, but still heterozygous for other markers in the same linkage group, have been recovered.

The existence of diploid cultures of *C. reinhardi* provides a new tool for both formal and biochemical genetic investigations with this organism. Studies of mitotic recombination, and of the evaluation of units of genic activity defined by complementation analyses, are now possible with this organism.

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## Improved in vitro Survival of Normal, Functional Spleen Cells

Abstract. *An enrichment method for improving the viability of monolayers of normal mouse spleen cells is described. These cells are capable of saving lethally x-irradiated recipient mice from death, presumably by proliferating and differentiating into mature blood cells.*

Attempts to culture normal, functional hemopoietic or antibody-producing cells have been discouraging (*1*). We now report a method for development of such cultures. This method involves a process of selection or adaptation analogous to the enrichment culture procedures used in microbiology. It has also been successfully applied to cancer tissue in developing several clonal lines of functional cells (*2*).

Mouse spleen cells were inoculated into culture bottles and, after a few days, the small fraction of cells which survived was harvested. Because these cells had been passed in vitro once we refer to them as passage-1 cells or $P_1$ cells (*3*). These cells were then injected intravenously into mice which had been irradiated with a lethal dose of x-rays. The injected cells proliferated in the x-irradiated animal, repopulated the depleted hemopoietic organs, and thus saved the host from x-irradiation death (*4*). After 2 or 3 weeks, when the animals had partially recovered from irradiation, they were killed and their spleen cells were inoculated into culture bottles. Again, after 4 days in vitro these cells ($P_2$) were harvested and injected into lethally x-irradiated mice. This whole process was repeated to obtain $P_3$, $P_4$, and $P_n$ spleen cells.

The choice of lethally x-irradiated recipients is a useful adjunct to this technique because it circumvents the host-versus-graft reaction. This process of serial in vitro and animal passage selectively enriches the cell populations which not only survive the conditions in vitro but also perform a life-saving function in the lethally x-irradiated recipients. Thus, briefly, two separate selective pressures are involved—one requiring viability in vitro, and the other requiring proliferation and differentiation in vivo into functional blood cells.

Parenchymal spleen cells of 8- to 10-week-old $LAF_1$ mice (Jackson Memorial Laboratory, Bar Harbor, Maine) were passed through a 40-mesh stainless steel screen and suspended in $F_{10}$ medium supplemented with horse serum (15 percent) and fetal calf serum (2.5 percent) (*5*). Stromal cells remaining on the screen were discarded. A portion of the cell suspension was stained with crystal violet, and cells were counted in a hemocytometer. Approximately $10^8$ nucleated cells were inoculated into 20 ml of medium in 150-ml tissue-culture bottles. The bottles were incubated at 37°C in a water-saturated atmosphere of 95 percent air and 5 percent $CO_2$. After 4 days, the small fraction of cells surviving was scraped from the bottles with a rubber policeman. Approximately $1.5 \times 10^7$

Table 1. Survival of lethally irradiated mice after intravenous injection of spleen cultures. All mice were irradiated with 770 to 850 roentgens and received $1 \times 10^6$ to $8 \times 10^7$ cells (most animals received 1 or $2 \times 10^7$ cells). Mice receiving the fewest cells had the highest mortality. All except one of the controls were dead by 21 days after irradiation. Most died from 10 to 14 days after x-irradiation.

| Origin of hemopoietic cells | No. of injected mice | | No. of uninjected controls | |
|---|---|---|---|---|
| | Total | Survivors | Total | Survivors |
| FSC* | 5 | 5 | 8 | 0 |
| $P_1$ | 19 | 12 | 7 | 0 |
| $P_2$ | 14 | 13 | 12 | 0 |
| $P_3$ | 13 | 12 | 6 | 0 |
| $P_4$ | 7 | 5 | 8 | 1 |

* Freshly isolated spleen cells.

Table 2. Comparison of peripheral blood erythrocyte and leukocyte counts of normal mice with lethally x-irradiated controls and lethally x-irradiated experimental mice harboring various spleen cells passaged in vitro. RBC, red blood cells; WBC, white blood cells.

| Origin of hemopoietic cells | Number of mice | $10^6$ RBC/mm³ | | WBC/mm³ | |
|---|---|---|---|---|---|
| | | Range | Mean | Range | Mean |
| $P_0$ (normal) | 5 | 8.5–11 | 10.2 | 4000–14500 | 13000 |
| $P_2$ | 5 | 6.6– 7.5 | 6.9 | 250– 4000 | 1200 |
| $P_3$ | 5 | 6.0– 7.5 | 6.5 | 300– 3500 | 1000 |
| $P_4$ | 4 | 6.0– 7.0 | 6.3 | 250– 900 | 450 |
| X-irradiated controls | 3 | 2 – 3.3 | 2.9 | | < 50 |