ANALYSIS OF RADIATION INDUCED MUTATIONS OF THE ARGININE PERMEASE LOCUS IN YEAST

by

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INTRODUCTION

What is the dependence of the mutation frequency on type and dose of radiation? How does the environment, such as growth condition before irradiation and a holding period before selection for mutants, affect the expression of mutant phenotype? Does the character of the mutant depend on the type of irradiation and the environmental condition? If so, what are the differences?

These questions may be approached experimentally by using a simple eucariobic organism such as Saccharomyces cerevisiae. This yeast is a convenient experimental organism with which much genetical research has been performed.

This yeast is capable of vegetative (mitotic) growth in both haploid and diploid states on a well defined medium. Transitions from one state to the other are easily induced. To answer the questions posed, one requires methods of measuring mutation frequencies, even at low levels and methods of determining the molecular nature of a given mutational gene. Such methods exist because of the effort of many workers and will be discussed more fully in the following specific discussions.

For this study a system, developed by W. L. Whelan (Ph.D. thesis 1972) was used. The ease of obtaining forward and reverse mutations was a very convenient feature of this system. It may prove generally useful in studies of mutations.

EXPERIMENTAL APPROACH

A. General Properties of Amino Acid Uptake in Yeast

S. cerevisiae transports amino acids by at least two major types of permeases (Grenson, Hou, Crabeel 1970) (Grenson 1966) (Grenson, Mousset, Wiame, Bechet 1966) (Gits, Grenson 1967). If the growth medium contains ammonium salts as the nitrogen source the cells contain a battery of permeases, each specific for one or for a small group of amino acids. Replacement of the NH_4^+ ions by proline as the nitrogen source results in the development of an uptake system of broader specificity and higher uptake rate. It was shown that ammonium ions inhibit the activity rather than the synthesis of the nonspecific permease.

Among the specific permeases is one which transports L-arginine (Grenson, Mousset, Wiame, Bechet 1966). Mutants, deficient in the L-arginine permease activity, are easily selected by their resistance to the toxic effects of the L-arginine analog L-canavanine. (In the remainder of the work the optical isomer of the compounds under discussion will not be specified each time. In all cases the L-isomer was used.)

Grenson and her coworkers showed that loss of arginine permease activity (caused by damage of the gene coding for it) was associated with resistance to L-canavanine. (For a different case see note on

allele can1-111.) The mutation behaved as a single gene defect when crossed to a wildtype strain and subjected to tetrad analysis. It served to define the CAN1 locus.

Canavanine was originally isolated by Kitagawa (1929) from Jack beans. The mechanism of killing by canavanine is not well understood. Its toxidity to yeast was shown by Srb (1956). It is usual to select for resistent mutants by plating canavanine sensitive (wild type) cells on a defined medium containing canavanine but lacking arginine. The concentration of canavanine is not critical; resistent cells grow at 60 mg/l while 10 mg/l completely abolishes growth of sensitive strains.

B. Design of the System

The system, as it was used in this study, was designed and the breeding of the strains performed by W. L. Whelan (1972). It was chosen because of the ease with which forward mutations could be selected and analysed. The use of a double block in the arginine pathway (arg6 arg8) before the metabolite ornithine, which also can be taken in directly out of the medium, insured a similiarly easy way of selection for reverse mutations. The cells cannot produce arginine from minimal medium but cells with intact permease can take it in directly or produce it from ornithine if this is supplied in the medium, while mutants only can exist on medium containing ornithine but not on medium containing arginine as the sole source.

Thus, a haploid strain wild type at CAN1 but containing arg6 arg8 can be grown in liquid medium containing ornithine and spread on agar plates containing canavanine to select for mutants defective at CAN1.

Mutant cells obtained in this way can be spread on agar containing arginine but no ornithine to select for cells which have reverted at CAN1.

Additionally two trp5 alleles (trp5-87; trp5-67C4) capable of complementation, were included into the genotypes to allow selection for diploid clones occuring in a mating mixture of the parental haploids. This is useful for many genetic methods as will be described below.

Thus selection for diploids can be performed by plating haploids of opposite mating types and different alleles at trp5 on agar lacking tryptophane. The complementation of the two alleles allows the diploids to grow while growth of the haploids is inhibited. A graphical summary of the system is given in Tables 1 and 2.

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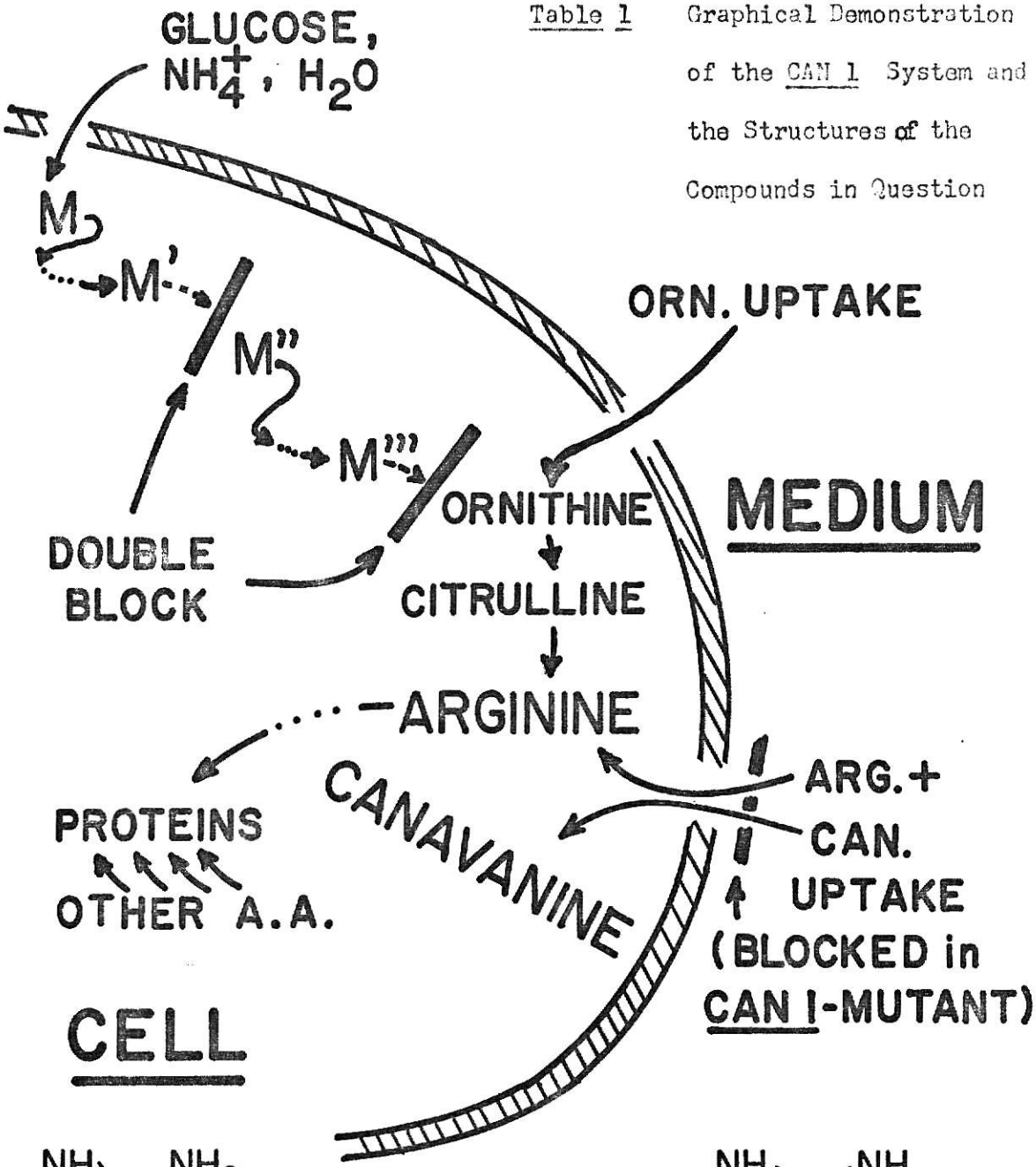
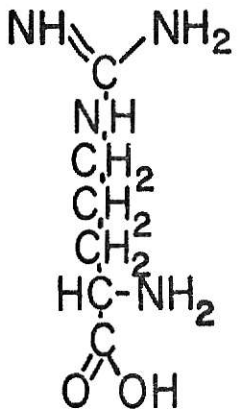
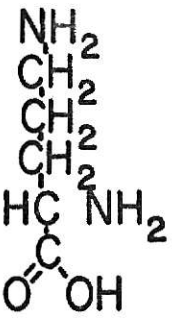
Table 1 Graphical Demonstration of the CAN 1 System and the Structures of the Compounds in Question

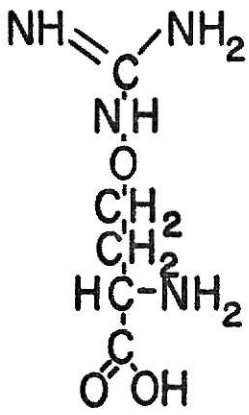




L-ARGININE



L-ORNITHINE



L-CANAVANINE

Table 2: Growth Response of the Strains

Strain*	Medium** MOT	MOTC	SC	State of strain	MOT	MO
wild type	+	-	+	haploid	+	-
mutant	+	+	-	diploid	+	+
revertant	+	-	+			

* All the strains have the above described lesions in the arginine and tryptophane pathways. Wildtype, mutant, revertant refer only to the CAN1 gene.

**MOT: Medium supplied with ornithine and tryptophane

MOTC: MOT plus canavanine

SC: Medium containing arginine but no ornithine

MO: Medium containing ornithine but no tryptophane

Media are more fully described in Material and Methods

MATERIAL AND METHODS

A. Yeast Strains

Most of the strains used in this study were kindly supplied by W. L. Whelan.

The CAN1 mutants are exclusively obtained by irradiating the haploid strain XW197-23 (a trp5-67C4 arg6 arg8 CAN1).

The strains used in the spot test for recombination are of the genotype trp5-87 arg6 arg8 can1-i where i = 40,39,35,23,34, 26,32,37. Their mapping position is described in the appropriate section.

As the tester for suppressibility a strain carrying can1-111 and SUP11 was used. The precise arginine genotype is not determined. (trp5-87 arg can1-111 SUP11)

The genealogy of the strains is described in W. L. Whelan's Ph. D. thesis.

B. Media

Media were prepared by dissolving the stated amounts of components on a weight/volume basis in distilled water and autoclaving the mixture for 15 minutes at 15 psi. For solidified media, agar was added to a concentration of 2%.

All supplements were Calbiochem A grade.

M (Minimal)--Difco Yeast Nitrogen Base w/o amino acids 0.6%, glucose 2%

MO--Minimal plus L-ornithine (200 mg/l)

MOT--MO plus L-tryptophane (20 mg/l)

MOAT--MOT plus L-arginine (20 mg/l)

MOTC--MOT plus L-canavanine (20 mg/l)

MAT--M plus L-arginine plus L-tryptophane (each 20 mg/l)

SC--M plus the following supplements: adenine (20 mg/l); L-arginine (20 mg/l); L-histidine (10 mg/l); L-leucine (30 mg/l); L-lysine (20 mg/l); L-threonine (150 mg/l); uracil (20 mg/l)

YEKAC (Sporulation medium)--Potassium Acetate (1%), Difco Yeast Extract (0.25%)

The purpose of each media will be detailed in the appropriate sections.

C. Conditions of Incubation

The cultures were incubated at 30° C. Liquid cultures were incubated on a shaker operating at about 250 rpm.

D. Irradiation Procedures

Ultraviolet light was delivered from a GE 15 Watt germicide lamp. The exposure rate at the level of the cells was estimated

as about 45 erg/mm^2/sec. (By comparison of the survival values of the strain X2180-1A with a calibration curve. The experiments performed in this study do not depend on absolute exposure or dose values.)

The cells were exposed either on the solidified medium shortly after plating or they were suspended in distilled water of less than 3 mm in depth. After irradiation the suspension was transferred into a 50 ml flask. 5X concentrated liquid medium was added to reach the concentrations indicated in section B. The cells were incubated in the dark. At given times part of the culture was plated as described in Chapter IV A.

Xrays were generated by a Machlett AEG 50-Xray tube with W-filament. It was operated at 50KV and 20MA with a thin beryllium window and no additional filtration.

The dose rate at the level of the cells was about 135 rads/sec, as measured by a beryllium window extrapolation ionization chamber. The cells were exposed on solidified medium. If it was necessary they were quickly washed off with liquid medium and incubated at 30°C.

The irradiation with protons was performed at the KSU Tandem van de Graaff accelerator. The beam current was about 100 p amps; the proton energy used here was 5 MeV. The cells were exposed on agar discs of 1.5 cm diameter. After irradiation the discs were transferred into liquid medium and vigorously shaken. After the discs were discarded, the suspension was incubated on a rotary shaker. Plating was performed after 24 hours.

E. Chemical Mutagens

N-methyl-n'-nitro-N-nitrosoguanidine (NG) was purchased from the Aldrich Chemical Company. Ethyl methane sulfonate (EMS) was a product of Eastman Organic Chemicals. These chemicals were used to produce reversion mutations as described in section H-5.

F. Permease Activity Assay

Uniformly labeled ^{14}C-L-arginine was purchased from the Radiochemical Centre, Amersham.

The assay was performed with cells incubated in liquid medium on a rotary shaker at either 30° C or at room temperature. At the start of the experiment ^{14}C-arginine was added to the medium (details are given in the appropriate section) and at given times 1 or 5 ml samples were removed and poured onto Millipore filters (pore size:0.45µ; 25 mm diameter), which were immediately washed several times with distilled water to remove not incorporated arginine. In a calibration experiment the washing procedure was checked to be sufficient for giving constant counts. The filters were placed in vials containing toluene plus Permafluor scintillation liquid (Packard Instrument Corp.).

The counting efficiency was tested by adding known activities of ^{14}C-toluene and was well above 90%. The concentration and the spec. activity of the ^{14}C-arginine were always 20 mg/l and .55 mCi/m mol).

G. Bioassay for Internal Free Arginine and its Precursors

To determine the amount of free arginine and/or ornithine and citrulline inside the cell the culture was centrifuged (3000 rpm) and washed two times, then boiled for 10 minutes in distilled water. The suspension was centrifuged again and the supernatant was poured into a 50 ml flask. Grenson and her coworkers showed that this procedure extracts about 90% of the internal free amino acids. Concentrated MT (minimal plus tryptophane) was added and the solution was inoculated either with the strain XW197-2B (arg^-, wildtype at CAN1) or with the strain carrying can1-111 (arg^-, mutant at CAN1).

After three days the cell density was determined by hemacytometer count. By comparison with calibration curves the concentration of arginine and ornithine/citrulline was calculated. These calibration curves were obtained by inoculating MT medium containing different concentrations of arginine or ornithine and counting the number of cells after 3 days. At first the concentration of ornithine in the cell extract was calculated from the density of the mutant. The mutant can only live from ornithine but cannot take up the arginine also present in the extract. From the calculated concentration of ornithine the limit of XW197-2B cells using the ornithine was determined. The growth above this number was attributed to uptake of arginine and comparison with the appropriate calibration curve yielded the concentration of arginine in the solution.

Since this procedure does not differentiate between the ornithine and the citrulline (See Table 1) the results bear a considerable uncertainty but nevertheless proved to be very useful. Assuming a similar uptake rate of citrulline as for ornithine the concentration of citrulline in the cell extract is contained in the value of the ornithine concentration.

The concentrations of the arginine and the ornithine plus citrulline were converted into mg/10^8 extracted cells.

H. Genetic Methods

The symbols representing genes and alleles used throughout are those proposed at the Fourth International Conference on Yeast Genetics.

(1) Tetrad Analysis - Diploid strains obtained by crossing the haploid parents on MOT and streaking for single colonies on MO. Part of this clone was transferred on YEKAC and incubated for 3 days to allow sporulation. The culture was examined microscopically for the presence of asci. The ascus walls were removed by treatment with an enzyme preparation (Glusulase, Endo Laboratories), and the spores of individual asci were distributed at intervals of 2 mm on a slab of MOT agar. This operation was performed with the aid of a micro-manipulator. The slab was placed on MOT agar and incubated for two to three days. The spore clones were then transferred to MOT agar and the phenotype of each clone determined by replica plating to the appropriate media (See Plate I).

(2) Spot Test for Recombination - Diploids heteroallelic at CAN1 were obtained by mixing the haploid parent strains on MOT agar and replica plating to MO agar to select for the diploids. After overnight incubation the strains were replica plated to YEKAC and incubated for three days to permit sporulation. Then a replica plating onto MOT agar was performed to allow the spores to grow. The next day this plate was finally replica plated to SC agar. The alleles were considered capable of recombination if sporulation resulted in a significant increase in clones capable of growth on SC (compared to both haploid parent strains). This procedure was designed specifically to detect deletion mutations (W. L. Whelan 1972) as described in section IV B.

(3) Xray Mapping Method - The method is that of Manney and Mortimer (1964). It was used in this study to determine the location of eight tester alleles (for the spot test for recombination). The purified clone (streaked for single colonies on MO) of a heteroallelic diploid strain, obtained by mating two of the haploid tester strains was inoculated into liquid MOTC (1 ml) and incubated overnight. Four ml of water were added and the cultures were centrifuged. The cells were washed once with water (5 ml), centrifuged again, and resuspended in water. This suspension, appropriately diluted, was spread on SC and MOT plates. Some were irradiated (within 15 minutes after spreading); the others were used to determine the background. This method is based on the fact

that Xray induced crossing over between two damages increases linearly with the dose, and the probability (given by the slope of the curve) is greater the larger the distance between the two damages. The crossing over results in a wild type progeny capable of growth on SC. Colonies appearing on SC and MOT plates were counted after 3 and 5 days. After 5 days the counts did not increase significantly; so these numbers were used for calculating the map distance between the two alleles. The Xray map unit is defined as number of revertants per rad per 10^8 surviving cells.

(4) Chemical and UV Induced Reversion - The method was designed to determine differences in the ability of mutants, obtained by either UV, Proton or Xray irradiation, to revert to wild type phenotype. The strains were tested in the haploid form by following these steps:

The mutant clone was transferred into water (about 2 ml) to a concentration around 10^7 cells/ml. A few drops of the suspension were spread on a sector of 1/8 of an MOT plate and incubated overnight. (So one plate contained 8 strains obtained from different clones.) The next day each MOT plate was replica plated to three other MOT plates and the cells were mutagenized in each of the three following ways:

(a) For NG mutagenesis, a few crystals of this substance were added to the center of the plate.

(b) For EMS mutagenesis, 10 microliter of this liquid was added to a small filter disc at the center of the second plate.

(c) For UV mutagenesis the third plate was irradiated for about 15 seconds. The outer part of each sector was shielded against the radiation.

After 24 hours each plate was replica plated to SC agar. The strains were scored after five days into one of the following sections:

Negative: less than about five colonies

Positive: more than about fifty colonies

Intermediate: colony numbers between the extremes.

For more details see section IV B-1.

(5) Response to Nonsense Suppressor - The strain carrying the suppressor allele is described in section A.

The strains to be tested were crossed with the tester strain on MOT agar. The following procedure is the same as described in the recombination spot test. Scored as suppressible were those strains for which sporulation resulted in a significant increase of colonies capable of growing on SC agar. These spores carry the mutant CAN1 allele but despite of that the wild type phenotype is expressed since the suppressor allele is also contained in the genetic material. The mechanism of this suppression is described in section IV B-3.

Confirmation of the validity of this assumption was obtained by subjecting several strains to tetrad analysis (section IV B-3).

IV. EXPERIMENTAL RESULTS

A. Mutations: Frequency

Since the first studies of the nature of the gene (reviewed by Dunn 1965) spontaneously occurring mutations have provided alternative (allelic) forms of a gene for study. Occasionally new alleles were found in defined laboratory stocks but the frequency of their occurrence was so negligibly small that a systematic study was impossible to perform.

Muller (1927) was able to demonstrate that the mutation frequency could be greatly increased by exposure of the organisms to Xrays. The capability of other radiations (e.g. UV, α, β rays) and certain chemicals to induce mutations was found in the following years. Additionally the use of microorganisms such as bacteria or viruses and sophisticated screening methods enabled the experimenters to examine huge numbers of individual organisms for mutations in relatively short times.

Clearly the probability of encountering mutants among a sample of organisms is strongly influenced by the restraints put upon the system. An environment which exerts a strong selective pressure causes a poorly fitted phenotype to vanish after a short period of time while a well fitted cell type will grow abundantly. The ease of detecting forward mutations at the CAN1 locus in yeast is due to the strong effect of canavanine on the metabolism of the cell. The

mutant strain has an immense selective advantage over the wild type cell if the medium contains the analog. This allows an easy determination of the mutation frequency even at low levels.

In the work performed by W. L. Whelan the spontaneous mutation rates during vegetative growth were compared to the rate among sporulated diploid cultures. In the present study we were interested in obtaining mutants induced by different radiations such as UV light, Xrays, and Protons. To be able to make valid comparisons of mutations (frequency and character) it is necessary to design a procedure of handling the cultures before and after irradiation as efficiently and uniformly as possible.

Obviously the number of mutational events depends largely on the radiation dose. Also the growth conditions before and after irradiation do influence the frequency. Kaudewitz (1955) and Zimmermann and his coworkers (1966) showed a considerable increase of mutant cells in a mutagen treated culture for a period up to 16 doubling times before selection for mutants was performed.

On the other side the mutation level after irradiation often decreases due to repair of the genetic damage. These effects could be observed in the present study and will be described and discussed below.

AA. Dose Response

Figures 1 and 2 show the change in mutation frequencies upon increasing the UV or Xray dose. In the lower part of the graphs the survival levels for each exposure are given. As it is known for haploid yeast cells of stationary phase (low percentage of budded cells), the Xray survival curves are, in the observed survival range, of pure exponential type (single hit curve) while the UV curves show a pronounced shoulder (multiple hit curve) (Elkind, Sutton 1959).

For low doses the mutation frequency curves are approximately linearly increasing for the Xray sample, while the UV irradiated cells show an increasing slope. This affirms that for a mutational event to occur several UV quanta but only one Xray quantum have to be absorbed. Nevertheless the curves show that for comparable survival levels UV is much more effective (by a factor of up to 10) in inducing mutations than Xrays. This is due to the fact that Xray irradiation causes death more often by gross damage of the genetic material rather than by inflicting mutations in genes essential for life. The same holds for Proton irradiation. Because of technical difficulties in irradiating large samples, only one dose point was taken (about 50% survival). The mutation frequency is about the same as for the comparable Xray dose.

Several variations in the radiation procedures were made, such as growing the cells before irradiation in different media (MCT, SC)and

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Figure 1

Section A shows the UV dose response curve after growth in MOT. The cells were plated on MOTC agar and irradiated at once. B shows the survival curve for the doses used (determined by irradiating the cells plated on MOT). C shows the dose response curves after growth in MOT (●) and SC (▲). The cells were irradiated in water; then transferred into liquid MO and plated after 24 hours holding. D shows the survival curves for plating shortly after irradiation (●, ▲) and for plating after 24 hours holding (○, △).

Figure 2

Section A shows the Xray dose response curve after growth in MOT. The cells were plated on MOTC agar and irradiated at once. B shows the curve after growth in SC. The cells were irradiated on pure agar, washed off with MO medium and incubated for 24 hours and then plated. The lower part of the figure gives the survival curves. (●): growth in MOT, irradiated on MOT agar; (▲): growth in SC, survival directly after irradiation; (△): survival after 24 hours in MO.

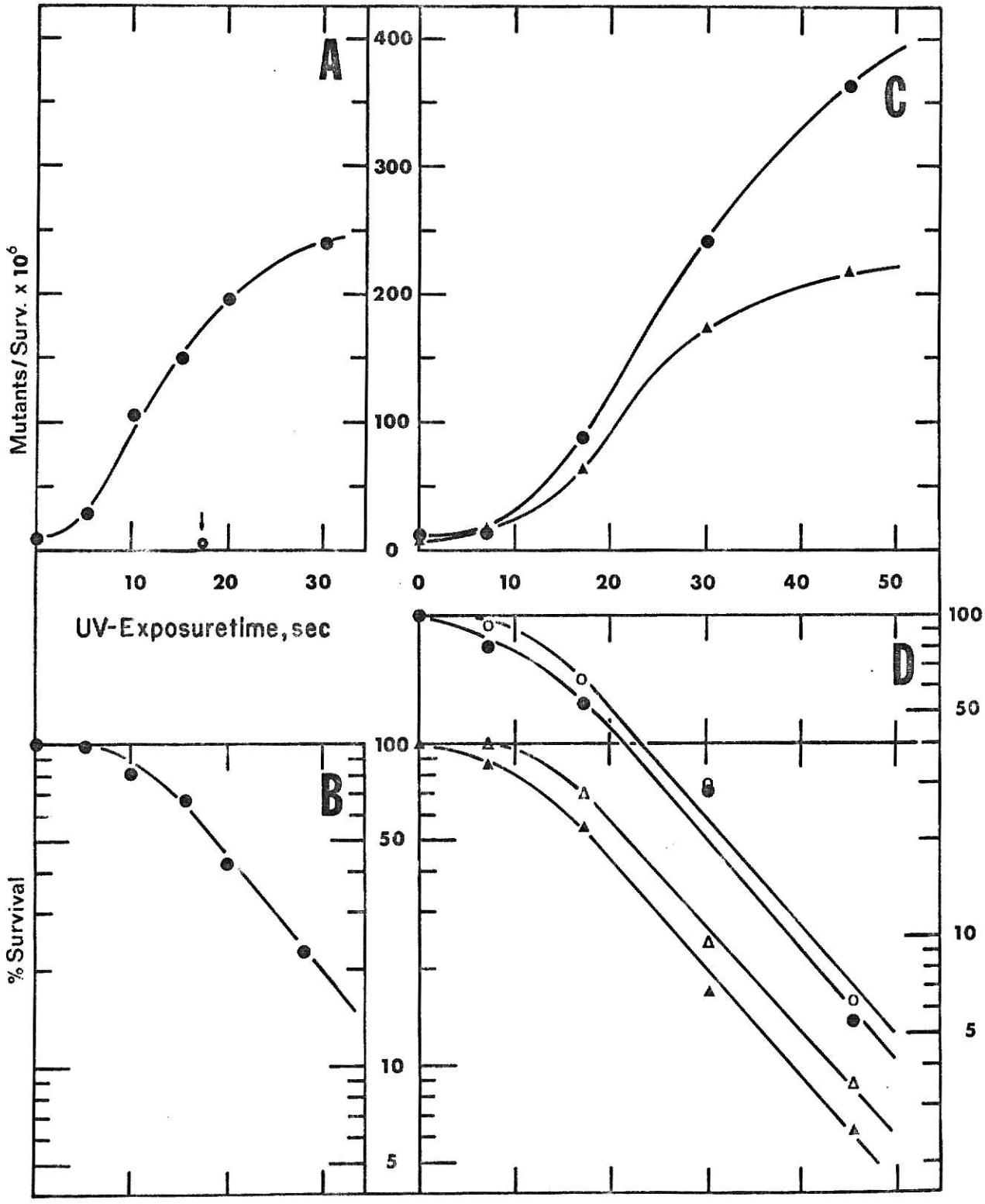

Fig. 1 UV Dose Response Curves

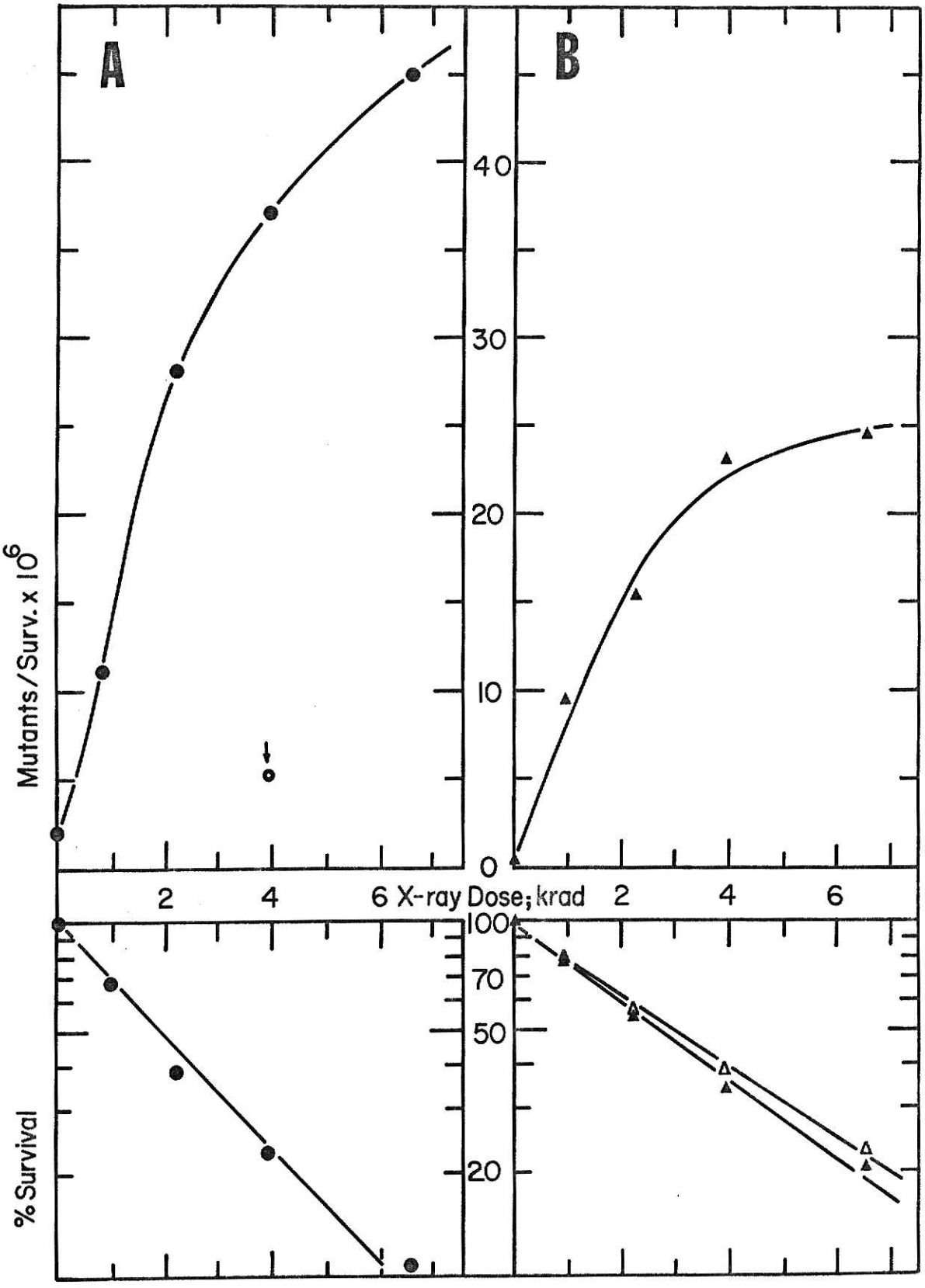

Fig. 2 Xray Dose Response Curves

holding irradiated cells in a nonselective medium but one that did not allow growth (MO) before plating to MOTC was performed.

AB. Influence of Environmental Conditions

Direct irradiation on MOTC was performed only with cells grown in MOT. Other experiments (see below) showed that irradiation of cells grown in SC medium resulted in a mutation level hardly above the spontaneous background if selection occurred shortly (within about 10 minutes) after irradiation. (See the open circles in Figures 14 and 24.) This is a most astonishing result since the DNA material (which is known to be the primary absorber of radiation) is in both cases the same. It is hard to believe that genetic damage is done to the cells grown in the one medium (MOT) but not to the cells grown in the other (SC). That this is really not the case will be shown below. Holding the cells for 24 hours in liquid MO - after irradiation but before selection - resulted in a considerable increase of the mutation frequency especially for the SC pregrown culture. An MOT pregrown culture, if treated after irradiation the same way as a SC pregrown sample, still shows a higher mutation frequency (Figure 16) but the difference is far less extensive than for direct irradiation on MOTC.

To reach a better understanding of the molecular mechanisms involved a series of experiments with slightly varying conditions

was performed. The result of the first experiment of this series is shown in Figure 3. MOT was chosen as the holding medium. In the lower part of the graph one can follow the growth of the irradiated yeast cells during the holding time. After an inhibition of growth for about five to six hours (as usually observed after UV irradiation) the cells start to divide in normal fashion.

In the upper part of the figure one can see again the dramatic difference in mutation frequencies directly after exposure. While the MOT pregrown cells show a mutation frequency greater than 10^{-4}, the SC pregrown culture shows hardly any increase above the spontaneous mutation level (about 10^{-6}). The picture changes vastly already within the time before the first cell division takes place. The frequencies of expressed mutations increase rapidly especially for the MOT pregrown cells and reach peaks at 2 and 7 hours after irradiation respectively. Thereafter the mutation frequency starts to decrease and eventually reaches a constant level as can be seen in the following figures.

I will attempt to discuss and explain the observed phenomena separately. The Figures 4 to 9 show the results of similar experiments as described above. References to these figures will be made at the appropriate places.

(1) <u>The Initial Mutation Frequency and the Following Rapid Increase.</u> The dramatic difference in the mutation frequency depending on the growth medium is the most surprising phenomenon observed in this part of the study. Also the quickness of increase after only short

Figures 3 through 8

The short form used in the legends is explained by the following example:

SC UV MO
MOTC/MOT
MOT UV MO

It means: The cells were grown either in liquid SC or MOT, then centrifuged and placed into water and irradiated by UV light for 17 seconds (about 50% survival). Then concentrated MO medium was added to reach the normal concentration of the nutrients. At different times samples were removed and the density of mutants and viable cells was determined by plating appropriate dilutions on MOTC and MOT plates. The mutant frequency is given by the ratio of the number of colonies on these plates X the dilution factor. It is plotted as a function of time. The lower parts of the figures show the growth of the cells during holding time as given by the colony counts on MOT.

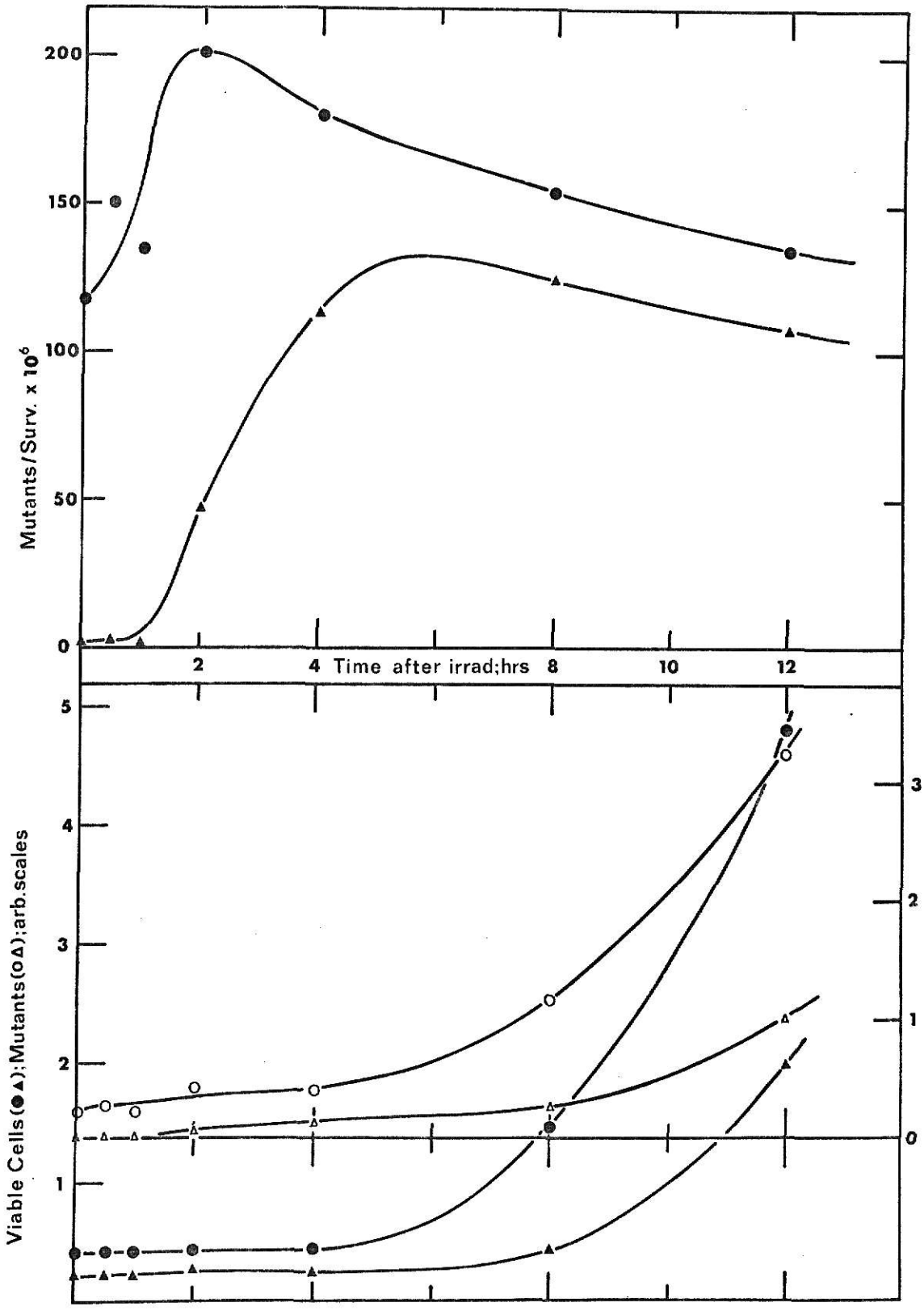

Fig.3 (●): MOT UV MOT

(▲): SC UV MOT

MOTC/MOT

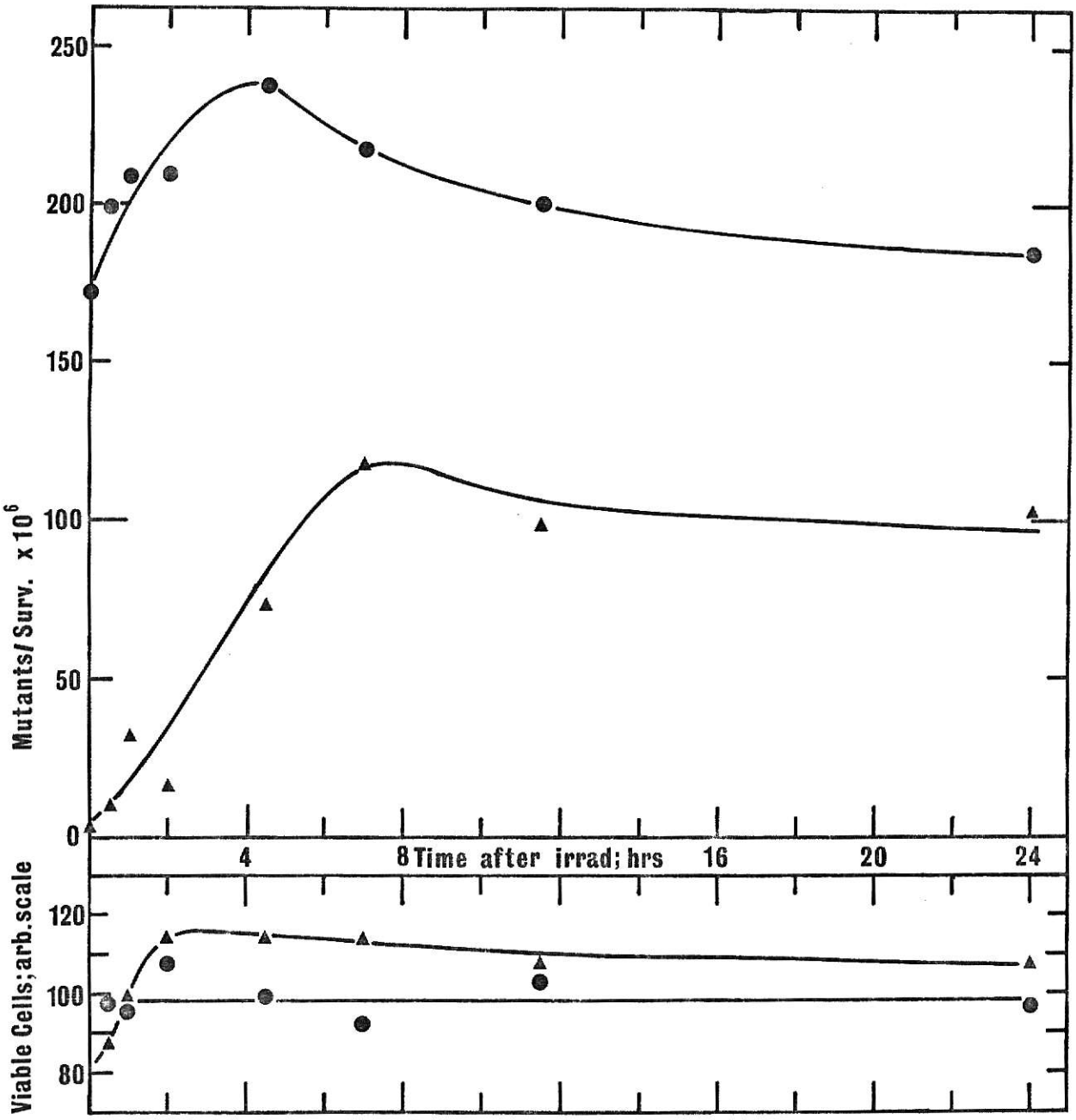

Fig. 4

(●): MOT UV SC-trp

(▲): SC UV SC-trp

MOTC/MOT

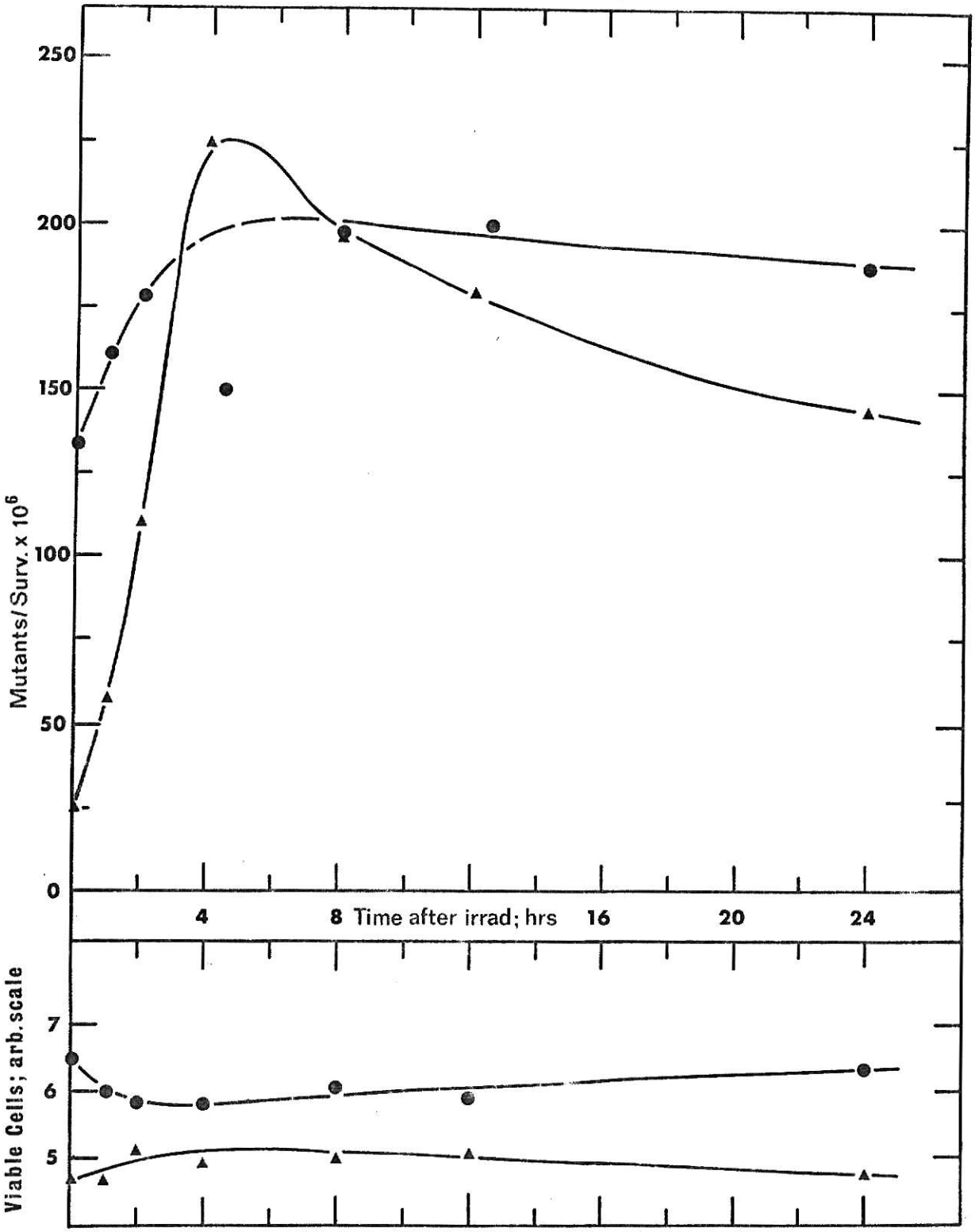

Fig. 5

(●): MOT UV MO

(▲): SC UV MO

MOTC/MOT

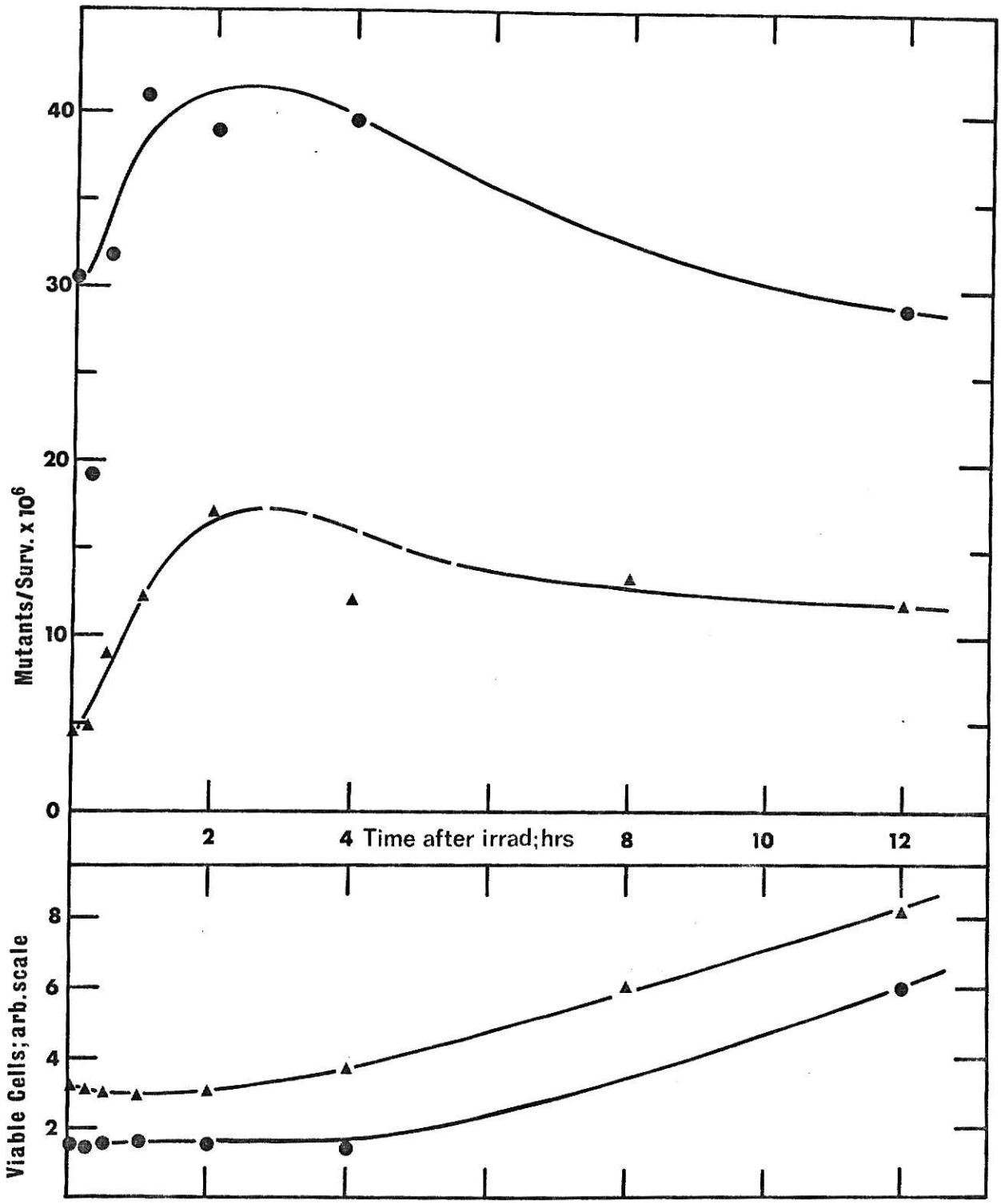

Fig. 6

(●): MOT Xray MOT

(▲): SC Xray MOT

MOTC/MOT

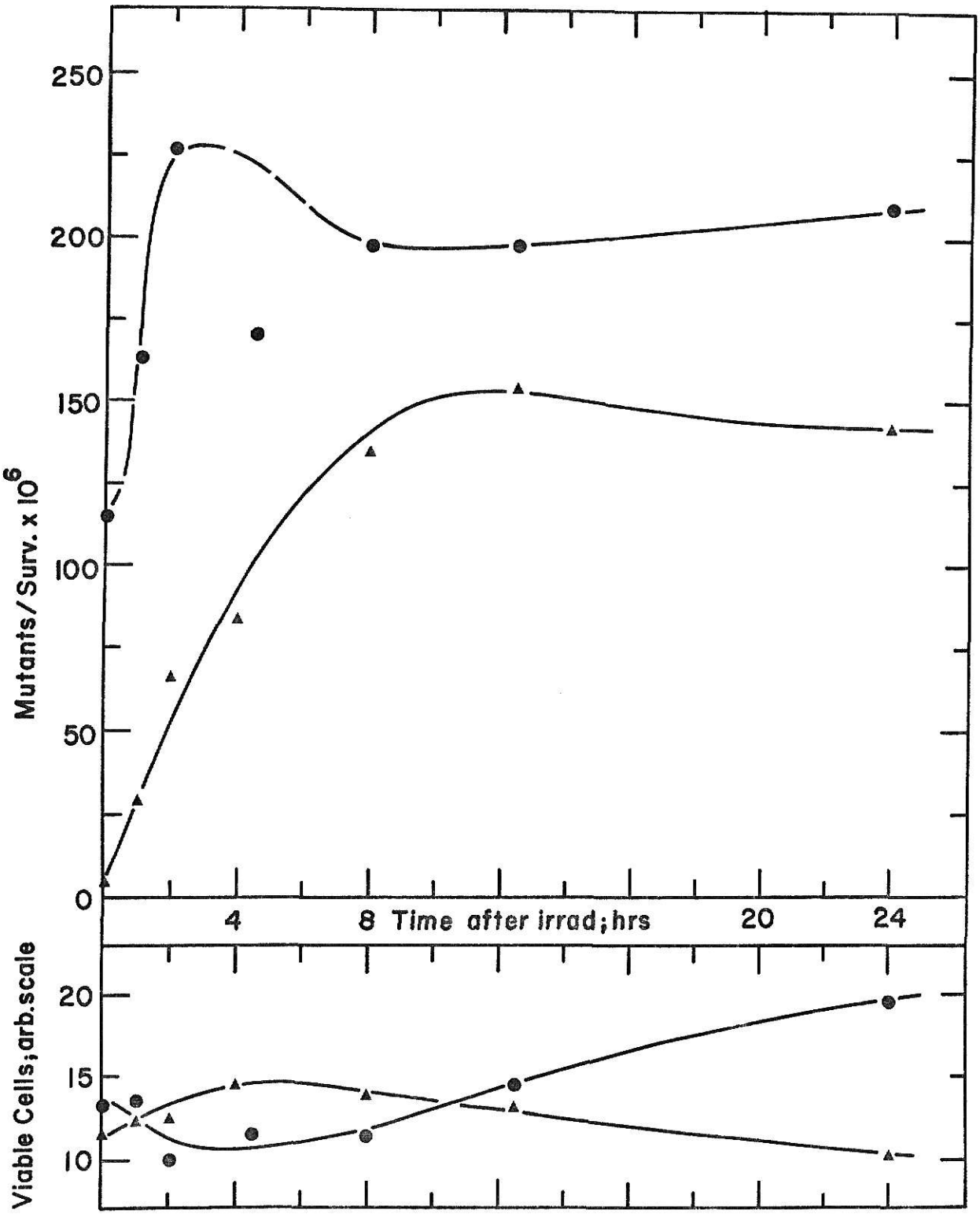

Fig. 7

(●): MOAT UV MO

(▲): MAT UV MO

MOTC/MOT

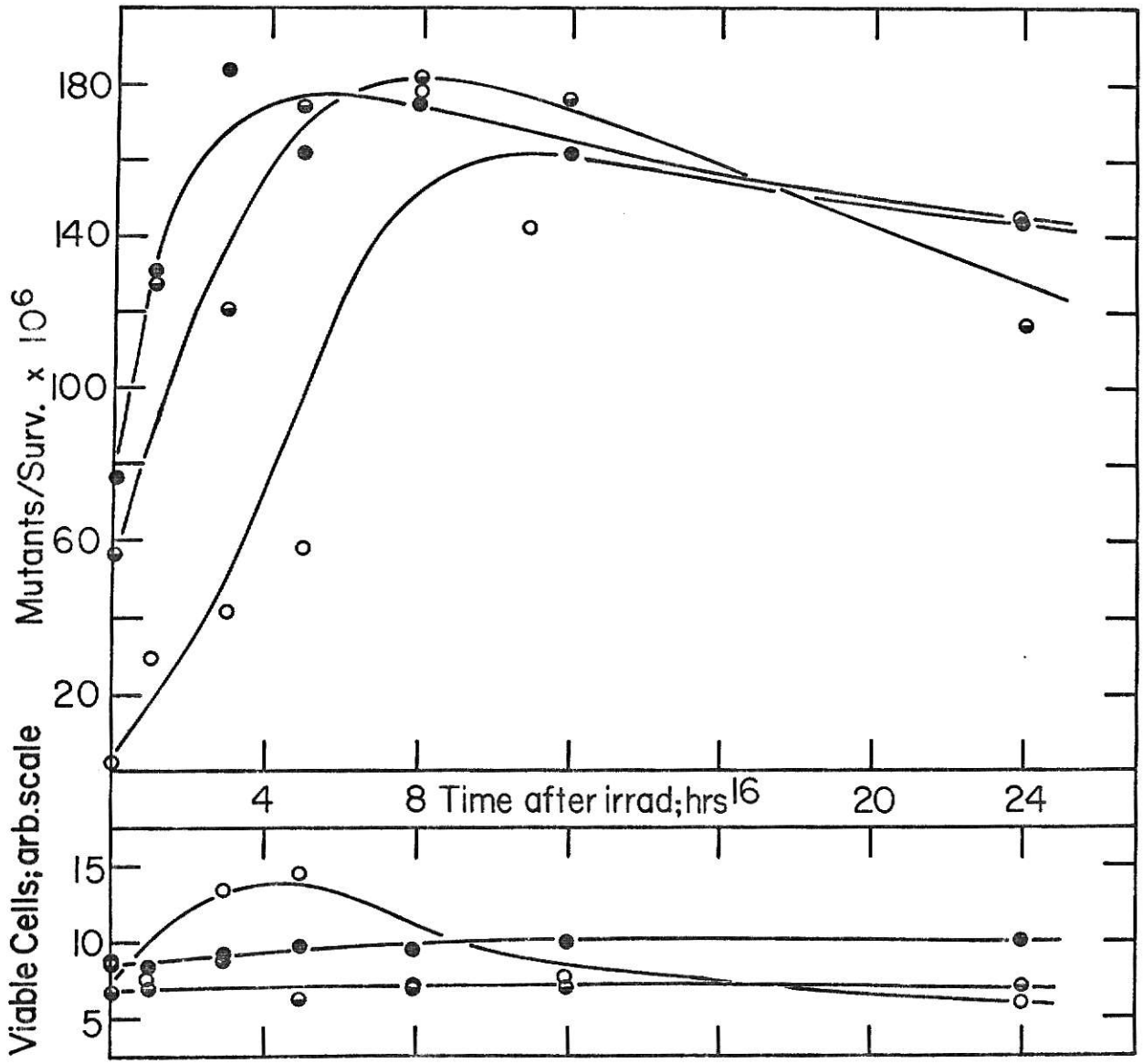

Fig. 8

(○): SC(20 mg/l arg) UV MT

(◒): SC(60 mg/l arg) UV M MOTC/MOT

(●): SC(200 mg/l arg) UV M

holding periods is rather astonishing. Variation of the holding medium did not change this feature appreciably (Figures 4, 5, and 8). There are several plausible explanations: The "potential" mutants (those for which irradiation resulted in a genetic damage at the CAN1 locus) still possess the arginine permease proteins. Although they are not able to synthesize this enzyme anew it is conceivable that the residual permeases take up enough canavanine so that the cell is poisoned before it is able to express its mutant phenotype. A decrease of the permease activity during holding time would result in a smaller uptake of canavanine and the cell would survive. The difference between the cultures grown in MOT and SC could be due to different permease activities. The cells grown in a medium containing ornithine as the sole source for the production of arginine were thought to have smaller amounts of active enzyme than the cells grown in a medium containing arginine. This would imply that external arginine induces the enzyme level to increase. The phenomenon of induction is a well established fact in microorganisms. (e.g. J. D. Watson, Molecular Biology of the Gene).

This theory was disproved when MOAT was chosen as pregrowth medium (Figure 7). The model would predict that the curve starts at a level similar to the SC or MAT curves, because the exogenous arginine would induce the permease level and canavanine would be taken in at the same rate as for SC or MAT pregrown cells. But the initial frequency obtained in this experiment is very much the same

as for cultures pregrown in MOT. So rather than the arginine, the presence or absence of ornithine seemed to be responsible for the difference in initial mutation frequencies.

On the other hand Figure 8 shows that the initial frequency becomes greater when the concentration of arginine is increased. To reach a better understanding of the observed phenomena it was necessary to abandon this type of experiments in favor of a more biochemical approach.

(2) Permease Activity. The use of ^{14}C labeled arginine for determining the rate of uptake (permease activity) seemed to be the most promising expansion of the studies. The scintillation counting method is extremely accurate and allows detection of minimal amounts of radioactivity inside the cell. The techniques are described in Material and Methods.

The first experiments were performed with exponentially growing cultures. As expected for enzyme activity the rate of uptake is constant within the limits of experimental error (Figure 2). By repeating the same experiment several times it was observed that the slopes of the curves were not very reproducible. The reason for this is not understood. But in almost all cases the cells grown in MOT and MOAT showed a considerably higher rate of uptake than the cells grown in SC.

In a second procedure (Figure 10) the cells were transferred after growth in MOT or SC into either MO or M. The lack of tryptophane and in the case of minimal medium, also the inability to

Figure 9

The wildtype cells were grown in MOT (●), MOAT (○) and SC (▲) to a density of about 10^8 cells/ml. At the beginning of the experiment labeled arginine is added. The experiment was performed at room temperature. The concentration of the arginine during the time of the experiment was 20 mg/l. At times samples were removed and the incorporated ^{14}C-arginine measured (Materials and Methods). For a comparison, the absence of uptake by the mutant strain can1-23 is shown (▼). The number of cells was determined by plating appropriate dilutions on MOT agar.

Figure 10

The cells were grown in the indicated media, then centrifuged and placed into holding media. After 3 hours holding at 30°C. concentrated tryptophane and ^{14}C-arginine were added (final concentration 20 mg/l each). At the indicated times samples were removed and the incorporated radioactivity and the viable cell density determined. The growth and holding media were:
(●): MOT MO (▲): SC MO (Δ): SC M

Figure 11

The cells were grown in MOT (●), MOAT (■) and SC (▲). At the beginning of the experiment they were placed into liquid MOTC containing also ^{14}C-arginine (see text). At times the incorporated activity was measured (section A) and the survival was determined by plating appropriate dilutions on MOT. The experiment was performed at room temperature.

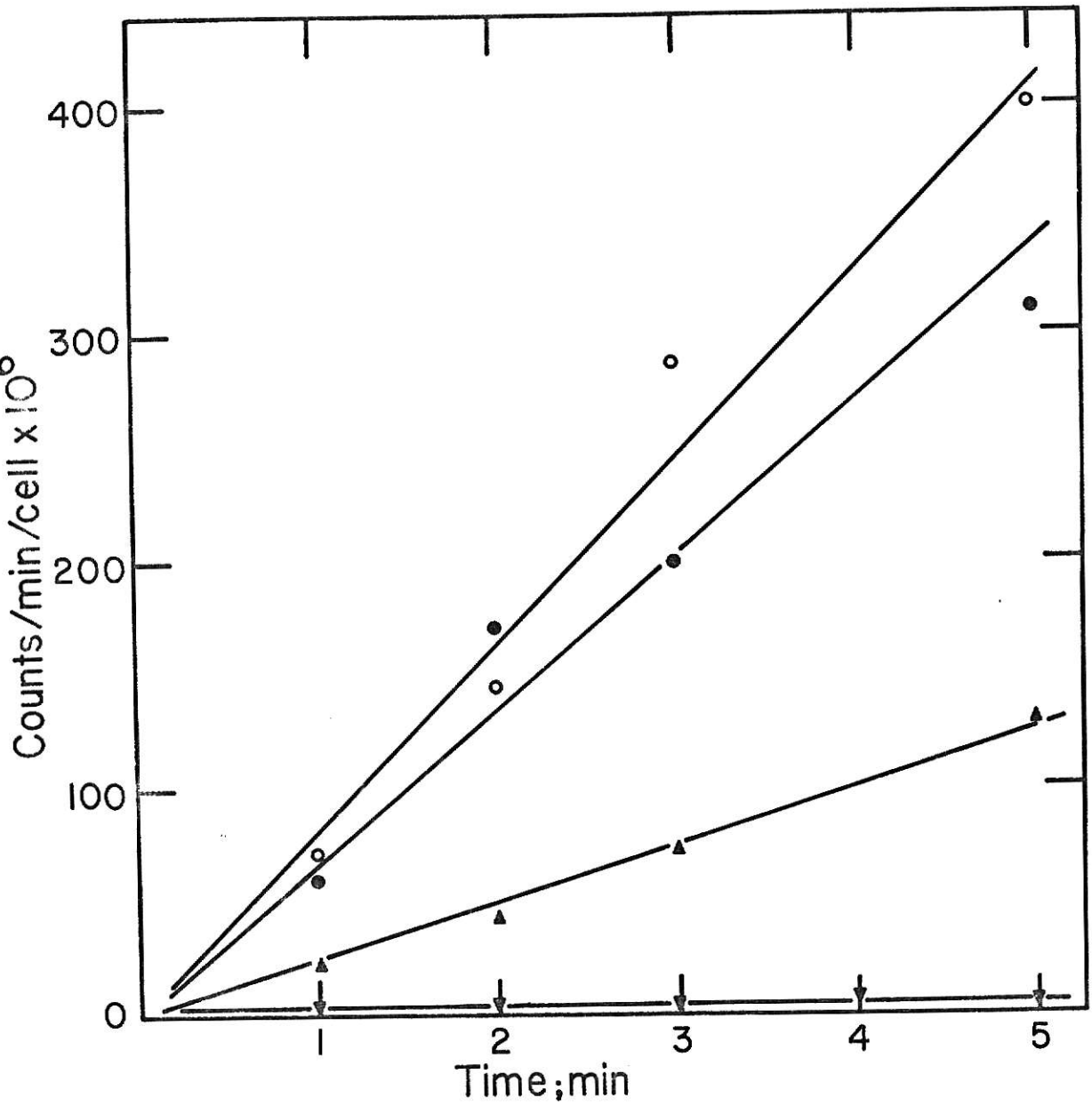

Fig. 9 The Uptake of Arginine by Exponentially Growing Cells

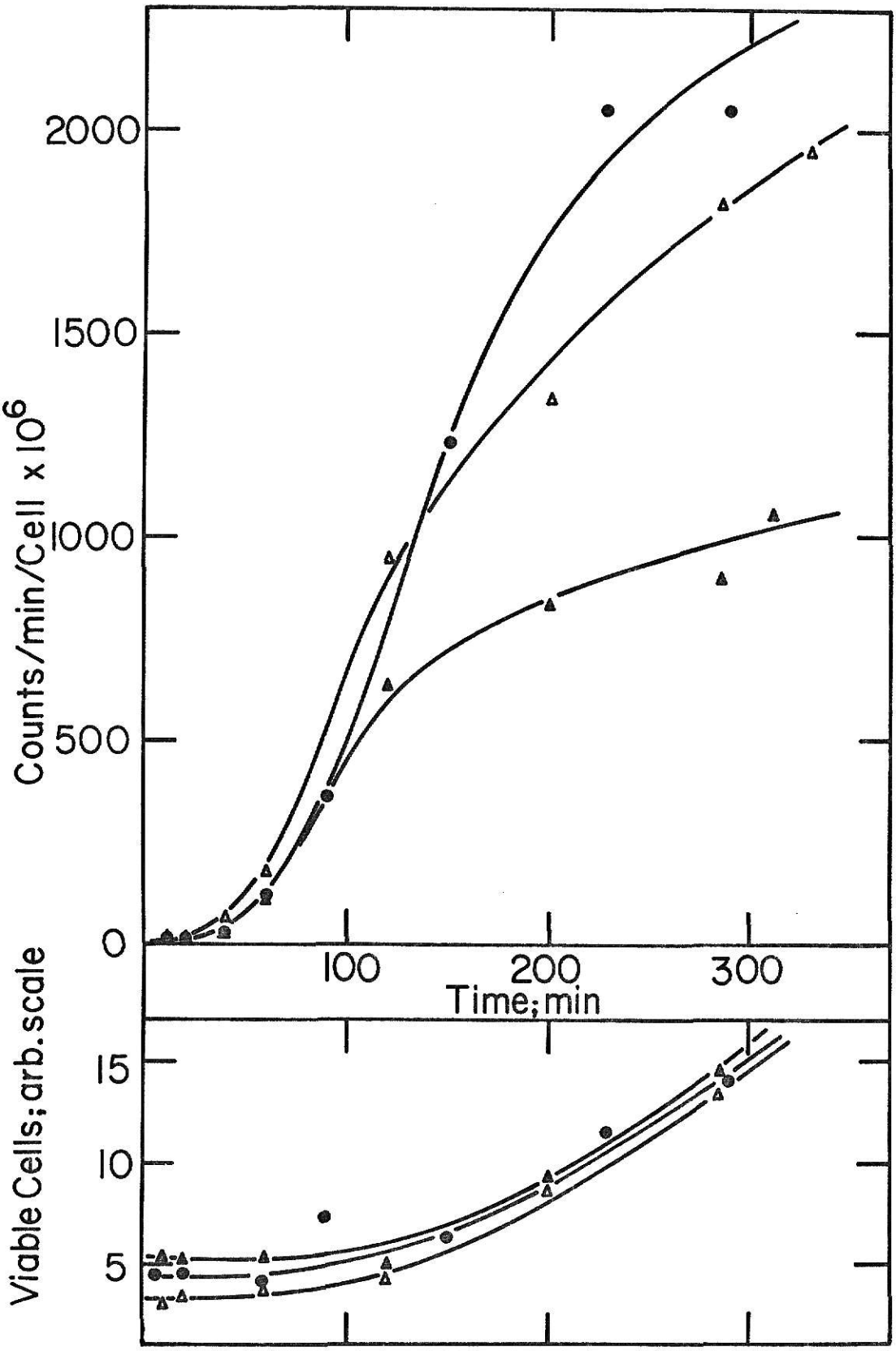

Fig. 10 Uptake of Arginine after Holding in Media Inhibiting Growth

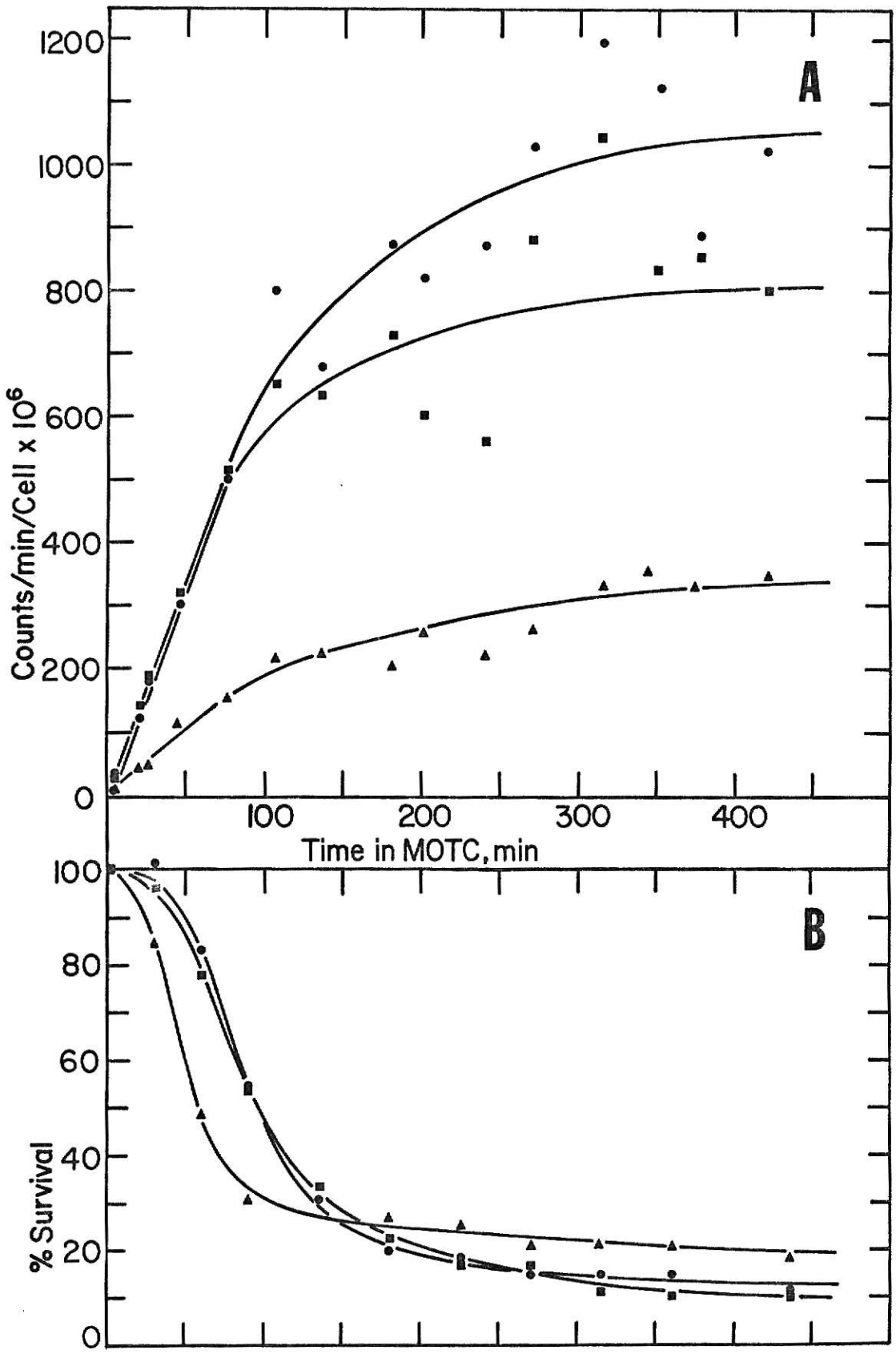

Fig. 11 Effect of Canavanine on the Wildtype Strain

produce arginine cause an inhibition of growth. After 3 hours ^{14}C-arginine and tryptophane (highly concentrated) were added to a final concentration of 20 mg/l. The cells started slowly to grow as can be seen in the viable cell count (lower part of the figure). The important result of the experiment is the obvious inhibition of enzyme activity for up to 60 minutes and the different final levels of incorporated arginine.

Several authors reported rapid decay of specific transport activity of amino acids in Neurospora and animal tissues after inhibition of protein synthesis by starvation for a required amino acid or by cycloheximide and other inhibitors (Elsas and Rosenberg, 1967) (Wiley and Matchett 1967) (Adamson, Langellutig and Anast 1966). It was suggested in these papers, as one or as the sole interpretation of the results, that protein synthesis is an obligate requirement for the maintenance of carrier proteins with short half lives (rapid turnover). In yeast, experiments seemed to suggest that inhibition of amino acid transport activity is due to a pool of endogenous amino acids which accumulate as a consequence of the inhibition of protein synthesis (Grenson, Crabeel, Wiame, Bachet 1968). The later explanation does not seem to fit here since the holding in minimal medium, where endogenous arginine cannot be accumulated because of the double block in the pathway and the absence of ornithine and arginine in the medium, caused an inhibition of uptake of very much the same extent. It cannot be definitely concluded, that the rise in the permease activity is caused by

re-synthesis, rather than by releasing of the inhibition, but other experiments (described below) strongly favor this explanation.

Both of the uptake experiments and also the one result shown in Figure 11A would suggest that potential mutants of the SC pregrown sample should be less affected (killed) by direct as well as delayed plating on the MOTC medium than the ones of the MOT or MOAT pregrown cultures. The rate of uptake and the final concentration of arginine is obviously smaller for SC pregrown cells and the same should be valid for uptake and final concentration of the poisonous canavanine.

That this is not the case became clear after performing an experiment where SC, MOT and MOAT pregrown cells were transferred into MOTC medium containing also very low concentration of labeled arginine of high specific activity (330 mCi/m mol). The purpose of this experiment was to determine the uptake of canavanine and at the same time follow the decrease in viability, which was tested by plating samples onto MOT at the times indicated in Figure 11B.

It would have been somewhat more satisfying to use labeled canavanine, which would allow calculation of the actual amount of the analog inside the cells; but since canavanine competes with arginine at a level depending on the ratio [can]/[arg] and this ratio was the same in all cases (well above 100), a valid internal comparison could be made. Again it is seen that cells grown in SC have a smaller initial rate of uptake. Nevertheless it is just this culture which is killed the quickest: 50% "killing time" for SC pregrown cells is about 55 minutes; for MOT and MOAT about 95 minutes. This phenomenon

was at that stage of study a rather surprising result. The reason will become clear in the following section.

C. F. Schactele and P. Roger (1965) showed that canavanine exposure of E. coli cells (arg^- as well as prototrophic) caused a logarithmic loss of viability (after a short bacteriostatic effect) over several decades. The concentration of internal free arginine had to be low. In Figure 12 one can see that the viability stops decreasing at about the same time that the uptake rate of canavanine starts to decrease. The loss of viability is obviously not logarithmic. It is suggested that the leveling off of the viability is due to a competition of the arginine produced from the ornithine of the MOTC medium. The ratio [arg]/[can] is assumed to stay about constant because no more canavanine is taken in (Figure 11A) and the probability of survival no longer decreases. A lowering of the concentration of ornithine in the MOTC is expected to cause a lower level of survival because the "pool" of endogenous arginine would decrease.

(3) Accumulation of Endogenous Arginine and/or Its Precursors.

After the complete failure of the theory of increased permease activity after growth in medium containing arginine the just mentioned model of different "pool sizes" proved to be very useful in the further study.

It is well known that certain metabolic molecules accumulate inside the cell under certain conditions. In yeast cells such pools of endogenous amino acids could be detected. (Grenson, Mousset, Wiame, Bechet 1966). Additionally for a poisonous analog (such as canavanine)

the absolute amount inside the cell is often less important for the killing effect than the ratio of the concentrations of the poison to the molecule it competes with (arginine). One could postulate that the poolsize of arginine and/or its precursors is several times greater in MOT or MOAT grown cells than in the cells pregrown in media not containing ornithine. Until the permease activity in potential mutants has decayed to a level which is not dangerous, a certain amount of canavanine has entered the potential mutant. This amount seems to be smaller for SC pregrown cells. But the assumption of a considerably smaller pool of endogenous arginine in just these cells would still predict a quicker killing of these cells compared to the ones pregrown in media containing ornithine. This could be observed in Figure 11B. So the potential mutants are killed before they are able to express their mutant phenotype.

The bioassay for actually measuring the amount of free endogenous arginine and the precursors ornithine plus citrulline is described in Material and Methods.

The results of this experiment gave strong evidence for the theory of different poolsizes as can be seen in Table 3.

Table 3

Cells Pregrown In	Internal free Ornithine & Citrulline ($mg/10^8$ cells)	Arginine ($mg/10^8$ cells)
MOT	24	1.3
MOAT	17.1	1.0
SC	<.1	<.1

Although the results of this experiment are not expected to be very accurate (See Material and Methods), the definite conclusion can be made that growth in media containing ornithine causes a dramatically higher concentration of endogenous arginine and its precursors than growth in media only containing arginine. The observations concerning the differences in initial mutation frequencies are now easy to explain:

(a) The concentration of ornithine in MOT or MOAT is actually ten times higher than the concentration of arginine in SC or MAT (200 mg/l vs 20 mg/l). The concentrations were chosen with regard to the poorer uptake of ornithine. For instance a concentration of 2 mg/l of arginine but only a concentration of 40 mg/l ornithine allows the wild type cell to grow up to a density of 7.5×10^7 cells/ml (other nutrients in normal concentrations). It could be shown that there are considerable pools of ornithine/citrulline. Since these two amino acids are eventually converted into arginine the effective ratio [arg]/[can] is especially large for these cells. This results in less extensive killing of the potential mutants - the cells are able to express their mutant phenotype.

(b) In the experiment described in Figure 8 the yeast cells were grown in SC with 1, 3 and 10 times the normal concentration of arginine, then UV irradiated and tested for their mutation frequencies. The higher the concentration the higher are the starting points of the curves. But they are still lower than for cultures pregrown in ornithine containing medium due to the lack of accumulation of the arginine percursors.

(c) Figure 12 (more fully discussed below) shows the ability (or inability) of a non-mutated cell plated on MOTC agar to perform cell divisions before death occurs. The numbers are obtained by microscopical count of cell colonies which did not result in visible clones. The initial numbers (of the cells plated directly after irradiation) show again a difference between MOT and MOAT on the one side and SC and MAT on the other. It is conceivable that the smaller the average multiplicity, the quicker the process of killing. Under this assumption one sees again, that SC and MAT pregrown cells are killed quicker by the canavanine that the ones grown in a medium containing ornithine.

(4) Decay of the Permease Activity. The quick rise of the mutation frequencies after only a short holding period could be explained by an accumulation of endogenous arginine during the time of holding. This would allow the potential mutant to perform several cell cycles when plated on MOTC. With each division the concentration of the permease protein inside the progeny cells would become smaller since it cannot be synthesized anew. This theory was disproved - at least as being the sole explanation-by using M or MT as holding media. These media don't allow an accumulation of the arginine but still the mutation frequency rises in the usual fashion (Figure 8). Also the average multiplicity (Figure 12) for this experiment shows that non-mutants are killed very quickly. An accumulation of arginine should enable the non-mutant cell to complete

Figure 12

The growth and irradiation procedures are the ones described in Figures 3 through 8. The pregrowth and holding media are indicated in the figure. The cells were plated at the given times on MOTC. After five days a microscopical cell and colony count of clones not visible by eye was performed. The Average Multiplicity is defined as:

Average Multiplicity = # of cells/# of colonies

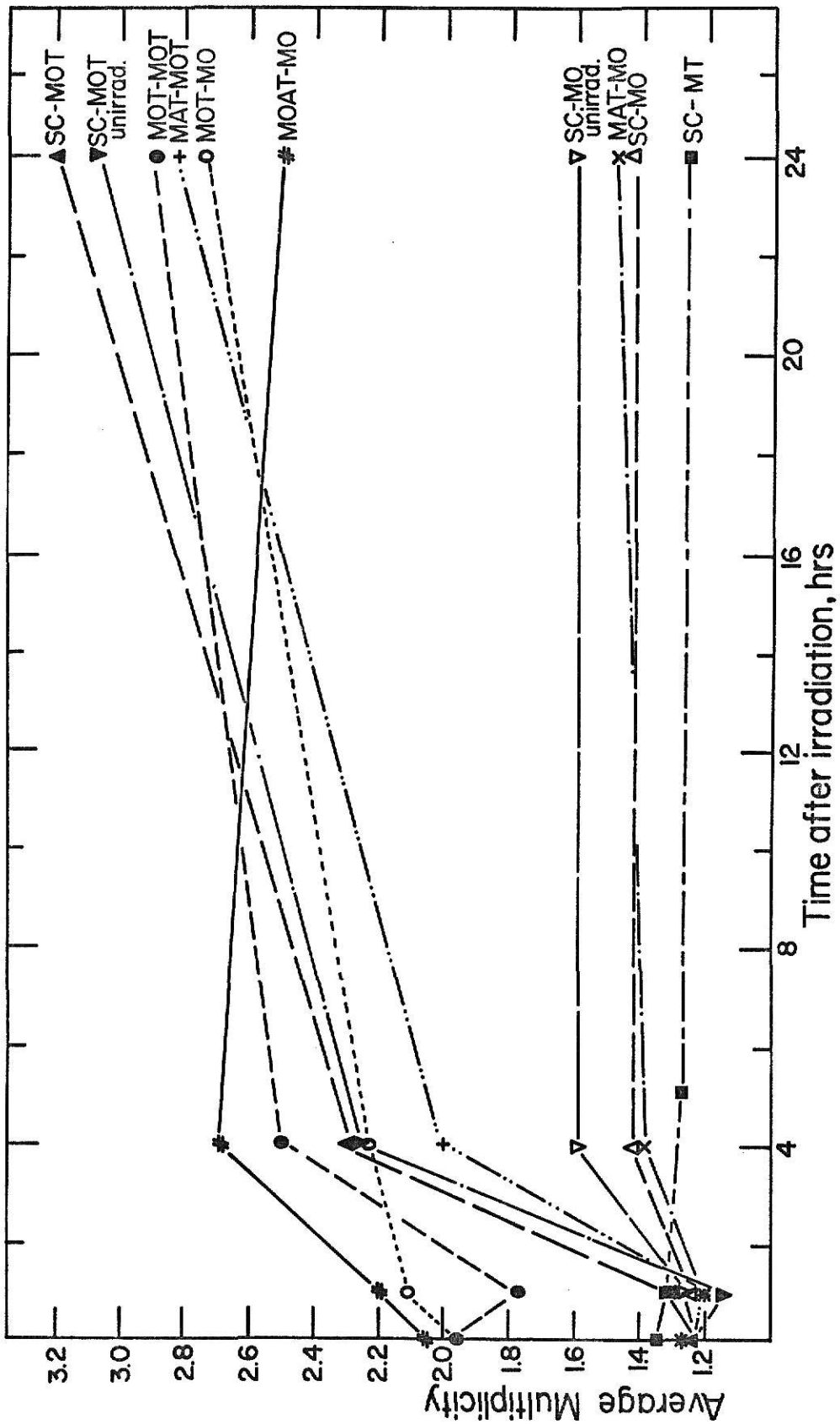

Fig. 12 Average Multiplicity of non Mutated Cells on MOTC Medium

several cell cycles before death occurs. The considerably higher multiplicity of the cells held in MOT or MO is thought to be caused by increased pools of arginine.

The theory of a temporary inhibition of a rather stable permease (See above, Figure 10A) is not entirely disproved. But one would expect under this assumption, that the great differences in the average multiplicity after 24 hours holding would result in appreciable differences of the mutation frequency. The SC - MT curve indicates that the inhibition is released rather quickly since death of the non-mutated cells occurs before hardly any cell division is completed. That says that the potential mutants too are not able to "dilute" the permease among their progeny to a non dangerous level. They should be killed before their phenotype is expressed. This was not the case (Figure 8). It is believed that the most logical explanation for the observed rise in the mutation frequency at delayed selection is a rapid decay of the permease enzyme or at least a necessary part of it in the potential mutants.

If the pool of endogenous arginine is large (for instance, after growth in MOT) the non dangerous level of permease activity is higher and the peak of the mutation frequency is reached quicker. If the pool is smaller (SC pregrown cells) the permease has to decay to a lower level to not be dangerous any more; the peak of the curve is reached later.

(5) Final Level of Mutation Frequency. In all cases (Figure 3 to 7) the growth in the media containing ornithine causes a higher

mutation frequency even after a holding period of up to 24 hours. The reason for this feature is not understood.

(6) Type of Radiation. Figure 6 shows the effect of substituting the UV irradiation by Xrays. As already observed in the dose response curves (Figures 1 and 2) the mutation frequencies are smaller by a factor of 5 to 10. The shapes of the curves are nevertheless very much the same. It can be concluded that the discussed phenomena are not due to the type of irradiation but are a property of the system and only influenced by the environmental conditions.

(7) Decrease of Mutation Frequencies After Peak. By using SC minus tryptophane and MO as holding media (Figure 4 and 5) it could be shown that the decrease of the mutation frequency cannot be totally explained by a selection against mutants. While the liquid MOT allows growth after irradiation, the tryptophane lacking media causes an inhibition of growth. Still the curves tend to decrease, although at a somewhat slower pace.

A successful repair of genetical damage in the holding medium would explain this phenomenon. An early plating would cause a selection for mutant phenotype and by that a fixation of the damage, while a longer holding period does not interfere with the repair process. Definite conclusions can certainly not be made at the present stage of study.

SUMMARY

The radiation induced mutation frequency shows a strong dependence on the medium the cells grow in before exposure and on the delay of selection after irradiation. The behavior could be largely explained by a combination of three factors:

(a) the accumulation of internal arginine and/or its precursors depending on the growth medium

(b) a rapid decay of the permease activity in the cells having a genetical damage at CAN1

(c) repair of the damage

The potential mutants still possess enough residual permease activity to take up a certain amount of the cell poison. Part of them were killed before being able to express the mutant phenotype.

Although the uptake rate of canavanine was tested as being smaller for cells grown in SC medium these cells were killed quicker than those pregrown in MOT due to the drastically smaller concentration of endogenous arginine. This caused an astonishing difference in mutation frequencies if the irradiated cells were plated on MOTC agar shortly after irradiation. The rapid decay of permease enzyme and the inability of the mutants to resynthesize the protein resulted in a quickly increasing chance of survival when selection was delayed. The values don't show so much difference.

At the time of the decay of the permease activity simultaneously a repair of the damages seems to occur in the holding medium. This

became observable after the permease activity had decayed to a non dangerous level. The frequency of mutants started to decrease over a considerable period of time.

B. Mutations: Characterization

The second part of this study was an attempt to develop a useful system for characterizing mutants. A widened knowledge of the type and location of genetical damage can provide a fuller understanding of the structure of the gene, its function and the genetics involved, as well as the mechanisms of interaction between irradiation and the DNA molecule. At this point it will be useful to distinguish between classes of mutations (Drake 1970).

Macrolesions - This class contains all the large changes, such as inversions, translocations, deletions or duplications of one or more genes. The first three abnormalities are usually detected by their effect on chromosome behavior. Duplications of one or more genes sometimes leads to a distinguishable change in phenotype.

Microlesions - This class contains aberrations of smaller extent. The DNA strand is altered over a length of only one or a few nucleotides. One can further distinguish between base changes (missense mutations) and insertion or deletion of one or a few base pairs (frameshift mutations). Most of the missense mutations will not result in an observable change of phenotype since very often the protein retains its activity. Frameshift mutations are detected

very efficiently since the enzyme loses in most cases the activity. Mutations of these types often cluster at one or at several sites along the gene (a hotspot) (Benzer 1961) (Yourno, Ino, Kohno 1971).

The method to detect the location of such microlesions used by Benzer was the deletion mapping method which will be described below. He could show that the distribution of the defects along the rII gene of the bacteriophage T4 differed for spontaneous and induced mutations of this gene (Benzer 1961) (Brenner, Benzer and Barnett 1958).

In the present study an attempt was made to distinguish populations of mutations induced by Xrays, UV light and Protons by several characterizations such as mapping, revertibility and suppressibility. The results were largely obtained as by-products of the search for a useful set of deletion mutants.

BA. Search for Deletion Mutations

W. L. Whelan found in his work by using the Xray mapping method that spontaneous CAN1 mutants isolated from haploid cultures have a different mapping distribution than the ones obtained from diploid cultures after sporulation. It was to be expected that mutants isolated after irradiation with UV, Xray or Protons would show differences, too.

The Xray mapping method (Manney and Mortimer 1964) is a very exact but rather laborious method for mapping a large number of alleles. So I set out to develop another method for the mapping of

the CAN1 locus. The deletion mapping method (Benzer 1961; see above) seemed to be especially promising.

Among the spontaneous mutants isolated by W. L. Whelan five independent alleles of CAN1 failed to recombine with any of nine test alleles distributed along most of the known length of this gene. This strongly suggests that the entire gene was deleted or inverted (which could not be distinguished) for these strains. No alleles with the properties of a deletion of less than the entire locus were found. But there was no reason known why an extended search should not yield a set of such mutations. It turned out that the effort was in vain. Not a single useful deletion could be found. Also it could be established that at least one of the above mentioned deletion mutants (allele can1-111) does not seem to be one. A possible reason for this will be discussed in the Note on allele can1-111.

Despite the failure of the search several observations of possible interest could be made. The screening procedure contained the following steps:

(a) The wild type strain was grown in liquid SC and irradiated by either UV, Xrays or Protons (5 MeV) as described in Material and Methods. The dose was chosen to yield a survival of about 50%. After irradiation the cells were incubated for 24 hours in liquid MO and then plated on MOTC. The cultures were treated as alike as possible to make comparisons more valid.

(b) The mutants were subjected to the test for revertibility. Extended deletions are expected not to revert.

(c) The class scored as "negative" for all three mutagens

(NG, EMS, UV light) was tested for recombination with eight tester alleles. Extended deletions should not recombine with several adjacent alleles.

(d) At the same time a test for suppressibility was performed. Suppressible strains are known to be not of the deletion type.

(1) Revertibility. It has to be noted that the results of this test were not very clear cut. In similar work done by W. L. Whelan homoallelic diploids instead of haploid cells were used. Because of a lower background reversion these results are less ambiguous. In general it can be said that the revertibility behavior of the mutants is about the same for induction by either of the mutagens used and differences were never very clear cut.

Table 4 - Revertibility

Mutagen	Response positive I	weak II	negative III	no response* IV	Forward mutation induced by
UV	16	37	46		Protons
NG	22	33	43	36	(5 MeV)
EMS	16	42	42		(99 strains)
UV	10	30	59		UV light
NG	16	36	48	34	(93 strains)
EMS	17	33	50		
UV	20	24	55		Xray
NG	20	18	59	50	(73 strains)
EMS	20	21	58		

*This column gives the percentage of strains which did not revert by any of the mutagens used.

The most pronounced difference between the samples obtained from the three different radiations is that 50% of the Xray mutants are scored in column IV but the two other values are only about 35%. An explanation for this is at the present time of study hard to give.

Since the test was only a first step towards identifying deletion mutations no further going experiments were attempted. The reversion frequency for such gross changes in genetic material as a loss of an appreciable part of the gene in question should be very low. So deletions are expected to be in column IV.

(2) Mapping. The strains scored in column IV were subjected to the recombination spot test. The tester strains carry mutations that are distributed along nearly the entire known CAN1 map. (W. L. Whelan 1972). The Xray mapping data are given in Table 5.

An extensive deletion or inversion among the alleles to be tested should show up by its failure to recombine with two or more tester alleles. Smaller changes (small deletions, inversions, base changes) might result in weak recombination with one of the tester strains if the two damages are close enough to decrease the probability of crossing over between them appreciably. As it turned out most of the strains tested gave reduced or no recombination with no more than one tester strain (Figure 13, Plate 1). There were some which did not recombine with two or three of the tester strains but this occurred always in the same area of the gene.

So the result we hoped to get, namely a set of rather extensive deletions at different places along the CAN1 map, was not obtained.

Table 5

Xray mapping distances between the tester alleles (W. L. Whelan's data). Because the sequence of alleles 34/26/32 was not unambiguous, several checks were performed (Data from this study).

W. L. Whelan's data:

alleles in cross	distance Xray map units
40/39	13.2
39/35	9.61
40/35	16.
35/23	11.3
39/23	17.7
23/34	4.85
35/34	12.2
34/26	1.95
23/26	9.52
26/32	5.33
32/37	11.2
26/37	8.85
23/37	20.1
35/26	21.4
23/32	13.5
39/26	44.8
40/26	29.6
40/23	16.8

Data from this study:

alleles in cross	distance Xray map units
23/37	20.1
23/34	16.3
23/26	12.8
23/32	10.4
23/35	10.1
23/39	13.5
23/40	26.8
26/32	4.5
26/34	5.3
32/35	17.9
35/39	6.6
35/40	10.9
34/37	4.
26/37	8.8
32/37	11.2

Out of these data a good map could be constructed. Figure 14 shows the sequence and approximate distances between the alleles.

On the other side it became clear that the recombination spot test seems to be rather well suited for mapping experiments (see discussion below).

Figure 14 shows that a rough mapping of the tested alleles was possible. It has to be remarked that only mutants scored as not revertible were subjected to this test. Still it is interesting to note some properties.

In all three cases (UV, Xray, Proton samples) far fewer mutants map in the area of allele 40 and 39 than in the other parts of the map. If this is a general feature of the radiation produced CAN1 mutants or a result of the revertibility screening procedure cannot be answered. The spontaneous mutants derived from haploid cultures show a relatively uniform distribution while those obtained after sporulation show clustering around allele 32 (W. L. Whelan 1972). The mutants described as non-revertible in the homoallelic diploid form also show a relatively uniform distribution.

Several mutants of the radiation induced samples gave weak recombination responses simultaneously with alleles 26 plus 34, 34 plus 37, or all three alleles. This seems to be partly due to the fact that the distances between these tester alleles are relatively small and that the "resolution" of the test is not sufficient to show appreciable differences in the numbers of recombinant colonies. However, the distances between alleles 32/26 and 35/39 are hardly bigger than between 26/37, for instance. But in no other area was simultaneous mapping at two adjacent sites found.

Figure 13

The irradiation procedures are described in the text. The reference alleles are drawn at the positions calculated from the Xray mapping method (data, Table 5). The width of the columns does not represent the uncertainty of the positions. For each radiation the numbers of strains which mapped at the area of the tester allele are indicated (for procedure see text). Horizontally extended columns indicate simultaneous mapping at the alleles covered. The numbers of strains tested is given. Also the strains for which no definite map position could be found by this test is indicated.

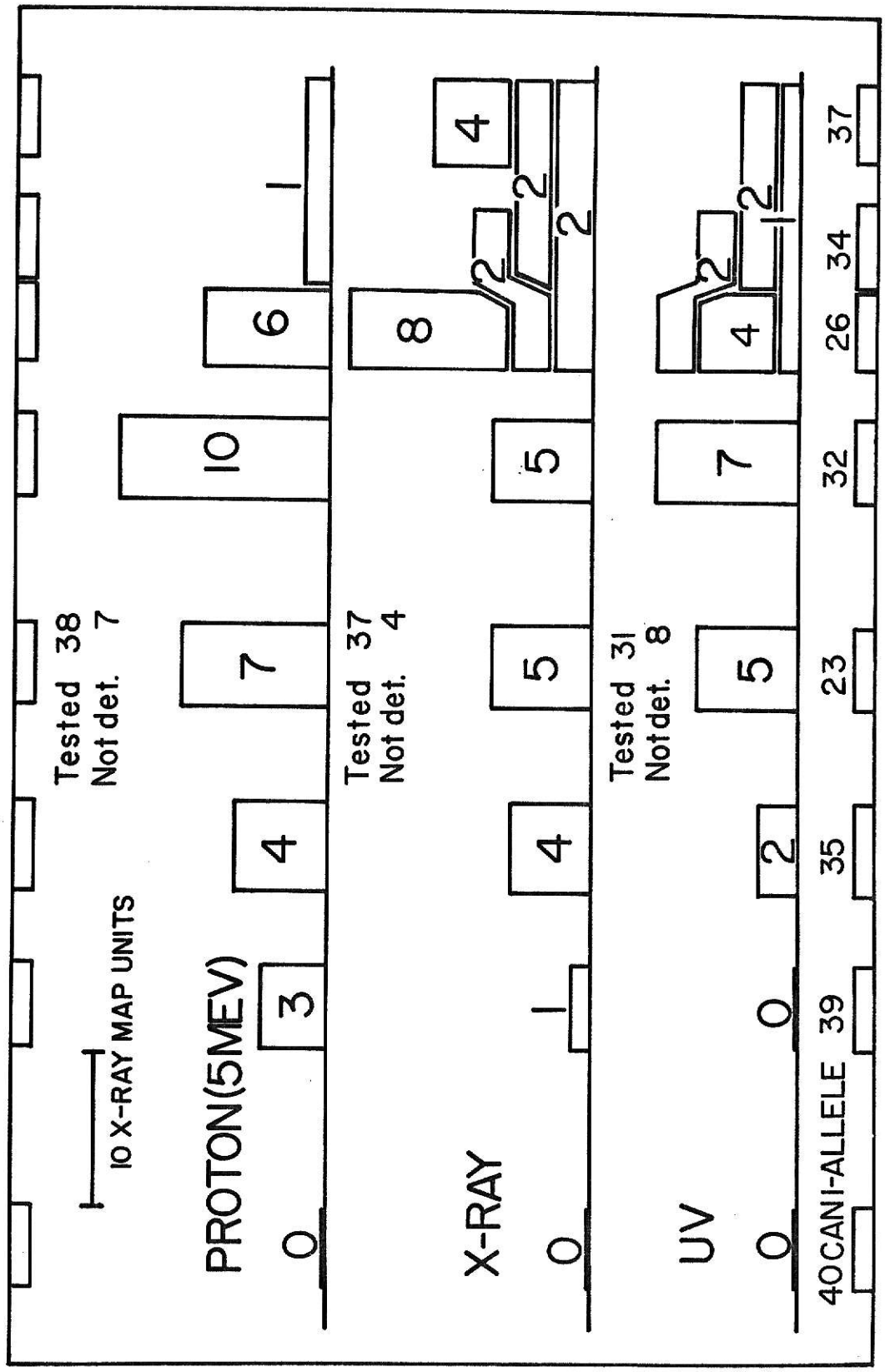

Fig. 13 Mapping Distribution of Irradiated Samples

Plate I

A. Spot Test for Recombination
The tester strains are streaked horizontally and crossed vertically by the strains to be tested. Places of no recombination are easily recognizable.

B. Dissection Slab of MCT agar with spores of 10 asci arranged vertically.

C. Tetradanalysis
Growth pattern of the spores of 5 asci (horizontally arranged) on SC agar. The ability of some spores to grow show that the parent strain is suppressible. The asci 1 and 4 are Tetratype. 3 and 5 are Non parental ditype. 2 is Parental ditype.

D. The same experiment for a non suppressible strain. No spore is able to grow on SC medium.

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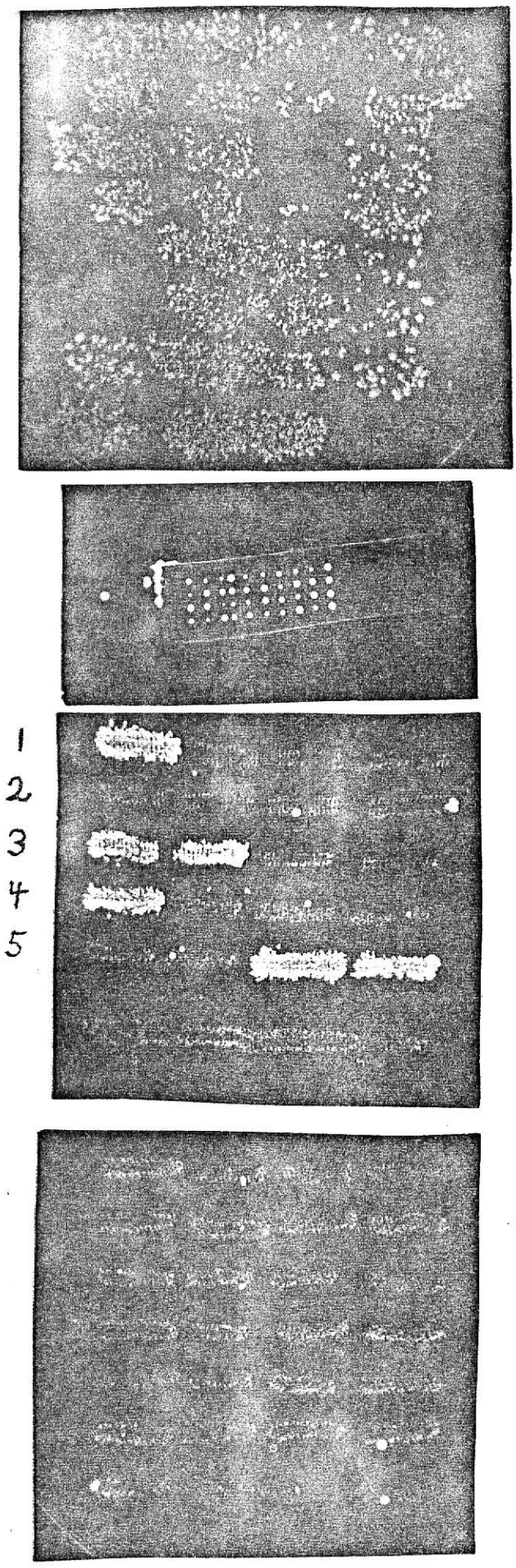
1
2
3
4
5

Although the number of mutants tested was too small to draw definite conclusions there seem to be some differences between the three groups. The Xray induced sample shows a somewhat higher clustering in the area of the alleles 26, 34, 37 while the proton irradiated sample seems to have a hotter "hot spot" around allele 32.

Especially interesting is that the proton sample has only one mutant which maps simultaneously at different sites (34,37) while there are for the UV and Xray samples several mutants which map simultaneously at 34 and 37, 26 and 34, 26, 34 and 37. If this is a real property of the irradiation procedures or just accidental can not be answered at the present stage of study. But the experiments certainly encourage an attempt to map a larger sample of mutants obtained by the different irradiation procedures.

Of special interest might be to look for possible differences in samples obtained by irradiation with heavy ion particles of varying LET values as can be produced at the Tandem van de Graaf accelerator.

It is felt that a doubling of the number of tester strains will practically eliminate cases where no definite mapping position can be determined. Instead of the meiotic recombination test one could use the mitotic Xray mapping method on a qualitative rather than a quantitative basis (Fink 1958). This method would probably also increase the "resolution" of the test.

(3) Suppressibility. Many types of mutations can be suppressed by a mutation at a second site. In the following section the suppression

suppression of nonsense mutations is discussed.

Suppression of this type of mutation was first found in bacterial systems. A mutant which carries beside the suppressible allele in question a suppressor gene, is able to synthesize the active protein the mutant gene codes for. Although the activity is usually somewhat reduced the phenotype of the strain is that of the wildtype cell. It is established that this suppressible allele is resulting from a change of a base triplet to a nonsense triplet (Stretton, Kaplan, Brenner 1966).

The super-suppressor (SUP11) used in this work was studied by Manney (1964) and shown to be capable of allowing translation of a nonsense (ochre) coding. SUP11 maps near the centromere of chromosome VI (Hawthorne and Mortimer 1968). The CAN1 locus is not linked to SUP11 since CAN1 is located on chromosome V.

Among the mutants of spontaneous origin tested for suppressibility (W. L. Whelan 1972) not a single positive response was found. So there was some doubt about the validity of the test since in other loci the percentage of nonsense mutations reaches up to 30% (Manney 1964). In preliminary experiments with UV induced samples eight positive responding alleles were found. To determine if these alleles are really of suppressible type a tetrad analysis was performed. Genetic analysis yields the following segregation for the spores of the cross

$$\frac{can1\text{-}X\ sup^-}{can1\text{-}111\ SUP11} :$$

Table 6

Type	Spores A	B	C	D
Parental Ditype (PD)	can1-X sup$^-$	can1-X sup$^-$	can1-111 SUP11	can1-111 SUP11
Non PD	can1-X SUP11	can1-X SUP11	can1-111 sup$^-$	can1-111 sup$^-$
Tetratype (T)	can1-X sup$^-$	can1-X SUP11	can1-111 sup$^-$	can1-111 SUP11

Can1-111 is not suppressible (W. L. Whelan). So both parent strains grow on MOTC but not on SC (See Introduction).

Assuming that can1-X is suppressible by SUP11 we get the following growth pattern on SC agar:

Table 7

Spores	Type PD	NPD	T
A	-	-	-
B	-	-	+
C	-	+	-
D	-	+	-

On MOTC the growth would be just the opposite.

The ratio PD:NPD:T for unlinked alleles is predicted as 1:1:4. So the probability for a spore to grow on SC medium is 6/24 = 1/4; while for a non-suppressible allele no spore at all should grow on SC.

The test was performed with eight strains that respond positively to the spot test and 2 which respond negatively. The first group contained 39 asci (3 to 5 per strain), and the second 16 (7 and 9 per strain). The spores were tested on SC and MOTC. While the picture on the SC medium is very clear cut, a rather strong residual growth

on MOTC hindered an unambiguous decision. This is due to the weakness of the suppression process (see Plate 1). The given results are based on the scoring on the SC plates.

Table 8: Tetrad Analysis of Positive Responding Alleles

Strain tested	PD	NPD	T	Asci tested
a	0	0	3	3
b	0	1	4	5
c	1	0	4	5
d	1	3	2	5
e	1	2	2	5
f	2	0	3	5
g	0	1	3	5*
h	0	0	4	4

* One of the asci yielded only three viable spores.

Of the 39 asci tested the number of spores capable of growth on SC was 40 (out of 155), the ratio (PD:NPD:T) of the 38 asci yielding four viable spores was 5:7:25. Of the second group (strains tested as non-suppressible by the spot test) not a single spore was capable of growth on SC. These results leave hardly any doubt about the validity of the spot test. The mutants obtained from the three irradiation procedures were tested for suppressibility:

Table 9: Numbers of Suppressible Mutants

Mutants induced by	No. of mutants
UV light	9 out of 93 (10%)
Xray	3 out of 73 (4%)
Protons(5MeV)	2 out of 99 (2%)

It seems that the CAN1 gene shows a scarcity of nonsense mutations if one compares these numbers with percentages up to 30% for other genes.

(4) Note on Allele can1-111. In the assay for permease activity it was found that this mutant has a distinct ability of transporting arginine. This is rather surprising since the mutant does not grow on SC but does on MOTC as expected for an arginine permease-less mutant. (The strain carries the double block in the arg⁻ pathway!) Although the rate of uptake is slower by a factor of about 4 than for wild type cells (Figure 14) it should clearly allow the cell to grow on medium containing arginine. Grenson and her coworkers showed that resistance to canavanine is accompanied by the loss of the arginine permease activity. The mutant carrying allele 23 show such a loss (Figure 15). The allele can1-111 obviously contradicts this theory. This allele also behaved in other experiments in an unusual fashion.

W. L. Whelan concluded from the failure of recombination (see above) that the gene was deleted. The slower growth of this mutant (termed as "semi lethality" by him) suggests that perhaps genes in the neighborhood of CAN1 were deleted too. These were assumed to allow more efficient metabolism. He showed that the "semi lethality" was clearly linked to the CAN1 locus and very probably a property of the allele. A satisfying explanation for this particular behavior is not easy to give.

As a preliminary explanation I suggest that the loss of viability on SC is due to a change in the arginine molecule after it is taken in by the permease in a way that it is not usable for protein synthesis. The poor growth on MOT could be explained by assuming that the intra-

Figure 14

The strains can1-23 (●), can1-111 (▲) and wildtype (▽) are grown in MOT. At the beginning of the experiment ^{14}C-arginine is added (final concentration 20 mg/l) and at the indicated times the incorporated radioactivity measured. The numbers of cells were determined by plating on MOT medium. The experiment was performed at room temperature.

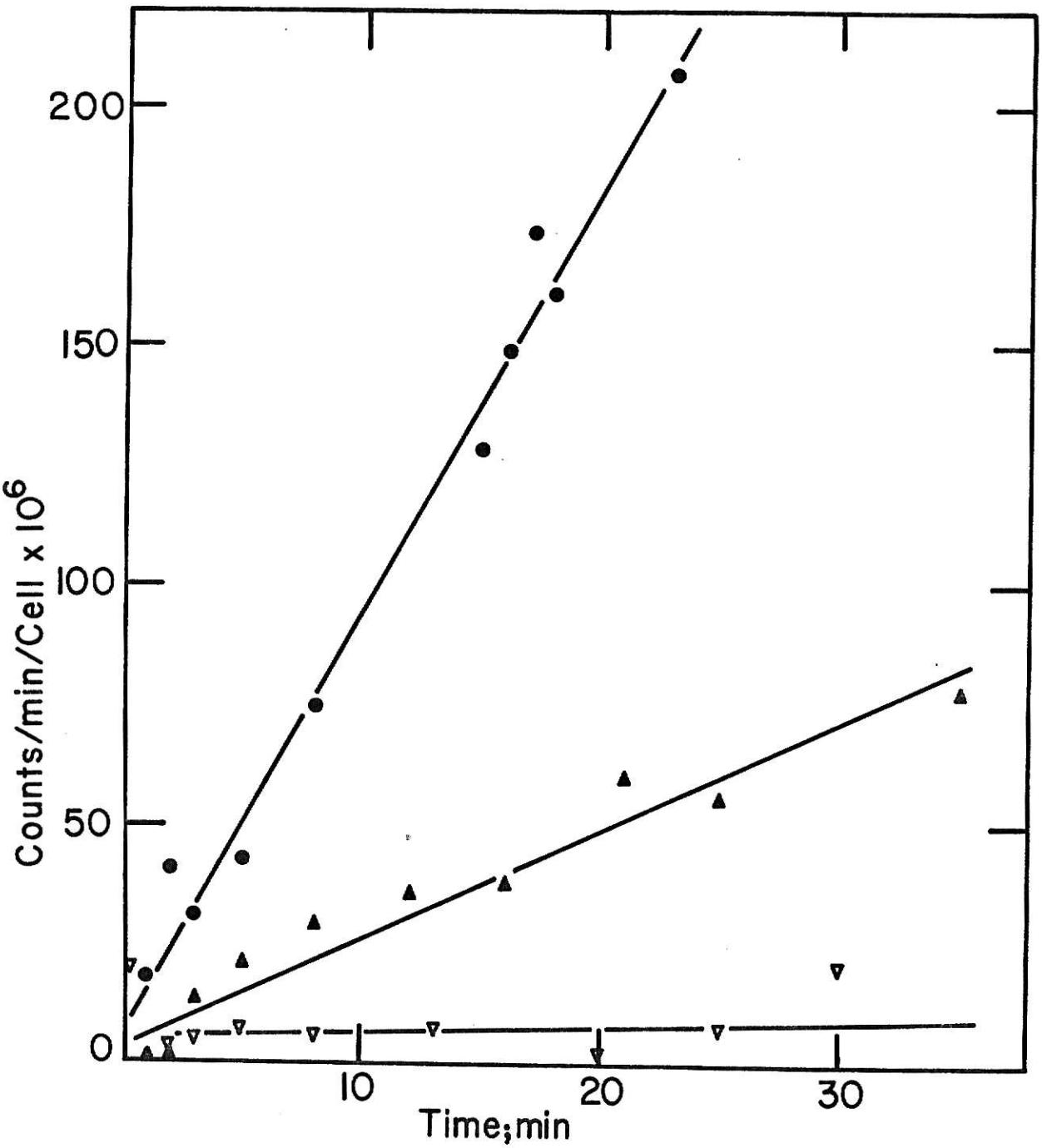

Fig. 14 Uptake of Arginine by Allele can1-111

cellular arginine produced from the ornithine is similarly affected. Some of these arginine molecules might get attached to the permease enzyme and be transformed into a molecule not capable of attachment to the arg-t-RNA. A similar process must occur to the canavanine taken in out of the MOTC medium. Obviously it is not incorporated into protein in a sufficient rate to cause the death of the cell.

This model is hardly satisfying. The failure of recombination cannot be explained without additional assumptions. A more thorough investigation of this allele should be of value in the understanding of the enzyme process.

(5) Scarcity of Nonsense and Deletion Mutations at CAN1. Both types of mutations have in common that the enzyme chains are of considerably smaller extent than in the wildtype or other mutant cells. W. L. Whelan found that the gene coding for the permease is of astonishing length. It is conceivable that the presence of the entire enzyme is essential for the cell (for instance, as a structural component of some cell membrane). The observed nonsense mutations might be in the area of the gene which is translated last, so that only a small part of the "end" of the enzyme is missing. This theory can be checked by mapping these mutants.

The quick decay of permease activity upon tryptophane starvation does not necessarily speak against this theory since it is possible that the decay is due to a removal of a small part of the enzyme (e.g. prosthetic group) and not to a degradation of the entire protein.

SUMMARY

This part of the study was an attempt to devise a system of deletions for use in a quick mapping procedure. It should be of interest to look for differences in the population of mutants obtained from different types of irradiation. Especially the use of heavy ion beams of different LET values, as can be produced by the Tandem van de Graaff accelerator, should provide better understanding of interactions of radiation and genetic material.

Although the goal of the study was not reached, it is felt that a further study in this area should be encouraged. The results indicate that other easy mapping methods can be designed. Differences in the character of the mutants (UV, Xray, Proton induced) were detected although definite conclusions at the present stage of study are not possible. The scarcity of deletions and suppressible mutations at the CAN1 locus seems to be of special interest. The detection of permease activity in allele can1-111 was a most surprising result. Further investigations of this allele should be of value for understanding the mechanisms of the enzymes involved.

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ANALYSIS OF RADIATION INDUCED MUTATIONS OF THE ARGININE PERMEASE LOCUS IN YEAST

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ABSTRACT

A system of strains and media, devised for easy selection of forward and reverse mutations of the CAN1 gene in S. cerevisiae, has been used to study the influence of the environment on the expression of mutations induced by irradiation. It could be shown that the expression of mutant phenotype (resistance to the amino acid analog canavanine) immediately after irradiation depends largely on the amount of intracellular free arginine or its precursors. A rapid decay of the activity of the product of the CAN1 gene (the arginine permease) enables a quickly increasing number of mutated cells to express their phenotype if exposure to canavanine is delayed.

In the second part of this study, an attempt was made to characterize mutants obtained from different types of radiations such as UV light, Xrays and Protons. It could be demonstrated that the CAN1 locus shows a scarcity of suppressible as well as deletion mutations. A possible explanation for this is discussed.