MUTAGENESIS OF HAPLOID CULTURED FROG CELLS[1]

LISELOTTE MEZGER-FREED

The Institute for Cancer Research

Fox Chase Center for Cancer and Medical Sciences

Philadelphia, Pennsylvania 19111

Running title: MUTAGENESIS IN HAPLOID CELLS

Send proofs to: Dr. Liselotte Mezger-Freed
The Institute for Cancer Research
7701 Burholme Avenue
Fox Chase, Philadelphia, Pennsylvania 19111

DISCLAIMER

ABSTRACT

Haploid cells afford an opportunity to test some of the assumptions from bacterial genetics which have been adopted by somatic cell geneticists. Haploid cultured cell lines derived from the grass frog Rana pipiens were compared to diploid cell lines in order to test a model which predicts that recessive mutations will be expressed in diploid cells with a frequency equal to the square of that in haploid cells. Haploid and diploid monolayer cultures were compared for (1) survival after exposure to compounds known to be mutagenic for bacteria (a measure of the frequency with which lethal mutations are expressed), and (2) the induction of drug-resistant variants (putative mutants) by such compounds. The proportion of cells which survived from diploid cultures was no more than ten times that from haploid cultures, a much smaller difference than predicted. Furthermore, the frequency of drug-resistant variants was independent of ploidy. Therefore, the validity of the follcwing assumptions is in question: (1) Haploid eukaryotic cells express mutations with a frequency comparable to that in bacteria; (2) molecules which increase the frequency of stable phenotypic variants in culture do so by inducing gene mutations; (3) stable phenotypic variants whose frequency in culture is increased by putative mutagens arise as the result of gene mutation.

Haploid vertebrate cell lines constitute an important material for somatic cell genetics since such lines should yield mutants at a frequency at least several orders of magnitude greater than that for the normal diploid condition (FREED 1962; FREED and MEZGER-FREED 1970; MEZGER-FREED 1972). According to bacterial models, recessive mutations at a locus with a mutation frequency of 10^{-6} would be expressed in haploid cells 10^{6} times as frequently as in diploid cells. The yield of mutants would be further increased by treatment with mutagens. The harvest resulting from such a combination of haploid cells and mutagens should, in the course of events, lead to an understanding of eukaryotic affairs as it has for bacteria and phage.

Predictions about the expression of mutation can most directly be tested by comparing survival in cultures of different ploidy after the application of mutagens since lethal mutations are for the most part recessive. Such ploidy comparisons are particularly powerful for haploid cells; a frequency difference between 10^{-6} (haploid) and 10^{-12} (diploid) can be more easily discerned in cell culture than one between 10^{-12} and 10^{-24} (diploid-tetraploid comparisons). Ploidy comparisons can also provide evidence for or against the origin of a specific phenotype as a gene mutation (DNA base change) (HARRIS 1971; MEZGER-FREED 1971; MEZGER-FREED 1972). For example, enzyme deficiencies would generally occur as a result of recessive mutations and therefore be induced by mutagens more frequently in haploid cultures.

In order to test these assumptions about gene mutation derived from micro-organisms, I have used the first vertebrate haploid cell lines; they were derived from embryos of the grass frog Rana pipiens (FREED and MEZGER-FREED 1970). These haploid cell cultures were compared to their diploid counterparts in regard to (1) survival after mutagen treatment, and (2) the production of phenotypic variants. Since the difference in survival was at most ten-fold and since the

frequency of two drug-resistant phenotypes was apparently a property of specific cell lines rather than their ploidy, we find ourselves in a dilemma familiar from investigations of carcinogenesis. Does a molecular compound act as a mutagen in producing a specific stable phenotype such as a transformation? Or, approached in another way, are these phenotypes the result of gene mutation and the compound (carcinogen) therefore a mutagen?

MATERIALS AND METHODS

The most useful of the haploid Rana pipiens cell lines has been ICR 2A which was initiated in 1968 from haploid embryos; such embryos are reliably obtained either by surgical removal of the maternal nucleus or by fertilization with genetically inactivated sperm. The haploid nature of the cells is attested to by the chromosome number (13), DNA content (5 pg) and volume (1200 μ^3) which are all half the diploid value. All of the ICR 2A cultures remained at least 95% haploid for 400 generations, after which some cultures began to show an increased proportion of diploid metaphases. Measurements of ICR 2A metaphase chromosomes were compared to a series of embryo metaphases; differences in length of less than 1% of the total genome were found for several chromosomes. Since genetically determined small, but significant, differences exist in normal individuals (i.e., human) (NEURATH, LIN and LOW 1972), and since the differences found for ICR 2A are small compared to those in pseudodiploid lines, we consider these cells to be not only haploid but euploid.

Two additional cell lines provided material for the haploid-diploid comparisons: ICR 21 and ICR 31. They contain genetic material from the same male frog although the enucleated eggs were from different females. Clones derived from each line constituted the haploid material. The haploid ICR A_1 and diploid ICR A_2 were each derived from ICR 2A as clones. For the ploidy comparisons of survival, all haploid cultures were over 90% haploid.

In order to increase the frequency of mutants, five compounds were chosen: MNNG (N-methyl-N'-nitro-N-nitrosoguanidine), EMS (ethylmethanesulphonate) and three acridine half mustard mutagens, ICR 191, ICR 340 and ICR 372. All have been shown to be mutagenic for bacteria. The three ICR compounds have been tested and shown to be mutagens in the Salmonella histidine reversion system (AMES and WHITFIELD, JR. 1966); they also have anti-tumor activity for mouse ascites tumors (CREECH, PRESTON, PECK and O'CONNELL 1972). Evidence that such compounds also act as mutagens for vertebrate cell culture systems is inconclusive, in part because the variants induced by them have not been proven to be mutants (MEZGER-FREED 1972; MEZGER-FREED 1973).

The mutagens were applied for 48 hours, during which time most cells in a culture would be expected to pass through a cell cycle, including DNA replication. Mutagen solutions were thawed just before application. The probable decay of mutagenic activity in a compound such as nitrosoguanidine was disregarded; such decay, as well as other interactions of cells, mutagen and medium, should be the same for both haploid and diploid cells and so cancel out in the comparison.

Mammalian cell culture procedures are applicable to the amphibian cell lines with minor modifications, e.g., in the osmotic pressure of the medium. At 25°C, the minimum population doubling time is 40 hours.

Media containing mutagens as well as media for selecting phenotypes are applied to monolayer cultures, which are usually at a density of 2×10^5 cells per 25 cm^2 flask. Low cell density or plating (cloning) conditions have generally been avoided, mostly for practical reasons such as the higher total number of cells which can be screened and plating efficiencies which are generally under 50% for this material.

Cell number is measured by counting the cells attached to the growth surface in a series of "windows" viewed through an inverted microscope. Thus the same, rather than replicate, flasks are followed throughout a period of time. If the

total cell number per 25 cm^2 flask is between 10^5 and 10^6, duplicate flasks usually differ by less than 10%. However, if the cell number decreases as a result of mutagen treatment, the count is less accurate, not only because of the smaller number, but because the cells become progressively less evenly distributed, a process which may ultimately result in colony formation.

Haploid-diploid comparisons of survival after mutagen treatment

The effects of the five putative mutagens on survival were determined for three haploid-diploid pairs: ICR $2A_1$ and ICR $2A_2$, ICR 21 and ICR $21C_8$, ICR 31 and ICR $31C_{12}$ (Figure 1). The haploid and diploid cells of each pair were isogenic to the extent that they were derived from the same cell line so that differences should be the result of ploidy. Survival, or the number of cells in a flask as a function of those present at zero time, was monitored during and after mutagen treatment until the population resumed the normal rate of increase, that of the control. Although it is not possible with this method to assess the relative contributions of lethal mutations, toxicity (non-genetic lethal effects) and cell division arrest in determining the number of cells, an estimate of the proportion of the initial population which survived can be made by extrapolating to zero time the slopes of the curves once normal growth rates are resumed (e.g., ALEXANDER and MIKULSKI 1961). Comparisons of these estimates for Figure 1 indicate that, although in no case did haploid cells show a higher proportion of survivors than diploid cells, the diploid cells had at most about a ten-fold better chance of surviving treatment by the putative mutagens. (The survivors in the haploid populations were not the progeny of the small percent of diploid cells as shown by a check of chromosome counts). Since these results are so far from the prediction of a 10^6-fold difference, one must conclude either that a large part of the lethal effect of these compounds is not the result of gene mutation or that the expression of gene mutation in these cells cannot be explained by the model for bacteria.

The observation that factors other than the number of chromosome sets influence cell survival in these experiments is supported by comparisons of the reactions of cell lines. For example, MNNG has a more lethal effect on ICR $21C_8$ than on ICR $31C_{12}$; both lines are haploid and so the difference cannot be due to ploidy. The same conclusions follow from a comparison of the diploids ICR 2A and ICR 31 after exposure to ICR 191.

The complexity of the interactions between cells and mutagens is further illustrated by the fluctuations in cell number in individual survival curves (Figure 1). For example, cells from the haploid line ICR $21C_8$ decrease in number when exposed to ICR 340, then multiply when returned to normal medium; another decrease is followed by a return to a normal growth rate two weeks after ICR 340 removal. The possible significance of these fluctuations is discussed in the next section.

The differences in the level of lethality of the three acridine mustard compounds (most lethal ICR 191 > ICR 372 > ICR 340) can be attributed to minor differences in molecular structure since they were all applied at the same molar concentration. Correlations between structure and antitumor activity and also mutagenesis have been made for a series of over 80 of these heterocyclic compounds (CREECH, PRESTON, PECK and O'CONNELL 1972) and may be instructive if extended to the haploid-diploid comparisons.

Clonal analysis of survival after mutagen treatment

If cell lines of the same ploidy composition differ in their ability to survive exposure to a putative mutagen (Figure 1), survival may depend on something other than the genotype. It then becomes important to determine if a single cell population is also heterogeneous since it would suggest that an increased frequency of a variant in "mutagenized" cultures could be the result of selection rather than mutation. Such an increased frequency would result if the variant phenotype was by

chance associated with a cell resistant to the mutagen or if the new phenotype was itself resistant. That such heterogeneity of mutagen resistance exists is illustrated by Figure 2; a population of cells from the line ICR B20, which happens to be a variant of ICR 2A, was sampled by cloning in Multiwell Microtest plates and cultures of these subclones were exposed to 1 µg/ml of compound ICR 191. The survival of the clones after ICR 191 treatment varies from resumption of a normal growth rate at 20 days to complete killing. If the proportion of the population that survived is estimated by extrapolation to zero time, it can be shown to vary significantly from clone to clone, yet these clones are haploid and should be substantially isogenic. The degree of resistance to ICR 191 is heritable since a cell line or subclone when retested shows a similar degree of resistance.

The fluctuations in cell number in the thirty days after exposure of the B20 subclones to ICR 191 were also noted for the independent cell lines shown in Figure 1. The reactions can be divided into four phases (MEZGER-FREED 1973) about whose significance we can as yet only speculate. The first, usually a decrease in cell number, occurs during exposure to ICR 191 and is considered to be a toxic (non-mutagenic) effect for several reasons: its appearance before a gene product might be depleted as a result of mutation expression and subsequent degradation; the variation among the subclones in the amount of cell death; changes in cell shape within hours after ICR 191 is applied; the return of some clones to normal growth rates after Phase I. In Phase II, the period after ICR 191 removal, most of the clones multiply. Thereafter, in Phase III, the cell number decreases in the period of time which would normally be three or more generations from the beginning of ICR 191 application. This phase may correspond to the period during which selective agents are most effective in isolating drug-resistant variants (CHU and MALLING 1968; ORKIN and LITTLEFIELD 1971a, 1971b); the time was correlated with the occurrence of three cell division cycles, whether or not these were delayed by the action of mutagen.

In ICR 191 such delays in cell division may occur to different extents in the subclones and also in the cells of a subclone and so account for the variations in the time of Phase III. Although the expression of a variant phenotype may require several cell divisions because it depends on the completion of two rounds of DNA replication (CHU and MALLING 1968), there are other possibilities. For example, it may be that ICR 191 has a toxic effect which is expressed only with an increase in cell surface such as occurs after cell division. Phase IV, the last phase, is a return to a normal rate of cell multiplication; since it takes place after the second decrease in cell number (mutation?) and several weeks after removal of ICR 191, it may reflect the proportion of cells with efficient DNA repair.

Factors affecting mutagen resistance

Since the differences between the haploid and diploid cultures in their reactions to mutagens are often small, it becomes more critical to assay the role of culture conditions in determining cell survival. Organic molecules such as mutagens are similar to, and in some cases identical to, compounds associated with drug resistance in cultured cells. Thus, cell survival may be dependent on drug concentration, duration of treatment, previous exposure to a drug, the age of the cell culture and cell density (HARRIS 1967; ORKIN and LITTLEFIELD 1971a). An analysis of these factors may determine conditions which are on the threshold of affecting the survival of a diploid cell line; in this situation, lethality not due to mutation should also be low for haploid lines barring differences of the kind found between subclones; under these conditions, differences in survival between haploid and diploid cultures, especially in Phase III, could with more confidence be ascribed to mutagenesis. For example (Figure 1e), ICR 372 decreases the cell number of the diploid lines relatively little compared to the controls; under the same conditions, there is a far more lethal effect on haploid cultures. However, with ICR 191, in order to obtain similar results with the diploid line, the molarity

of the solution must be reduced to half (Figure 3); at this concentration, the haploid survival also is like that in ICR 372 at 1 μg/ml. Another factor, cell density at the time of mutagen application, also affects the proportion of cells which survive; ICR 191 applied to 10^5 cells per 25 cm^2 flask is several times as lethal as it is for 10^6 cells. Previous exposure to ICR 191 increases resistance to a second treatment with ICR 191, perhaps as the result of selection. Although the sensitivities of various stages of the cell cycle have not been determined in these experiments, the age of the culture, i.e., the number of hours that have elapsed after trypsinization, is a factor in determining lethality. All three of the ICR compounds are more lethal when applied to line ICR B20 twenty-four hours after cultures have been subdivided by trypsinization than if applied immediately after trypsinization (MEZGER-FREED, unpublished observations). Such a result is hard to explain in terms of gene mutation; the cell coat may be involved in this difference.

More extensive analyses of the interactions of cell cultures and the ICR acridine mustard series of compounds may lead to more valid ploidy comparisons not only for the study of mutation but in order to use the haploid-diploid comparisons as a test for the mutagenicity of compounds. The possibility that these compounds may induce phenotypic variants by interacting with components of the cell other than the genome should not be excluded.

The induction of variant phenotypes by mutagens

The induction by putative mutagens of phenotypic variants in culture can provide circumstantial evidence for the origin of these variants as gene mutations. Phenotypes involving resistance to drugs are particularly suitable for such studies since they can be selected for by treatment with the drug in question and because they are often associated with a loss of enzyme activity or permeability. For forms of resistance involving loss of an enzyme or a transport protein, many of the mutants

would be expected to be recessive; therefore, the haploid-diploid comparison should reveal substantial differences in frequency.

Resistance to the protein synthesis inhibitor puromycin was the first system chosen for study (MEZGER-FREED 1971). The five mutagens already described were applied to cell lines ranging from 95% to 48% haploid to give the results in Table 1. For one cell line, none of the five mutagens increased colony frequency; in another line, ICR $21C_8$, all four mutagens employed increased the number of colonies. If any of the compounds are acting as mutagens for these frog cells, an increase in frequency would be expected for each cell line or for none. Instead, the ability to form colonies seems to be a property of a particular cell line and not its ploidy; the observations suggest that the compounds are effecting the expression of a phenotype in some way other than by changing the genome.

Our studies have shown that various levels of resistance to puromycin exist and that resistance is a function of the capacity to exclude the drug. Furthermore, although a certain level of resistance is characteristic of a population, each one consists of cells with a wide range of resistance. A line of cells resistant to relatively high concentrations of puromycin remains resistant even after 150 generations in the absence of puromycin although there is evidence that during this time, a small number of less resistant cells accumulates which are immediately eliminated when the culture is again exposed to puromycin. The puromycin resistance trait behaves as though it is determined by many units and since permeability is involved, I have speculated that self-determining properties of membrane units rather than gene mutation might be responsible for the puromycin resistant phenotype.

Haploid-diploid comparisons have also failed to support the origin of another permeability variant as a gene mutation (MEZGER-FREED 1972). Resistance to 10^{-4} M bromodeoxyuridine (BUdR) in frog cells is associated with a decreased uptake of BUdR or thymidine as it is in Chinese hamster cells (BRESLOW and GOLDSBY 1969);

a transport system specific for thymidine and its analogs and saturating at 10^{-4} M is non-functional in the resistant variants (FREED and MEZGER-FREED 1973). The phenotype is stable and, in fact, no revertants have been obtained even after mutagen treatment. Such a phenotype is consistent with a permease mutation similar to those in bacteria (COHEN and MONOD 1957). However, the results of mutagen application followed by selection in BUdR (Table 2) indicate that: (a) Mutagens which increased the colony frequency in one haploid line did not increase the frequency in a second haploid line; (b) the number of cell generations between treatment by mutagen and selection by BUdR affected the yield of colonies. Since BUdR-resistant cells have a normal growth rate in BUdR-free medium and since colony frequency increased with time after mutagen treatment in some cases and decreased in others, selection against the variant does not explain the observation; (c) the frequency of colonies in a (pseudo)diploid line was higher than in the haploid lines; this is not explained by assuming dominance since compounds which increased the frequency in the haploid line actually decreased the frequency in the diploid line.

The results of the haploid-diploid comparisons are therefore not consistent with a mutational origin of transport-deficiency BUdR resistance in frog cells.

A more classic kind of BUdR resistance is that associated with thymidine kinase deficiency, in which the absence of the enzyme prevents a lethal incorporation of BUdR into DNA (KIT et al. 1963; LITTLEFIELD 1965). In attempts to obtain thymidine kinase deficient cells from the frog cells, a total of 3×10^7 haploid ICR 2A cells, including 0.8×10^7 mutagen treated ones, have been exposed to BUdR at concentrations of 10^{-4} M or higher without yielding a single colony. In addition, 2×10^8 ICR 2A cells have been treated with 5×10^{-5} M BUdR; 25 of the resulting colonies have been tested for thymidine kinase activity and all found positive (FREED and MEZGER-FREED 1973). Although no TK- variants have been obtained from the wild type ICR 2A cells, ICR B20, a transport-deficient variant of ICR 2A, yields TK- colonies at a frequency

of 10^{-6} which is increased 20-fold by prior exposure to ICR 191. The properties of the variants include thymidine kinase activity equal to 5% of the wild-type, resistance to 10^{-3} M BUdR, the medium in which they are selected, and stability of the phenotype in the absence of BUdR. In mammalian cultured cells with a similar phenotype, the stepwise progression to high resistance (10^{-3} M BUdR) from intermediate (10^{-4} M BUdR) has been explained by postulating that the intermediate is heterozygous (LITTLEFIELD 1965), but in the frog cells all three states are haploid and isogenic to the extent that all were derived from ICR 2A. Furthermore, the low resistance phenotype is qualitatively different (i.e., transport deficient) from that for high resistance (thymidine kinase deficient). Culture conditions are probably not the reason for the inability to obtain TK- colonies from ICR 2A since artificial mixtures of as few as 10 TK- cells per 10^5 cells of ICR 2A allows recovery of TK- colonies after selection in 10^{-3} M BUdR. Although other reasons for the requirement for a transport deficient intermediate for thymidine kinase deficiency cannot be ruled out, it is difficult to explain on genetic grounds. Therefore, the idea that thymidine kinase deficiency, a stable phenotype, represents a loss of gene expression rather than mutation should be considered. A clonal analysis of the transport deficient phenotype now in progress indicates that the cells differ in their capacity to yield phenotypes resistant to 10^{-3} M BUdR (MEZGER-FREED 1973).

Mutation in somatic cells

We have reviewed several lines of evidence from experiments on amphibian cell cultures which question whether mutations in cultures of somatic cells are expressed with the frequency predicted from bacterial models: (a) the small differences in the survival of haploid compared to diploid cell cultures after treatment with compounds known to produce mutations in bacteria; (b) the lack of correlation of ploidy with production of drug-resistant variants for puromycin and for 10^{-4} M BUdR; (c) the absence of thymidine kinase deficient variants in wild-type haploid cultures,

even after mutagen treatment, although this phenotype can be obtained from a transport deficient variant. Whether these results reflect experimental difficulties or fundamental differences in gene expression between prokaryotes and eukaryotes remains to be established. DNA repair processes may erase many of the mutations which occur, perhaps enough to mask part of the difference between haploid and diploid cells. A second explanation is that many gene products concerned with cell viability are represented by more than one gene per haploid set. These multiple loci could code for isozymes or for the same enzyme. The large amount of DNA in vertebrate cells, 10^3 times that in E. coli, is enough to deal with both greater complexity (more loci) and redundancy. OHNO (1972) has postulated that much of the extra DNA is "non-functional" for coding, thus protecting against the consequences of excess mutation by reducing the number of mutable loci. However, multiple gene copies would be more likely to prevent the expression of recessive or codominant mutations than would inert DNA. The combination of DNA repair and multiple gene copies may enable an organism to avoid some of the hazards resulting from the Watson-Crick molecule while at the same time benefiting from its properties.

Our discussion has implied that some of the questions raised by the cell culture studies are applicable to the intact organism. On the practical level of testing for environmental mutagens, the haploid-diploid comparisons indicate some reservations about the use of cell culture systems for this purpose. A haploid-diploid system has an advantage because of its innate controls for factors such as toxicity, metabolism of putative mutagens and whether the variants used as endpoints are the result of mutation. On a more fundamental level, it should be pointed out that cells in culture generally do not have gene products peculiar to the differentiated cells of an organism but contain products required for cell multiplication and maintenance which are present in most cells from the beginning of development. Such loci would be most subject to the pressure of selection during evolution since mutations would be expressed in all the progeny of a mutated cell. One of the responses to such

selection may have been the evolution of multiple gene copies for ubiquitous proteins compared to fewer or single copies for those proteins which appear in more specialized cells. If this is true, then the frequency of mutation expressed in cultures even of haploid cells may be less than that in somatic cells in vivo.

The observations on survival after mutagen treatment of haploid and diploid cultures which suggest an infrequent expression of mutation are incompatible with reported frequencies for phenotypic variants obtained in studies with cultured mammalian cells as high as 10^{-3} (LITTLEFIELD 1965; BRESLOW and GOLDSBY 1969). This discrepancy, as well as the results on drug resistance at different ploidy levels, would be explained if at least some of the variants are not the result of gene mutation but of non-genetic processes perhaps similar to those which produce stable phenotypes in differentiation.

Unfortunately, rigorous tests for gene mutation in cultured cells are not at present available. An increased frequency of variants after treatment by compounds shown to be mutagens in another system is not evidence for mutation; that a wide variety of organic and inorganic molecules can promote stable epigenetic changes has been extensively documented by experimental embryologists (NEEDHAM 1942). Stability of phenotype is characteristic of differentiated cells as well as mutated cells. Low frequency as a criterion of mutation is inadequate; it may mean that changes in the control of transcription and translation involve very specific and rarely-combined conditions of cell environment and cell competence which infrequently occur in cell cultures. Cell hybridization, which combines all of the components of two cells, is not comparable to breeding tests which involve the fusion of specialized cells after an orderly segregation. Even the appearance of an altered protein may be the result of changes in gene expression rather than mutation.

A unique test for the mutational origin of a variant is possible in the amphibian system. A nucleus from the cultures can be recycled through the early part of development using the Briggs and King nuclear transplant technique; the early gastrulae

which develop can be put into culture (MEZGER-FREED, unpublished work). The continued expression of the mutant phenotype would indicate its origin as a gene mutation.

Fortunately, the knowledge to be gained from cells in culture does not depend on whether variants originate as mutations or as non-genetic changes. If eukaryotic organisms have evolved a greater capacity for preventing the expression of gene mutations and at the same time have evolved mechanisms for the production of stable epigenetic changes, our understanding of them will depend on knowing about both kinds of phenomena.

LITERATURE CITED

ALEXANDER, R. and Z. B. MIKULSKI, 1961 Differences in the response of leukaemia cells in tissue culture to nitrogen mustard and to dimethyl myleran. Biochem. Pharmacol. 5: 272-282.

AMES, B. N. and H. V. WHITFIELD, JR., 1966 Frameshift mutagenesis in Salmonella. Pp. 221-225. In: Cold Spring Harbor Symposium Symp. Quant. Biol. 31: 221-225.

BRESLOW, R. E. and R. A. GOLDSBY, 1969 Isolation and characterization of thymidine transport mutants of Chinese hamster cells. Exp. Cell Res. 55: 339-346.

BRIGGS, R. and T. J. KING, 1952 Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc. Nat. Acad. Sci. U. S. 38: 455-463.

CHU, E. H. Y. and H. V. MALLING, 1968 Mammalian cell genetics. Chemical induction of specific locus mutations in Chinese hamster cells in vitro. Proc. Nat. Acad. Sci. U.S. 61: 1306-1312.

COHEN, G. N. and J. MONOD, 1957 Bacterial permeases. Bacteriol. Rev. 21: 169-194.

CREECH, H. J., R. K. PRESTON, R. M. PECK and A. P. O'CONNELL, 1972. Antitumor and mutagenic properties of a variety of heterocyclic nitrogen and sulfur mustards. J. Med. Chem. 15: 739-746.

FREED, J. J., 1962 Continuous cultivation of cells derived from haploid Rana pipiens embryos. Exp. Cell Res. 26: 327-333.

FREED, J. J. and L. MEZGER-FREED, 1970a Culture methods for anuran cells. Pp. 19-47. In: Methods in Cell Physiology. Edited by D. M. PRESCOTT. Academic Press, New York.

FREED, J. J. and L. MEZGER-FREED, 1970b Stable haploid cultured cell lines from frog embryos. Proc. Nat. Acad. Sci. U.S. 65: 337-344.

HARRIS, M., 1967 Phenotypic expression of drug resistance in cell cultures. J. Nat. Cancer Inst. 38: 185.

HARRIS, M., 1971 Mutation rates in cells at different ploidy levels. J. Cell. Physiol. 78: 177-184.

KIT, S., D. R. DUBBS, L. J. PIEKARSKI and T. C. HSU, 1963 Deletion of thymidine kinase activity from L-cells resistant to bromodeoxyuridine. Exp. Cell Res. 31: 297-312.

LITTLEFIELD, J. W., 1965 Studies on thymidine kinase in cultured mouse fibroblasts. Biochim. Biophys. Acta 95: 14-22.

MEZGER-FREED, L., 1971 Puromycin resistance in haploid and heteroploid frog cells: gene or membrane determined? J. Cell Biol. 51: 742-751.

MEZGER-FREED, L., 1972 Effect of ploidy and mutagens on bromodeoxyuridine resistance in haploid and diploid frog cells. Nature 235: 245-246.

MEZGER-FREED, L., 1973 The effect of mutagens on BUdR resistance in haploid cells: genetic or epigenetic? In: Model Studies in Chemical Carcinogenesis. Edited by P. O. P. TS'O and J. A. DI PAOLO. Marcel Dekker, New York (In press).

MEZGER-FREED, L., 1973 On the question of mutation in haploid vertebrate cell cultures. Int. Rev. Cytol. supplement. (In press).

NEEDHAM, J., 1942 Biochemistry and Morphogenesis. University Press, Cambridge.

NEURATH, P. W., P. S. LIN and D. A. LOW, 1972 Quantitative karyotyping: a model twin study. Cytogenetics 11: 457-474.

OHNO, S., 1972 Gene duplication, mutation load, and mammalian genetic regulatory systems. J. Med. Genet. 9: 254-263.

ORKIN, S. H. and J. W. LITTLEFIELD, 1971a Nitrosoguanidine mutagenesis in synchronized hamster cells. Exp. Cell Res. 66: 69-74..

ORKIN, S. H. and J. W. LITTLEFIELD, 1971b Mutagenesis to aminopterin resistance in cultured hamster cells. Exp. Cell Res. 69: 174-180.

FOOTNOTE

[1] This work was supported by U.S.P.H.S. grants CA-05959, CA-06927 and RR-05539 from the National Institutes of Health, contract AT(11-1)3110 with the U. S. Atomic Energy Commission (report no. COO-3110-7), and by an appropriation from the Commonwealth of Pennsylvania.

FOOTNOTES TO TABLES

TABLE 1. The frequency of colony formation before and after mutagen treatment of cultures exposed to puromycin. Mutagen treatment of cell monolayers lasted 48 hours at a mutagen concentration found in preliminary experiments to decrease the cell population by half. Mutagen concentrations were: MNNG (N-methyl-N'-nitro-N-nitrogoguanidine), 1.5 - 2.0 µg/ml; EMS (ethylmethanesulfonate), 300 - 500 µg/ml; three acridine half-mustard ICR mutagens, 372, 0.5 - 1.0 µg/ml; 340 A, 0.75 - 1.50 µg/ml; 191 C, 0.5 - 1.0 µg/ml.

TABLE 2. The frequency of colony formation before and after mutagen treatment of cultures of different ploidy exposed to 15 µg/ml BUdR. See legend for Table 1 for mutagen treatment.

Table I

Puromycin Resistance

Colony Production by Haploid Cell Lines Before and After Mutagen Treatment

Cell Line	Puromycin Conc. µg/ml	Mutagen treatment: None Col./10^6 cells	None Total cells	EMS Col./10^6 cells	EMS Total cells	MNNG Col./10^6 cells	MNNG Total cells	ICR 191C Col./10^6 cells	ICR 191C Total cells	ICR 340A Col./10^6 cells	ICR 340A Total cells	ICR 372 Col/10^6 cells	ICR 372 Total cells
2A 95% haploid	1	85	$5x10^6$	9[illegible]	$5x10^6$	92	$5x10^6$						
	2			0	$2x10^6$	0	$2x10^6$						
	3	0	$2x10^6$	0	$2x10^6$	0	$2x10^6$						
	5	0	$10x10^6$	0	$30x10^6$	0.1	$40x10^6$						
21 80% haploid	2.5	0	$30x10^6$	.02	$53x10^6$			1.5	$50x10^6$	.04	$47x10^6$	.44	$34x10^6$
$21C_8$ 92% haploid	2.5	0	$52x10^6$	0	$45x10^6$	0	$40x10^6$	0	$46x10^6$	0	$48x10^6$	0	$38x10^6$
31 48% haploid	2.5	0	$26x10^6$	0	$52x10^6$	0.07	$58x10^6$	0	$39x10^6$	0.2	$39x10^6$	0	$37x10^6$

Table 2

Cell line	Mutagen	Generations between mutagen treatment and selection	Time of colony appearance (weeks)	Number of Cells treated with BUdR $\times 10^6$	Colonies 10^6 cells
2A	None	---	4	40	.03
Haploid	MNNG	3	---	24	0
	MNNG	50	4	8	4
	EMS	26	10	10	0.1
	EMS	52	3	18	12
	EMS	56	4	10	10
	ICR 372	7	4	10	1.4
	ICR 372	34	---	9	0
	ICR 340A	6	4	9	1.6
	ICR 340A	34	4	9	2.1
	ICR 191C	8	4	15	0.3
	ICR 191C	31	---	12	0
21	None	---	5	10	0.1
Haploid	EMS	45	---	12	0
	ICR 340A	12	---	10	0
132	None	---	10+	30	0.7
Pseudo-	MNNG	3	10	25	0.4
diploid	MNNG	7	---	9	0
	MNNG	30	---	10	0
	EMS	7	12	10	0.2
	EMS	30	---	10	0
	ICR 372	7	10	9	0.4
	ICR 372	30	---	10	0
	ICR 340A	30	---	10	0
	ICR 191C	7	9	7	0.3

FIGURE LEGENDS

FIGURE 1.---The effect of putative mutagens on survival of haploid and diploid cell lines. Changes in cell number as a function of time for three pairs of haploid and diploid cell lines, ICR 2A (90% haploid) and ICR $2A_2$; ICR $21C_8$ (92% haploid) and ICR 21; ICR $31C_{12}$ (92% haploid) and ICR 31, after 48 hours exposure to: a) 1.5 µg/ml MNNG b) 100 µg/ml EMS c) 1 µg/ml ICR 191 d) 1 µg/ml ICR 340 e) 1 µg/ml ICR 372. Open symbols represent haploid cultures; closed symbols represent diploid cultures; smaller symbols represent control cultures.

FIGURE 2.---The effect of ICR 191 on survival of clones from a single cell line. Changes in cell number as a function of time for three cell lines and 16 clones derived from them after 48 hours exposure to 1 µg/ml ICR 191. ICR B_{20}191 are cultures derived from line ICR B_{20} which had previously been exposed to ICR 191.

FIGURE 3.---The effect of concentration of ICR 191 on survival of a haploid and a diploid cell line. Changes in cell number as a function of time for haploid and diploid cell lines, ICR $21C_8$ and ICR 21, after 48 hours exposure to ICR 191 at different concentrations. The curves are arbitrarily placed along the ordinate to facilitate comparison.

Fig 1a

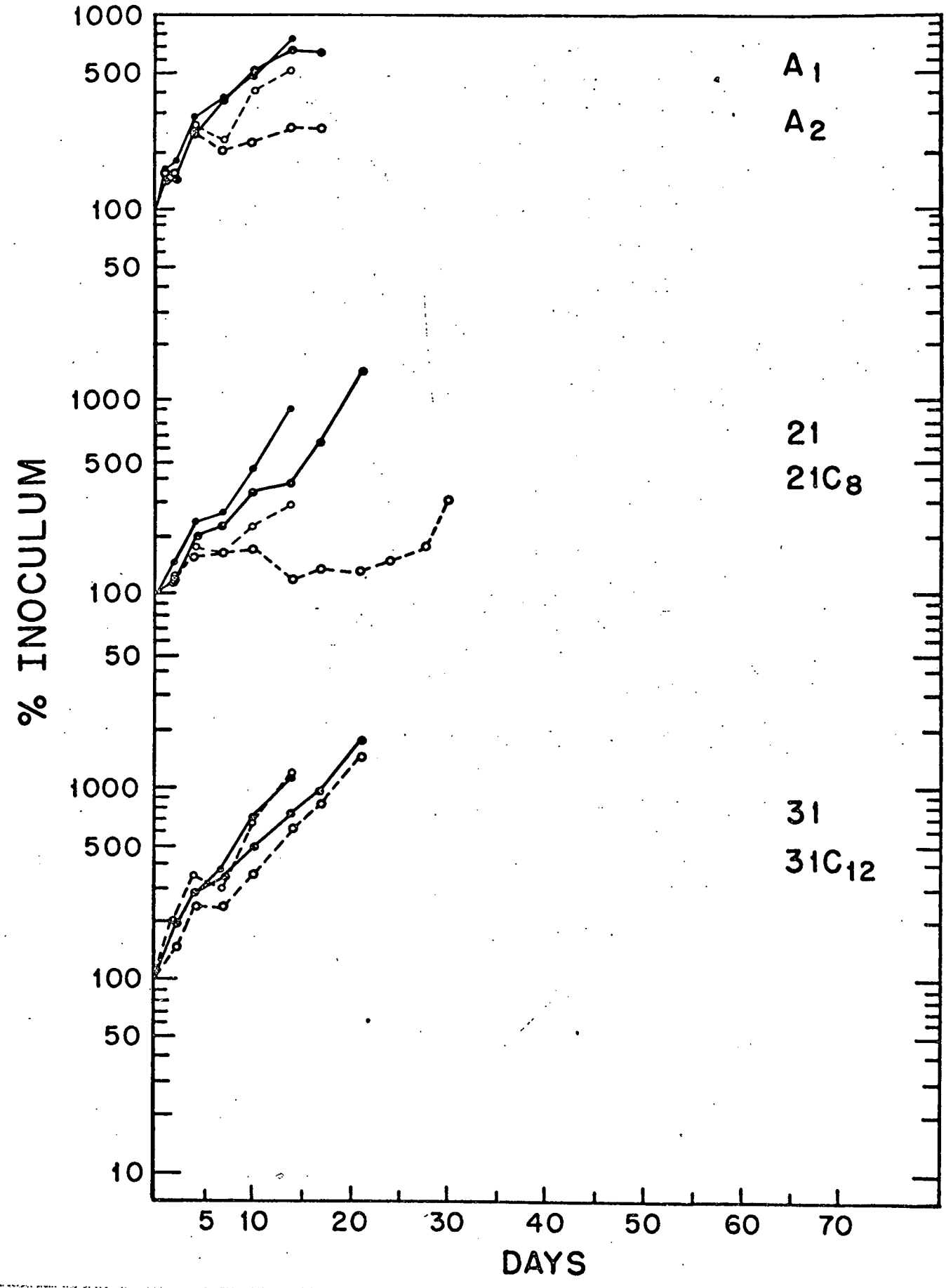

1000
500
100
50
1000
500
100
50
1000
500
100
50
10
% INOCULUM
A1
A2
21
21C8
31
31C12
5
10
20
30
40
50
60
70
DAYS

Fig 16

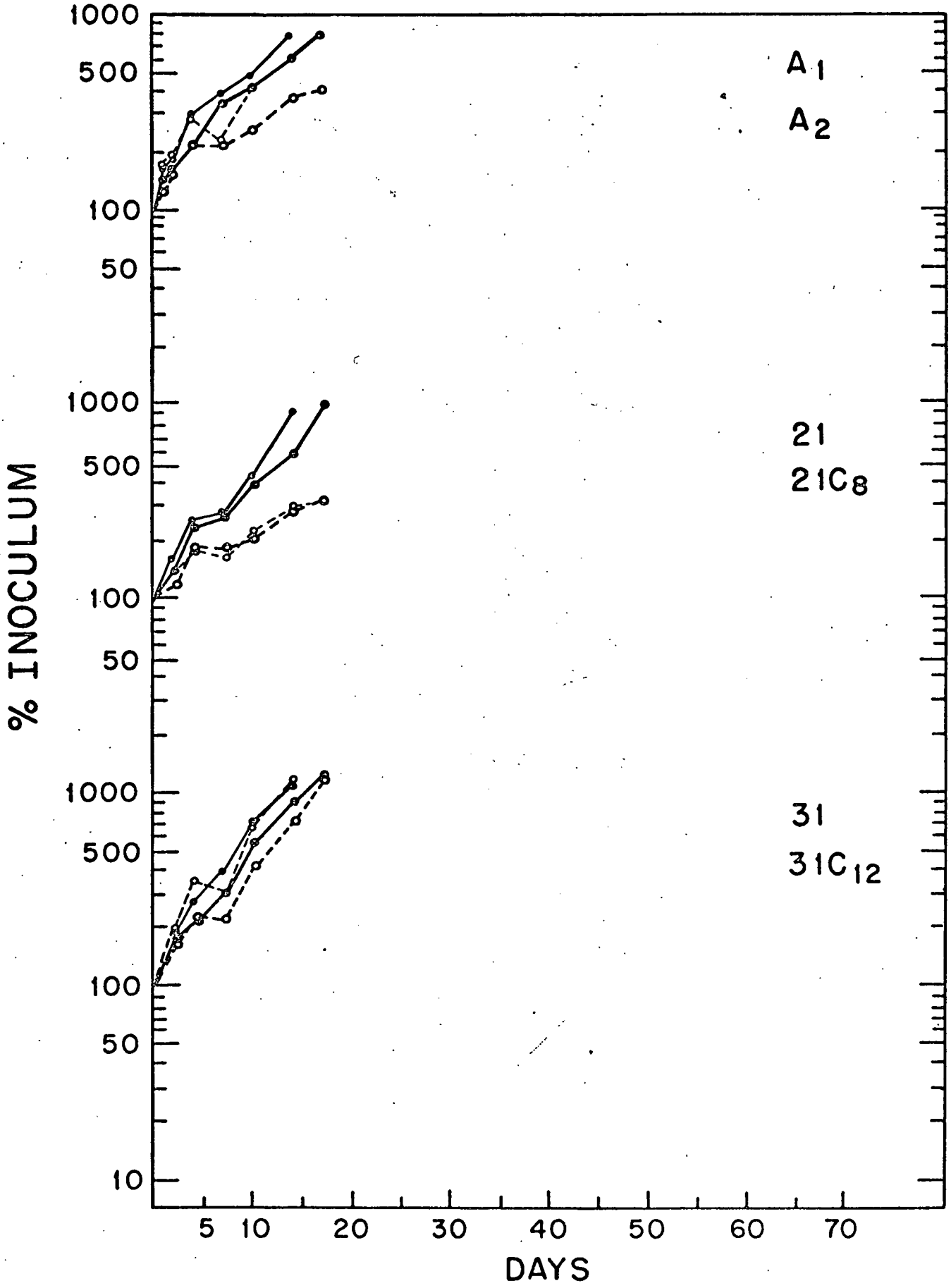

1000
500
100
50
1000
500
100
50
1000
500
100
50
10
% INOCULUM
A1
A2
21
21C8
31
31C12
5
10
20
30
40
50
60
70
DAYS

Fig 1c

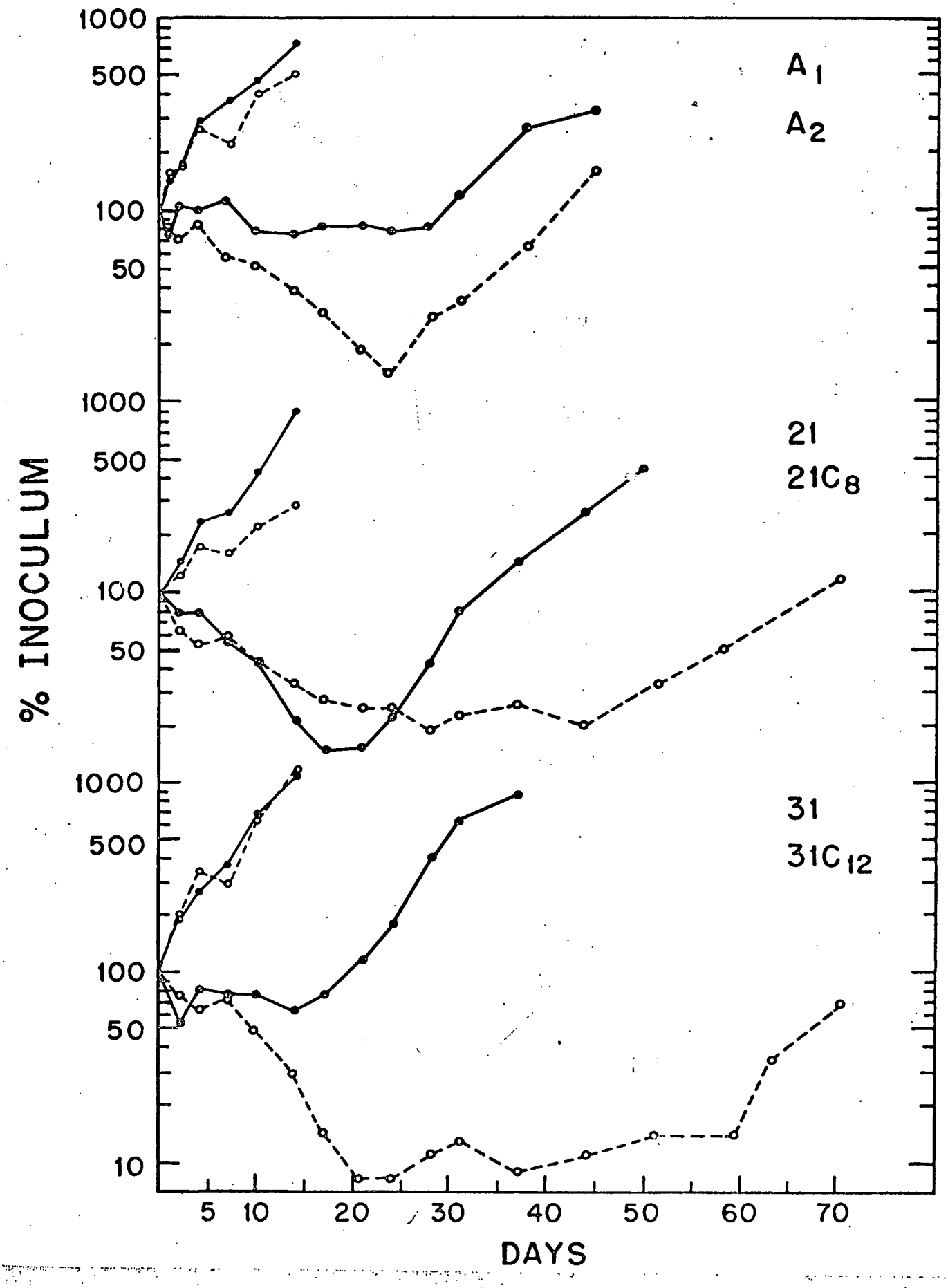

1000
500
100
50
1000
500
100
50
1000
500
100
50
10
% INOCULUM
A1
A2
21
21C8
31
31C12
5
10
20
30
40
50
60
70
DAYS

Fig 1d

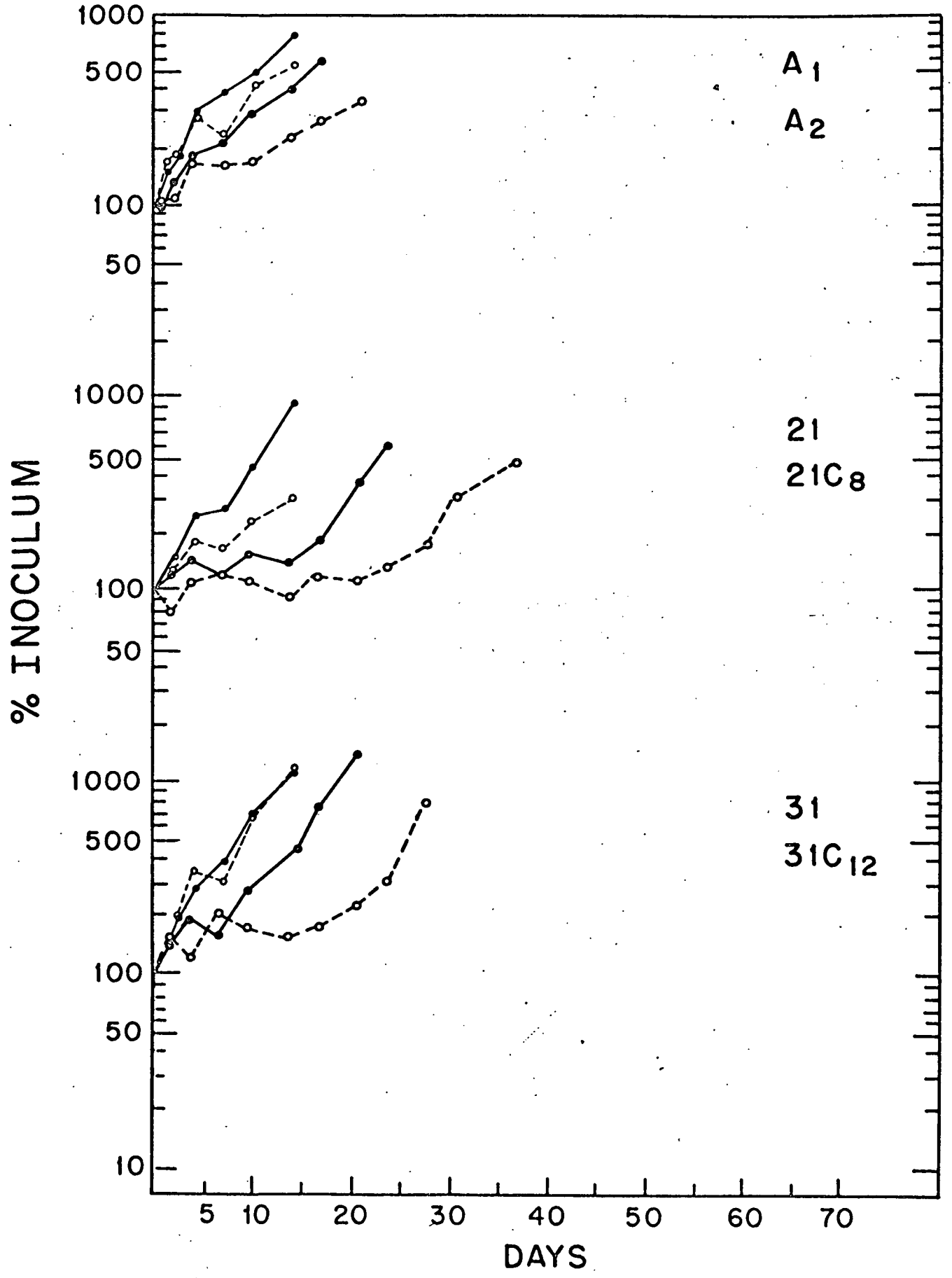
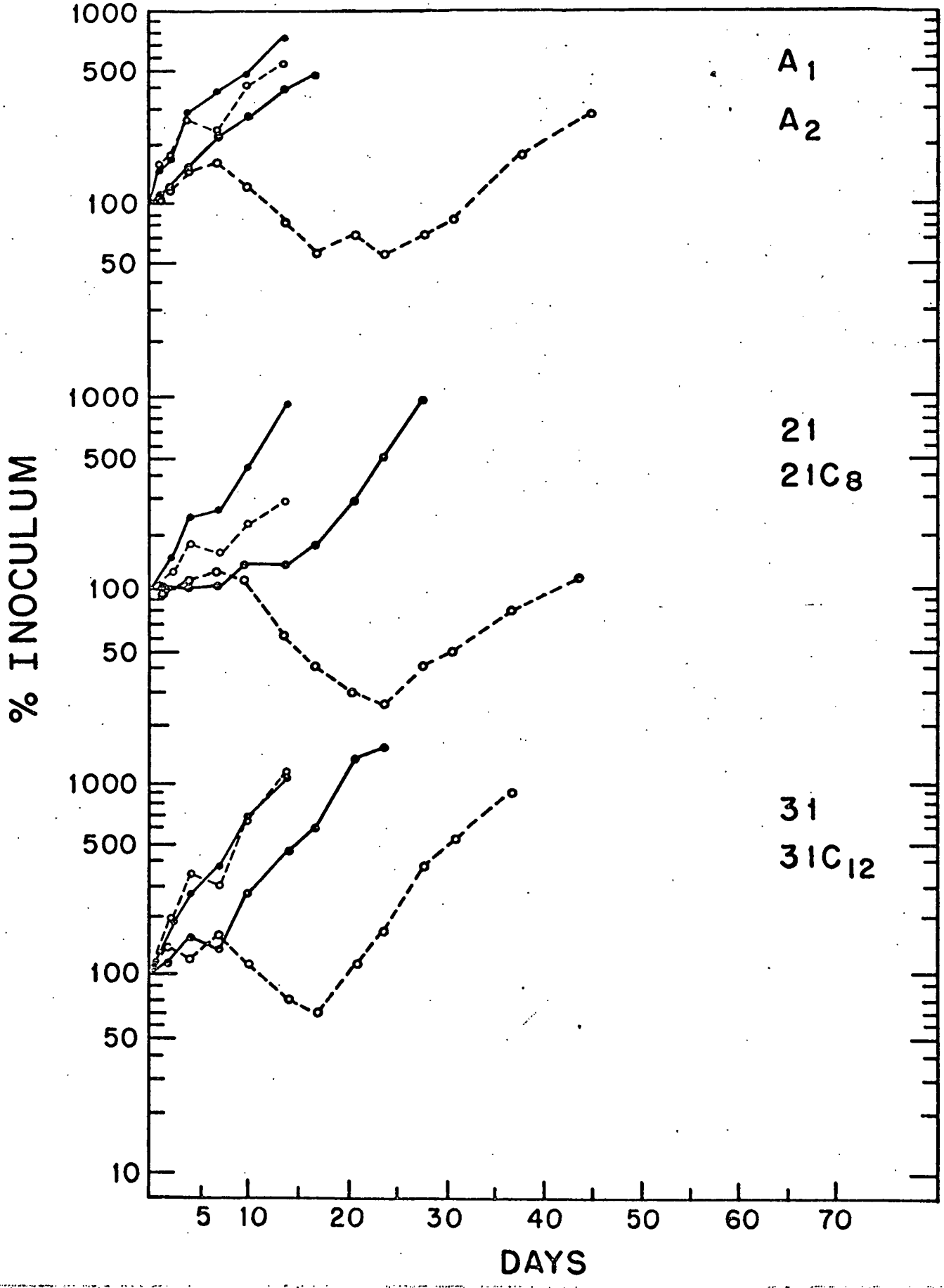

1000
500
100
50
1000
500
100
50
1000
500
100
50
10
% INOCULUM
A1
A2
21
21C8
31
31C12
5
10
20
30
40
50
60
70
DAYS

Fig 1e


1000
500
100
50
1000
500
100
50
1000
500
100
50
10
% INOCULUM
A1
A2
21
21C8
31
31C12
5
10
20
30
40
50
60
70
DAYS

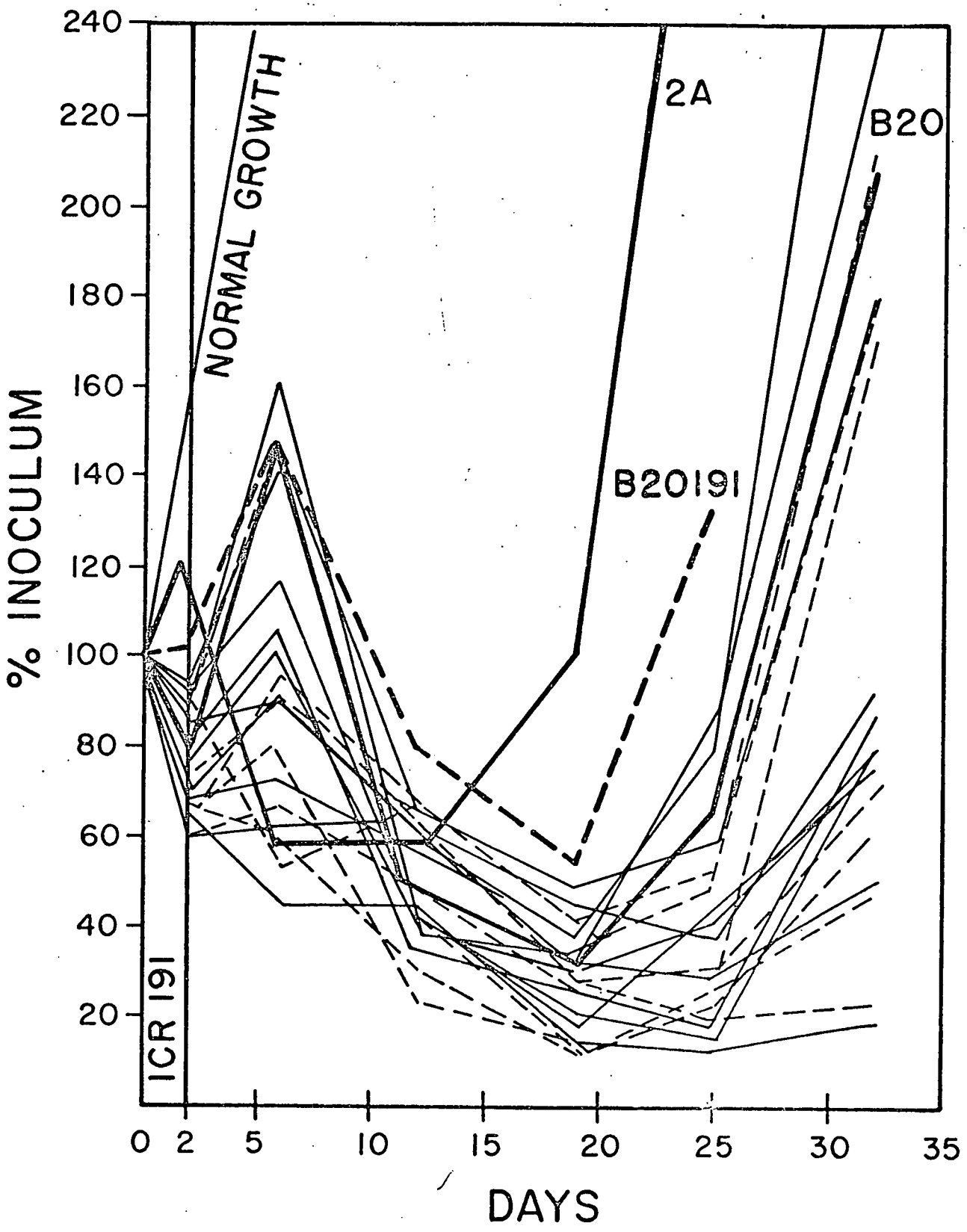
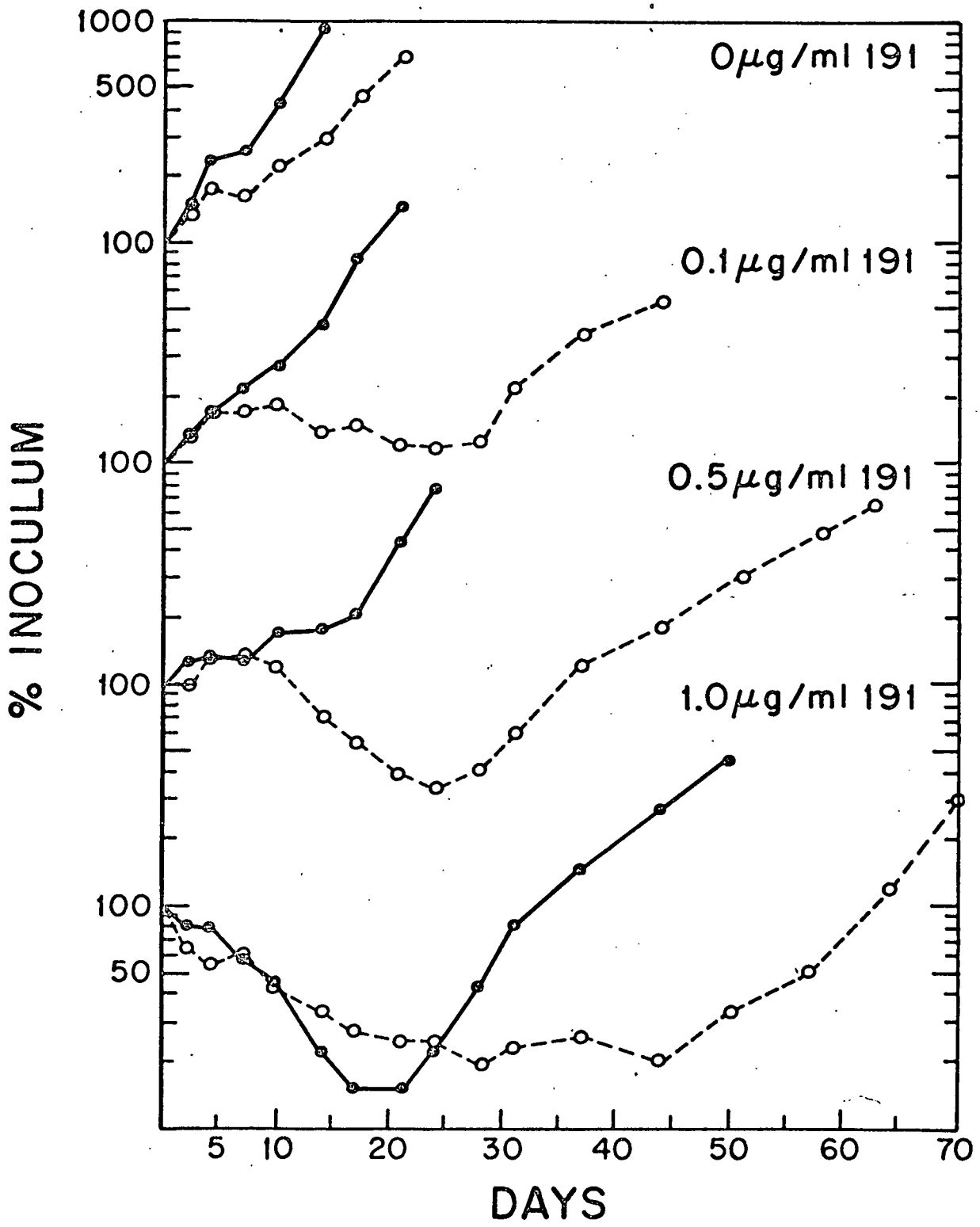

240
220
200
180
160
140
120
100
80
60
40
20
% INOCULUM
NORMAL GROWTH
2A
B20
B20191
ICR 191
0
2
5
10
15
20
25
30
35
DAYS

Fig 3


1000
500
100
100
100
100
50
% INOCULUM
0μg/ml 191
0.1μg/ml 191
0.5μg/ml 191
1.0μg/ml 191
5
10
20
30
40
50
60
70
DAYS