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Saccharomyces cerevisiae strain XS-380, a heteroallelic diploid strain, carries six genetically analyzed loci on the left arm of chromosome VII. Nitrosoguanidine (NMG) at low concentrations was observed to cause allelic recombination (either mitotic recombination or gene conversion) of the heteroallelic loci with low lethality.

The NMG-inducible heteroallelic system was used in studies of the relationship of mutagenic action to DNA replication in both randomly and synchronously growing yeast cultures. Studies of asynchronous cultures in balanced logarithmic growth, in the early stationary phase, and in the early phases of amino acid starvation indicated that each locus had a characteristic recombinant response. Two of these conditions, amino acid starvation and early stationary phase, produced a drastic reduction in numbers of induced recombinants. Moreover, after onset of amino acid starvation, a

timed series of samples could be individually induced by pulse mutagenesis to show that the mutagen response decreased by the time that DNA synthesis stopped. Similar results were also obtained by substituting cycloheximide treatment for the amino acid starvation conditions in the time course experiment. However, a definite relationship of mutagenic response to DNA replication could not be demonstrated by amino acid starvation or cycloheximide inhibition, because coordinate protein synthesis may be necessary for the maintenance of DNA synthesis.

Synchronous cultures also produced ambiguous responses to NMG-mediated pulse mutagenesis. The frequency of inducible mitotic gene conversion increased rapidly before initiation of a discrete synchronous doubling of total DNA. In contrast, high levels of inducible mitotic recombination could also be obtained during the period of DNA replication with decreasing levels toward the end of the first round of DNA doubling. Most of the markers on the chromosome arm displayed similar cyclic variations in recombination frequency. It was suggested that the cyclic activity of DNA repair enzymes may be responsible for the variations of inducible prototroph frequencies during synchronous growth. No definite direction of recombination or polarity could be observed on the chromosome.

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EFFECTS OF PHYSIOLOGICAL STATE UPON NITROSOGUANIDINE-MEDIATED ALLELIC RECOMBINATION IN *SACCHAROMYCES CEREVISIAE*

INTRODUCTION

The way in which cells replicate their chromosomes has intrigued biologists for a long time. Recently, clockwise sequential replication from a fixed origin in the single chromosome of *Escherichia coli* has been demonstrated. These experiments, which utilized different strains of *E. coli*, established the sequence and direction of replication by differing methods, including phage transduction, induced enzyme synthesis, and pulse mutagenesis by nitrosoguanidine. Obviously before such experiments could be attempted, a well-defined genetic map had to be constructed in order to provide a fixed sequence of genetic markers against which replication processes could be measured.

Although genetic maps exist for eukaryotes, the manner in which these higher cells duplicate their sets of numerous chromosomes is not very well understood. For example, it has been proposed that mammalian cells possessing long chromosomes must have multiple sites of initiation in each chromosome to finish replication during the relatively short period of observed DNA synthesis. Yeasts are among the most simple organisms that have a nuclear membrane. The chromosomes of these organisms are probably quite short, if the

relatively small amount of DNA per cell is compared to the number of chromosomes or linkage groups determined by both genetic and cytological observations. In our studies of DNA replication, the yeast *Saccharomyces cerevisiae* was used. Numerous genetic studies have established 18 linkage groups for the diploid stage of the yeast life cycle; this map may comprise approximately one-half of the total genetic information carried by this species. Although the yeast chromosomes are difficult to detect microscopically, 18 chromosomes have been seen at certain stages of the life cycle.

Moreover, in studies of *S. cerevisiae*, either haploid or diploid stages of the life cycle can be used for experiments. The vegetative cells of this organism are primarily diploid when isolated from nature. However, under unfavorable growth conditions, these organisms will sporulate forming two mating types of haploid ascospores by meiosis. Four ascospores in random arrangements are generally formed. As soon as germination of ascospores occurs, the two mating types, if allowed to remain in close proximity, will conjugate to form a diploid clone. Although the haploid cells can be propagated by mitotic division, in nature the haploid state is probably only of brief duration.

In diploid *S. cerevisiae*, only one method proved experimentally feasible for tracing the course of DNA replication. Although induced enzyme synthesis occurs at certain time intervals throughout the yeast cell cycle, DNA replication occurs only during the first quarter

of the cycle. This implies that a sequential transcription unrelated to active DNA replication could be occurring. In bacteria, on the other hand, both DNA replication and induced enzyme synthesis had been observed to proceed together during the entire cell cycle under the growth conditions normally used for such experiments. Phage transduction also cannot be used for yeast replication experiments because no virus specific for yeasts has been found. Therefore, investigating DNA replication by pulse mutagenesis with nitrosoguanidine was the logical choice. Nitrosoguanidine has been shown to be very effective in inducing mutation in regions of actively replicating DNA in bacteria and in inducing mutations in eukaryotic cells.

Preliminary investigations established that haploid yeast strains could not be used for the proposed experiments. Neither a distinct response in forward mutagenesis nor synchronous growth could be obtained in haploid yeast strains under the necessary experimental conditions. In addition, a back-mutation system producing prototrophic haploids could not be used, since it had been previously observed that these reversions were primarily the result of suppressor mutations. Therefore, the relationship between DNA replication and induced mutations was primarily investigated in auxotrophic heteroallelic diploid strains, which could be synchronized and which could produce a clearly defined genetic response by prototroph production after mutagen treatment.

The prototrophs generated by treating such heteroallelic diploid cells were produced mainly by mitotic non-reciprocal recombination or gene conversion. Most recent models of gene conversion postulate that some form of DNA replication is necessary to complete the process of intra-allelic recombination. Consequently, use of nitrosoguanidine, which is very specific for regions of DNA replication, could indicate whether active DNA replication is important in gene conversion.

In this investigation an examination was made of the relationship between DNA replication and mutagenesis (as shown by mitotic gene conversion) by: 1) examining the effects of mutagenesis in logarithmic and stationary phase; and 2) determining whether amino acid starvation had the same effect upon both DNA replication and mutagenesis in yeast as in the bacteria. Additional information could be gained on the effects of nitrosoguanidine-mediated pulse mutagenesis in synchronous cultures by learning: 1) whether the effects of mutagenesis occurred primarily during the period of DNA replication; and 2) whether a displacement in time of different genetic markers on the same chromosome could be observed, indicating a timed sequence of replication.

REVIEW OF LITERATURE

In *S. cerevisiae*, genetic mapping by linkage and tetrad analysis produced 14 linkage groups which were estimated to contain about one-half the total genetic information (Mortimer and Hawthorne, 1966a). Evidence for four additional chromosomes will be reported soon (Hawthorne, 1970). Linkage relationships may be determined by segregation of known centromere-linked genes that were deliberately included in hybrid crosses. Second division segregation frequencies may also be used to determine centromere linkage.

In addition, the specific location of a marker on a chromosome can be determined by trisomic analysis and mitotic recombination or segregation. In trisomic analysis, a hybrid cross of mutant by disomic (A/A/a) will produce 4:0 and 3:1 segregations, if the marker is on the disomic. If a 2:2 segregation is obtained, then the marker is not on the disomic chromosome.

Subsequently, radiation-induced mitotic segregation in heterozygous diploids may be used to determine the marker sequence on a chromosome arm. These events were detected in colonies of heterozygous diploids by sectoring, which operated primarily through mitotic cross-over for two or more adjacent genes. For a single gene, non-reciprocal sectoring was observed. Linkage for markers on a given chromosome arm was shown by the percent coincidence of sectoring of

the markers. The frequency of coincident sectoring determined the degree of linkage and indicated the position of a given marker. For our studies we used six markers on the left arm of chromosome VII. The left arm of chromosome VII so far has been shown to carry seven known loci. For these loci, the frequencies of sectoring were linearly related to their distance from the centromere by mitotic segregation.

Chromosome VII

	18.7	42.3	22.4	18.3	8.5	54.5	
a	le_1	tr_5	ac_2^r	met_{13}	ty_3	ly_5	$ad_{5,7}$	0
α	LE_1	TR_5	AC_2^s	MET_{13}	TY_3	LY_5	$AD_{5,7}$	

These distances were confirmed by tetrad analysis (Nakai and Mortimer, 1969). Moreover, tetrad analysis is used in fungal genetics to determine meiotic linkage between any two loci A and B after crossing AB by ab. After sporulation, the following possible combinations of ascospore markers can occur: 1) parental ditype (PD) AB, AB, ab, ab; 2) non-parental ditype (NPD) Ab, Ab, aB, aB; 3) tetratype (T) AB, Ab, aB, ab. Linkage is indicated if PD:NPD is greater than one (Mortimer and Hawthorne, 1966b).

Furthermore, fine structure mapping of genes in *S. cerevisiae* can be carried out by allelic recombination studies of heteroallelic diploids. These diploids carry two independent mutants in repulsion at a single locus ($a_1+/+a_2$). Normal 2:2 segregation yielded two

(a_1+) spores and two (+a_2) spores in each tetrad. Prototrophic revertants (++) can also be isolated. Most of these prototrophs resulted from 3^+:1a segregations (gene conversion or non-reciprocal recombination), but some were also the product of reciprocal recombination between the input alleles. The meiotic prototroph frequency induced by sublethal doses of x-ray was directly proportional to x-ray dose. The value of the slope depended on the pair of heteroalleles that were compared. For example, in the arg_4 locus, three mutant alleles mapped in linear order: ar_{4-4}, ar_{4-5}, ar_{4-2}. The slope of ar_{4-2}/ar_{4-4} was greater than ar_{4-2}/ar_{4-5}. The distance between these loci is proportional to the value of the slope. The above slopes of homoallelic mutant alleles all approach one (Manney and Mortimer, 1964). The mechanisms of non-reciprocal recombination will be discussed later.

In addition to genetic mapping, transcriptional events in bacteria can also provide information about the manner in which the bacterial genes are replicated. Several experiments with synchronous cultures of bacteria have indicated that a relationship exists between replication of genes and their mapping position and transcription. In these experiments, periodic induced enzyme synthesis can be directly linked through sequential transcription to sequential replication of the genome.

In bacteria, the maximal rate of induced enzyme synthesis was

observed to be a function of the number of gene copies coding for that enzyme in the cell. The observed rate of induced enzyme synthesis reflected the number of gene copies as well as repressor and effector levels. This potential rate of enzyme synthesis doubled in the cell cycle depending on the map position of the given gene. The genome of E. coli was continuously available for transcription except in the region of active DNA replication (Kuempel, Masters and Pardee, 1965). There are some exceptions to continuous transcription in the bacteria. For example, spore outgrowth in Bacillus subtilis was observed to be discontinuous (Masters and Pardee, 1965).

In S. cerevisiae, discrete changes in rates of induced enzyme synthesis were also noted, although a direct relationship to the replication of the yeast genome was not established. In contrast to continuous replication of the bacterial genome, the replication of the yeast chromosomes occurs during the first 27% of the cell cycle (Williamson, 1965), but the periodic transcription of different genes continues throughout the yeast life cycle.

In addition to periodic transcriptional events, DNA synthesis also oscillated in yeast. Williamson (1966) described nuclear events during vegetative growth in S. cerevisiae. The first 25% of the cell cycle was occupied by DNA replication and simultaneous bud formation. A short period of mitosis was followed by nuclear migration and fission. The final step was cell division. With the exception of an experimental

hybrid of *Saccharomyces dobzanskii* x *Saccharomyces fragilis*, DNA synthesis occupied about 10-30% of the cell cycle of various yeast strains (Williamson, 1965, 1966; Bostock, 1970). The hybrid strain exhibited DNA synthesis during 75% of the cell cycle (Halvorson *et al.*, 1964). Mitochondrial DNA synthesis was also shown to occur during a discrete part of the cell cycle, but was timed differently than the nuclear DNA synthesis in cultures derepressed for biosynthesis of mitochondria (Smith *et al.*, 1968).

Besides cyclic patterns of DNA synthesis, the DNA polymerase of *S. cerevisiae* also displayed a well-defined peak pattern in synchronous culture. Polymerase activity was at a maximum before the beginning of DNA synthesis and then declined during replication to a minimum. The peaks were not directly related to DNA replication because cyclic DNA polymerase activity was seen in cultures that were blocked in DNA synthesis and cell division by x-ray treatment (Eckstein, Paduch and Hilz, 1967).

Mitchison (1969) suggested several patterns of periodic enzyme synthesis in synchronous cell cultures of bacteria and yeasts. There were step and peak patterns as well as continuous exponential and continuous linear patterns. The difference between the continuous exponential and the linear pattern required further explanation. The exponential pattern reflected a continuous synthesis of an enzyme. In contrast, the linear pattern was marked by a sudden change in slope to

a new linear rate of synthesis indicating changes in rates of transcriptional events or changes in the number of gene copies per bacterium. Most rates of enzyme synthesis were classified as step patterns. These patterns were proposed to reflect changes in enzyme stability or a change from inactive enzyme to active enzyme. Examples of periodic enzyme synthesis in yeast are described below.

Most of the enzymes studied in synchronous cultures of various species of *Saccharomyces* had discontinuous patterns (Mitchison, 1969), but linear patterns of induced enzyme synthesis were detected in *Schizosaccharomyces pombe*. Mitchison and Creanor (1969) maintained that these linear patterns could be related in the latter yeast species to a delayed transcription of the genome during the same cell cycle.

A model has been proposed to account for periodic enzyme synthesis in *S. cerevisiae*. Tauro *et al.* (1969) postulated that nuclear control was exerted over enzyme synthesis by sequential transcription occurring at discrete time intervals during the yeast cell cycle. A series of experimental observations supported their hypothesis. First, the level of inducer did not produce any changes in the timing of enzyme synthesis. This observation seemed to exclude the argument that periodic enzyme synthesis was due to an unstable regulatory system. Secondly, gene products were expressed as a function of their position on the genetic map. Thirdly, closely linked structural

genes exhibited changes of enzyme synthesis at about the same point in the cell cycle. Fourthly, the induced level of enzyme synthesis in allelic genes was a function of gene dosage. Fifthly, unlinked non-allelic structural genes were transcribed and expressed in discrete time periods. Also each structural gene had a particular time of expression depending on chromosome location. The Halvorson group concluded that their data supported models of end-to-end ordered transcription as well as models of regional or bidirectional transcription of individual chromosomes.

Unlike DNA and induced enzyme synthesis, total RNA or protein synthesis does not show abrupt changes in rates of synthesis during synchronous growth. Schweizer, MacKechnie and Halvorson (1969) suggested that continuous RNA synthesis was the result of an ordered transcription of unlinked or redundant cistrons coding for RNA synthesis.

For the thesis problem, the effects of amino acid starvation upon macromolecular synthesis in S. cerevisiae were of interest. For a haploid strain of S. cerevisiae after amino acid starvation, total DNA was observed to be synthesized resulting in a total increase of 11-17% under conditions where net RNA and protein synthesis stopped (Wehr, 1970). The increase in total DNA content of the cells after amino acid starvation suggested that cells engaged in DNA synthesis at the introduction of starvation conditions completed replication of the genome but did not initiate new rounds of synthesis. These results

were qualitatively similar to those obtained in bacteria with some specific differences especially under starvation conditions (Maaloe and Kjeldgaard, 1966). Some of these differences in *S. cerevisiae* were thought to be due to the large amino acid pools which were found in yeast.

There is some evidence that yeasts and other eukaryotes may be more limited than the bacteria in completion of DNA synthesis after amino acid starvation conditions are imposed. In yeast, Grossman, Goldring and Marmur (1969) have shown that there are only minor increases in nuclear DNA after inhibition of protein synthesis by amino acid starvation, cycloheximide, or by shifting temperature-sensitive mutants deficient in protein synthesis to non-permissive temperatures. In contrast, mitochondrial DNA showed major increases during inhibition of protein synthesis comprising 5-15% of the total DNA. These increases were dependent upon the choice of carbon and energy sources.

DNA synthesis in some eukaryotic cells has also been observed to have requirements for concurrent protein synthesis. In HeLa cells, the lysine-rich and arginine-rich histone fractions were made during DNA synthesis, but the synthesis of all the histone fractions was only partially dependent on DNA synthesis (Sadgopal and Bonner, 1969; Hodge *et al.*, 1969). In the fungi the presence of histone fractions was only recently established. Histone fractions were found

in both *Physarum polycephalum* and *Allomyces arbuscula* (Stumm and van Went, 1968; Mohberg and Rusch, 1969). Tonino and Rozijin (1966) also found basic proteins associated with the nuclear fractions of *S. cerevisiae*, but the lysine-rich fractions typical of histones in the higher cells were not found. These data on yeast histones were complicated by the incomplete separation of the nuclear fractions from the other cell components.

Gene conversion was described previously as a technique useful for mapping sites within a gene. However, experiments detecting simultaneous conversion events in different genes have also been performed. In synchronized yeast cultures, Esposito (1968) reported that both induced intragenic recombination (gene conversion) and intergenic recombination (reciprocal) frequencies were at a maximum before the onset of DNA replication and declined to a minimum for a number of heteroallelic loci during replication. Furthermore, Parry and Cox (1968) found that cell death and induced intergenic recombination occurred during the period of DNA synthesis. However, induced intragenic recombination frequencies were maximal toward the end of total DNA synthesis in synchronous cultures. Both of these studies suggested that DNA synthesis might be related to gene conversion.

Historically, gene conversion was first discovered in yeast by Lindegren (1953). His discovery was soon confirmed by others in studies of *Neurospora* and *Sordaria* (Mitchell, 1955; Olive, 1959). As

a result of genetic analysis of allelic recombination in several fungi, recombinational events were attributed to 3:1 segregations or non-reciprocal recombination (Fogel and Hurst, 1963; Stadler and Towe, 1963; Kruszewska and Gajewski, 1967).

Besides further development of the definition of gene conversion, models of recombination are also presented to deal with the relationship between gene conversion and reciprocal recombination. In addition, mechanisms are provided by which reciprocal and non-reciprocal recombination events can occur separately or together. For our experiments the role of DNA synthesis in these models was of interest.

Recombination can occur both in a reciprocal and a non-reciprocal manner. A cross-over mechanism can produce recombination using a breakage-reunion of two of the four strands of the meiotic bivalent. Recombination can also occur without crossing-over. This non-reciprocal form of recombination differs in that the reciprocal products of recombination are absent or rare. Recombination within a cistron may be non-reciprocal for one or both the input alleles 1 and 2 in a diploid represented by $\frac{+1+b}{a+2+}$, where 1 and 2 are two alleles of independent origin within a locus, and a and b are linked proximal and distal markers relative to the centromere. Alleles 1 and 2 or both may segregate $3^{+}:1^{-}$ or $1^{+}:3^{-}$ while all the other segregate in the classical Mendelian $2^{+}:2^{-}$ fashion. The $1^{+}:3^{-}$ segregation gave double

mutant segregants. The flanking markers a and b may or may not be recombined (++) or (ab) in the convertant strand or chromatid (Hurst and Fogel, 1967). Irregular $4^{+}:0^{-}$ and $0^{+}:4^{-}$ ratios are also found. However, Roman (1963) has shown that these aberrant segregations probably originate from other causes in yeast. Polarization or polarity was observed in many gene conversion studies. Polarization has been defined as the consistent excess of proximal allele (1+) convertants over distal (+2) convertants within a locus (Hurst and Fogel, 1967).

Recombination processes have been linked to either active DNA replication or dark repair mechanism in several models. The copy-choice model, one of the earliest proposed mechanisms, proposed to link non-reciprocal recombination with DNA replication. Homologous chromosomes were paired at certain points at the time of replication. The replicase copied one parental strand of the chromosome in the first region. In the region of effective pairing, the replicase could switch strands and copy from a similar strand in a homologous chromosome. The switch did not need to be reciprocal. This hypothesis could explain the observed coincidence of reciprocal and non-reciprocal recombination in meiosis. The direction of replication could explain the polarity in gene conversion. Replication was assumed to occur at the chromatid level (Freese, 1957).

However, later observations did not fit the copy-choice mechanism. The homologous chromosomes were not paired at the time

of replication because DNA replication occurred prior to meiosis in eukaryotic cells. Gene conversion could occur without cross-over in flanking markers (Roman, 1963). In some phages, genetic recombination occurred without genome replication, although repair replication could not be excluded (Kellenberger, 1961; Meselson and Weigle, 1961).

In contrast to the copy-choice model, the models of gene conversion by Whitehouse (1963) and Holliday (1964a, 1968) were based on the dark repair mechanisms found in bacteria. Both models had several features in common. One of the main features was a repair enzyme that recognized mismatched bases in "hybrid DNA" sequences and exised them. After repair the mismatched region became homozygous. Repair was carried out in several steps. First, single strand breaks were produced in two homologous chromatids; second, the free ends of each broken strand were released, followed by pairing (H-bonding) with the complementary unbroken strand of the other chromatid (if a segregating site fell within this region of hybrid DNA, improper pairing could result, which could be corrected in the subsequent steps); third, a segment of the strand, which included the improperly paired site, was exised; fourth, excision was followed by repair replication including rejoining by DNA ligase.

The models of Whitehouse and Holliday cited above were able to

explain many of the experimental observations pertaining to gene conversion and reciprocal recombination. Both models could generate non-reciprocal recombination without or with outside marker exchange. Gene conversion and reciprocal recombination occurred after genetic replication at a time when the chromatids were visibly paired during meiosis.

Map expansion was also explained by the models of Whitehouse and Holliday. In map expansion, recombination between mutant alleles several intervals apart in a locus was consistently greater than the sum of the intervals. However, in normal reciprocal recombination the recombination frequencies were equal to the sum of the intervals so that map units could be calculated. Both gene conversion and reciprocal recombination gave linear relationships of mutant alleles in a locus. To explain map expansion, Holliday proposed that the distance between two mutant sites on a fine structure map of a gene depended not so much on the frequency of a recombinational event occurring between the sites, but rather on the degree of inhibition upon the processes of pairing of hybrid DNA by the mutant sites themselves.

The polarization phenomena found in certain fungi were hard to explain within the context of the Whitehouse-Holliday models. Initially, polarization was intensively investigated in spore color mutants of Ascobulus exhibiting 2:6 segregations. The frequencies of such asci

were used to map mutants of a series into a linear order. A series could then be subdivided into polarons. In each polaron, mutants could be assigned a left to right placement within the polaron consistent with the recombinational map of the mutants, so that the mutant on the left was present twice as frequently as the mutant on the right. The mutant alleles within such a polaron could recombine by gene conversion. However, both non-reciprocal and reciprocal recombination could occur between different polarons (Lissouba _et_ _al_., 1962).

Subsequently, Whitehouse proposed fixed opening points to account for polarization in his model. These points were postulated to act as points of recombinational discontinuity at the ends of genes or cistrons. Thus gene conversion would be maximal near an opening point and then would decline resulting in polarization (Whitehouse, 1966). Murray (1969, 1970) found genetic evidence in _Neurospora_ for such discontinuities between contiguous genes as well as reversal of polarization in different genes. However, the recent evidence for polarization has become contradictory. Rossignol (1969) reported no simple polarity relationships from left to right in gene 75 of _Ascobulus_. But in previous work, Lissouba _et_ _al_. (1962) had proposed polarity in other genes of _Ascobulus_. Stadler and Kariya (1969) have recently reviewed their own findings as well as those of others to illustrate the contradictory data for polarization.

The recent experiments of Stadler and Kariya as well as those

of Fogel and Mortimer (1969), have indicated that gene conversion may well be a repair process in which mismatched bases in hybrid DNA are exised and replaced with identical information contained in nucleotide strands hundreds of nucleotides in length from a corresponding section of a non-sister homologue. Enzymatic studies have also shown complete fidelity in transfer of information between homologues. Zimmerman (1968) found that wild type and prototrophic enzymes were identical in nine different properties measured in studies of threonine dehydrase activity.

Fogel, Hurst and Mortimer (1970) have shown how conversion operated upon two different mutant alleles of the same gene. In these gene conversion events, alleles that were widely separated in a locus produced primarily single-site conversion. If the mutant alleles were close, double site conversions predominated. Intermediate distances between alleles produced both single and double site conversions. Thus double site conversions were a function of physical separation between mutant alleles. Moreover, symmetrical co-conversion may extend to include heterozygous sites in adjacent cistrons encoding different polypeptides. In yeast, therefore, there are no discontinuities observed at the initiation or termination sites of single genetic units or cistrons that would correspond to the "fixed opening points" mentioned previously. Thus, the fixed opening points responsible for polarity may lie outside the locus containing the mutant alleles.

In addition to the repair and copy-choice models of gene conversion, there are models postulating additional local syntheses of DNA and non-reciprocal breakage and reunion exchanges between homologous chromatids. Generally these models implied fixed pairing regions for homologues rather than fixed opening points as in the dark repair model.

In the recent model proposed by Stahl (1969), additional local syntheses of DNA occurred in homologous regions of paired chromatids. Two cross-overs took place between non-sister arcs of the "sex circles" by a breakage and reunion with formation of short heteroduplex overlaps and survival of only one of the two recombinant arcs. A restriction had to be imposed in the second exchange. The product of the cross-over which was retained was the one permitting the reconstruction of a continuous pair of chromatids.

The model of Paszewski (1970) was also quite similar to the Stahl model. Gene conversion was produced by breakage and reunion and by partial degradation of a nucleotide chain from one chromatid, followed by its resynthesis with a chain from the homologous chromatid as template. Gene conversion would result if this newly synthesized chain and template were then inserted in the first chromatid displacing the previously unbroken chain of the latter. The single-stranded gap left in the other chromatid would then be repaired.

Both the Stahl and Paszewski models were criticized recently

for failure to account for many of the experimental observations regarding gene conversion in the fungi. One of the critical points was that the polarity proposed by the Stahl model relied on fixed pairing regions. The pairing mechanism would imply that gene conversion frequencies would be maximal in the middle of a pairing segment with a decline toward the ends. This did not agree with the experimental observations in the fungi. In these organisms, the observed polarization effects actually seemed to start from the ends of the genes. Also map expansion could not be explained by either the Stahl or the Paszewski models (Holliday and Whitehouse, 1970). In S. cerevisae, Fogel et al. (1970) and Hurst, Fogel and Mortimer (1970) did not find that the recombination data fitted either the Holliday or the Stahl models. Both models, however, were able to generate reciprocal recombination events 50% of the time in association with meiotic gene conversion.

Boon and Zinder (1969) have proposed another model of recombination based on work with phage f_1. In this model recombination frequencies are also a function of DNA replication processes. One parent and one recombinant are generated in a single recombinational event between two DNA duplexes. Basically three events are necessary for recombination to take place: 1) an initiation which requires breakage and joining of strands (single- and double-stranded breaks in the two parental chromosomes); 2) a replication which proceeds through a

biparental replication fork generated in initiation; and 3) a return event also involving breakage and joining of strands dependent on the type of DNA molecule (linear or circular). The model implies that the presence of a replicating structure could have an influence on the location of the return point relative to the position of the initiation point. In the f_1 phage system, using circular DNA molecules, a return event must involve breakage of the same three strands broken during initiation, and the return event must occur before the replicating fork reaches the initiation point. In other words, the return event could take place ahead of the initiation fork or could alternatively be an integral part of the active replication mechanism.

Application of the above model to recombination of eukaryotic chromosomes poses several problems not described by Boon and Zinder. For example, a large number of initiation points have been demonstrated in eukaryotic chromosomes. Also it is not known whether eukaryotic chromosomes are linear or circular.

In addition to the literature dealing with the relationship between meiotic gene conversion and models of recombination, there is an equally important body of work dealing with gene conversion during vegetative growth (mitotic gene conversion). As shown by Roman (1963), mitotic gene conversion was characterized by aberrant segregations such as 1:3 and 3:1 segregations in contrast to the 2:2 segregations that were characteristic of reciprocal recombination.

Non-reciprocal recombination could not be proven until other deviations from the 2:2 ratio were excluded. Other processes producing aberrant segregations included variation in chromosome number, mutation at the mating type locus, extra division in the ascus, mitotic reciprocal recombination, and modifying genes. In yeast, the frequency of 3:1 and 1:3 segregations of wild-type alleles versus mutant alleles was generally equal in either direction. No obvious correlation was found between the distance of the locus from the centromere and the frequency of gene conversion.

Mitotic gene conversion was studied by mitotic segregation of heterozygous markers as well as in heteroallelic markers. For example, Roman (1963) studied 29 mutant alleles of the ad_6 locus. Any two mutants in repulsion at this locus were adenine-requiring; either homoallelic or heteroallelic strains could be produced. For the heteroallelic combination ad_{6-2}/ad_{6-1} the corresponding homoallelic strains were ad_{6-1}/ad_{6-1} and ad_{6-2}/ad_{6-2}. In these tests the adenine requiring recombinants would not be isolated, and only the prototrophic recombinants could be detected on the selective media. The normal test for mitotic gene conversion compared the different rates of reversion to prototrophy in auxotrophic segregants isolated from the prototrophic recombinant. In these tests, the haploid auxotrophic segregants were mated with parental tester strains carrying either mutant allele ad_{6-1} or ad_{6-2}. The spontaneous frequency of reversion to prototrophy of

of the homoallelic strain and mutations of the haploid strains was much lower (10^{-7} to 10^{-8}) than the frequency of the heteroallelic reversion (10^{-4} to 10^{-5}). In analysis of revertant prototrophs from heteroallelic loci, the product of reciprocal recombination, the double mutant, was found in the same daughter cell with wild type alleles. Absence of the double mutant product after recombination indicated that prototrophs resulted primarily from mitotic gene conversion at the locus.

The coincidence of mitotic reciprocal recombination with mitotic gene conversion at a locus can be checked by using recombination of closely linked and flanking heterozygous markers. Roman and Jacob (1958) reported that mitotic reciprocal recombination and mitotic gene conversion do not necessarily occur at the same time in heteroallelic diploid yeast strains. Kakar (1963) reported that there was a coincidence in the occurrence of mitotic gene conversion at the heteroallelic locus (is_{1-1}/is_{1-2}) with reciprocal recombination of outside markers. Fogel and Hurst (1963) found a marked coincidence in recombination frequencies for linked outside markers and mitotic gene conversion, but conversely not every gene conversion event was associated with outside marker recombination.

Radiation and a wide variety of mutagens have been used by many different workers to induce mitotic gene conversion and mitotic recombination. X-ray and ultraviolet radiation (UV) were used to

induce both mitotic gene conversion and mitotic recombination (Roman and Jacob, 1957; Mortimer, 1957; Schwaier, 1968). Mitomycin C, EMS, and other alkylating agents were also similarly used (Holliday, 1964b; Yost, Chaleff and Finerty, 1967).

Mitotic reciprocal recombination has been induced in heterozygous yeast strains with nitrous acid (NA), diethylsulfate, 1-methyl-3-1-nitrosoguanidine (NMG), N-nitroso-N-methylurethane (NMU), and other nitrosamides. Zimmermann, Schwaier and Laer (1966) tested for mitotic recombination by induction of homozygosis in ad_8hi_8 heterozygous loci. These two markers were in linkage on the same chromosome arm with AC_4^r. After induction the homoallelic strains were tested for concommitant segregation of heterozygous markers on other chromosomes. After tetrad analysis, the results indicated normal 2:2 Mendelian segregation of these unlinked markers in the homoallelic strains. Tetrad analysis of the linked heterozygous markers (hi_8, ad_8, and AC_4^r) showed that the strain was homoallelic for these markers. With the exception of NA, all of the mutagens induced similar frequencies of mitotic recombination and mutation. The mutation frequency of the ad_8 locus could be measured separately by working with ad_2 homozygotes showing high frequencies of reversion or complementation. The stable ad_2 homozygotes were excluded because these could be due to either mitotic gene conversion, mutation, or mitotic recombination.

Zimmermann and Schwaier (1967) were also able to induce mitotic gene conversion with nitrosamides including NMG at hetero-allelic loci in doses producing little lethality. They reported that the frequency of homozygosis taken as an index of mitotic recombination was not correlated with the spontaneous or nitrosamide-mediated gene conversion in four out of five loci linked with the heteroallelic is_{1-a}/is_{1-b} locus of S. *cerevisiae*. The particular nitrosamide for these studies was NMU. In all cases after induction with this compound, the frequency of homozygosis of the heterozygous linked markers (frequency of mitotic recombination) was very low compared to the frequency of mitotic gene conversion of the is_1 locus. The is_1 mutant alleles shown above have also been used by other workers in studies of induced allelic recombination in yeast (Roman and Jacob, 1958; Kakar, 1963; Sherman and Roman, 1963).

Tetrad analysis of the is_1 prototrophic recombinants indicated that mitotic gene conversion was the primary event at this locus, because the double mutant segregant ($is_{1-a,b}$) could not be isolated. The conversion event occurred without recombination of outside markers in 16/23 convertants. Also, both of the mutant alleles (is_{1-a} and is_{1-b}) survived at equal frequencies. Mutational events at the heteroallelic locus could be excluded because of the stability of the is_{1-a} and is_{1-b} alleles when placed in either haploid or homozygous recessive diploid strains. Furthermore, Ebbs (1967) has shown that

NMG at pH 5 in 0.2M acetate buffer induced primarily mitotic gene conversion at the heteroallelic locus trp_{5-1}/trp_{5-2} of *S. cerevisiae*. Similar studies of mitotic segregation in heterozygous yeast strains carrying the AD_7/ad_7 locus also indicated that non-reciprocal recombination was the main event in mitotic recombination events.

To produce specific induction of mitotic gene conversion and other mutations, nitrosamides form one or more species of alkylating agents *in vivo* as well as *in vitro*. The main sites of action in the cell include the N-7 of guanine in the nucleic acid fraction, in addition to the N-1 and N-3 positions of histidine and sulfhydryl groups in the proteins (Miller and Miller, 1966). Direct *in vitro* reactions of diazomethane, one of the proposed intermediates in methylation of nucleic acids, yielded methylated products as well as cleavage of the phosphodiester bonds. Methylation of salmon sperm DNA with diazomethane in ether yielded 7-methylguanine and 3-methyladenine derivatives of the deoxyribonucleotides (Kriek and Emmelot, 1964). Holy and Scheit (1966) found that alkaline diazomethane-ether solutions and diazomethane in ether at 0 C reacted with dinucleoside phosphates to produce ribonucleoside 2'(3') methyl phosphate, ribonucleoside 2'(3') cyclophosphate as well as methylated ribonucleotides. These reactions indicated that diazomethane was also active in cleavage of the phosphodiester bonds of the dinucleosides which *in vivo* presumably would result in single- or double-stranded breaks in DNA.

Other *in vitro* reactions of NMG also suggested diazomethane and carbonium ion as the main forms of reaction intermediates. NMG reacted at pH 5.5 to form 1- and 3-methyladenine from adenosine and AMP. Another reaction product, 7-methylguanine, was obtained by NMG with either adenosine, GMP, or xanthine (Rau and Lingens, 1967). Reactions of nucleosides, nucleotides, and bases with ^{14}C-CH_3-1-nitroso-3-nitroso-1-methyl-guanidine yielded the same methylated purine derivatives by measuring methyl group incorporation (Lingens, Rau and Suessmuth, 1968). Production of nitrous acid in reactions of NMG with DNA at pH 5.5 indicated deamination reactions. Craddock (1969), however, found no evidence for *in vitro* deamination by NMG.

The *in vivo* reactions of NMG also gave some evidence for the proposed operation of diazomethane intermediates in studies of NMG-mediated mutagenesis and lethality in bacteria. The tritiated methyl group labeled NMG was incorporated into bacterial DNA. A striking parallel between the percent NMG uptake and the percent mutation rate was observed in this experiment. In addition, survival showed first order kinetics. Resistance to NMG could be induced through alterations of cellular permeability or uptake mechanisms, but not through methylation of all available sites in the DNA. Secondary effects of NMG also occurred at the transcriptional and translational levels. Transcription initially seemed to be affected by

the methylation of DNA rather than RNA (Suessmuth *et al.*, 1969). *In vivo* and *in vitro* protein synthesis, however, was directly affected at the ribosome level by NMG (Lingens *et al.*, 1967).

In addition to methylation of DNA, NMG also produced scission of DNA strands. Olson and Baird (1969) detected single-strand breaks in bacterial DNA after mutagenesis with NMG. Labeled DNA of *E. coli* was subjected to alkaline sucrose density centrifugation after mutagenesis. At first, the cells showed single-strand breaks by a shift of the single-stranded bands toward the denser region of the gradient, indicating smaller pieces. However, if the NMG-treated cells were incubated in broth for three hours before DNA extraction and density gradient centrifugation, the experimental DNA bands shifted back to the gradient region characteristic of the untreated DNA control. This shift indicated that the single-stranded short sections had been rejoined into longer sections. No loss in colony-forming ability was observed during the three hour incubation of cells treated with NMG. These single-stranded breaks in the DNA of NMG-treated cells also appeared faster than would be expected by loss of methylated bases through formation of apurinic acids.

Cerda-Olmedo and Hanawalt (1968) have also proposed a diazomethane intermediate in studies of NMG induced lethality and mutagenesis in normal and excision-defective ultraviolet-sensitive (UVS) strains of *E. coli*. Lethality and mutagenesis were found to be

pH dependent with a maximum at pH 5 to pH 5.5. The UVS mutant showed greater cell death during NMG treatment but exhibited a similar level of induced mutations as the cells with normal dark repair mechanisms. The difference in lethality between the UVS and the wild type strains indicated that NMG induced both repairable lethal lesions and non-repairable mutagenic lesions in bacteria with wild-type dark repair mechanisms. Cerda-Olmedo and Hanawalt (1967) also observed repair synthesis after treatment of bacteria with NMG. Tritiated bromouridine (^{3}H-BU) was incorporated into DNA prelabeled with ^{14}C-thymidine followed by CsCl density gradient centrifugation. Further incubation of bacteria labeled with ^{3}H-BU in medium containing cold BU showed that eventually the repaired DNA was able to replicate and produce the bands of hybrid DNA usually observed in semi-conservative replication.

In _S. cerevisiae_, the effects of NMG-induced back mutations were studied in an adenine-requiring haploid strain carrying a mutation at the ad_{6-45} locus. The conditions of maximal induction of reversion by NMG were determined. Analogous compounds lacking the nitroso group of NMG did not produce any of the single base changes associated with mutation by NMG. It was again suggested that diazomethane was the reaction intermediate in mutagenesis (Marquardt, Zimmermann and Schwaier, 1964). In another study, pH dependence of the same system was shown; maximal induction of reversion

occurred from pH 4-6. In contrast to a spontaneous frequency of $1/10^7$, maximal induction of revertants by NMG yielded 84-89% mutation. Chemical tests of the nitrosamide solutions established the presence of nitrous acid under the experimental conditions. It was proposed that both nitrous acid and diazomethane may be involved in mutagenic action of the nitrosamides (Zimmermann, Schwaier and Laer, 1965).

Moreover, NMG mutagenesis under conditions of minimal lethality was shown to be highly specific for the replication points of bacterial chromosomes. This specificity suggested a new means of mapping the course of DNA replication.

The first chromosome mapping with NMG was attempted in *Staphylococcus aureus* using antibiotic markers (Altenbern, 1966, 1968). These investigations, however, were hampered by lack of adequate synchronization procedures and lack of a complete genetic map. Subsequently, Cerda-Olmedo, Hanawalt and Guerola (1968) found that the majority of revertants induced by NMG in auxotrophic strains of *E. coli* TAU-bar were located in the replication point region of the chromosome in studies of cultures that were chromosome-aligned by animo acid starvation. NMG treatment of successive samples from one of the cultures showed a maximum of a given type of mutant at the time the corresponding gene was replicated. A replication map describing the pattern of replication of genes was

constructed from these data. This map agreed with the genetic maps obtained by interrupted conjugation and transduction experiments. Pulse mutagenesis by NMG also located the origin of replication at a fixed portion of the chromosome and determined a clockwise direction of replication.

Ward and Glaser (1969a) obtained results similar to those of Cerda-Olmedo _et al._ using sequential pulse NMG mutagenesis in synchronized cell cultures of _E. coli_ B/r. Auxotrophic strains of B/r were used to measure reversion rates induced by NMG. If the rate of reversion of a given auxotrophic marker was a function of cell age, the cell age at which the given gene was replicated could be determined as shown in the previous experiment.

The two experiments differed primarily in the physiological conditions and the strains. In the experiments of Ward and Glaser, synchrony was induced by the membrane elution technique which permitted continuous growth of the cells during the procedure while permitting elution of a cell population of the same cell age. In the experiments of Cerda-Olmedo _et al._, the cells were allowed to complete their rounds of DNA replication by using amino acid starvation. Ward and Glaser (1969b) also found that multiple growing points could be detected by the pulse mutagenesis method. NMG-mediated forward mutagenesis can also be used to demonstrate the location of replication forks on completed chromosomes (Botstein, 1969). In this

experiment, non-random map distributions of forward mutations were obtained in stationary phase cultures; however, random map distributions were obtained in log phase cultures.

Effects of NMG-induced mutagenesis have also been studied in the fungi. Malling and de Serres (1970) observed the effects of NMG by using a system of point mutations in the ad_{3A} and the ad_{3B} loci of *Neurospora crassa*. Multilocus deletions in the ad_3 region and recessive lethal mutations of the whole genome were also used. Point mutations were induced by NMG with high efficiency in the ad_3 locus at doses of low toxicity. Forward mutagenesis was proportional to the square of the time of treatment with NMG. The spectrum of complementation patterns among ad_{3B} mutants induced by NMG was compared to similar spectra induced by other mutagens. It was proposed that the majority of NMG-induced mutants could have a GC base pair at the mutant site.

Using NMG to induce dose-response curves for the ad_{6-45} back-mutation system, Schwaier (1965) demonstrated an initial sharp increase in response followed by a drop and gradual increase in revertant number at higher mutagen concentrations. The initial sharp peak was correlated with a drastic drop in survival, which then leveled out for increasing concentrations of NMG. These dose response data were interpreted to show the existence of two populations of cells with different sensitivity to NMG. However, Lingens

and Oltmanns (1966) did not find populations of different sensitivity in similar studies of forward NMG mutagenesis in yeast. They did find that most of the mutations induced by NMG were leaky and that the mutants had poor viability.

Suessmuth and Lingens (1969b) found a direct correlation between the rate of revertant production and the rate of ^{3}H-methyl-NMG uptake at pH 5-6 for both E. coli and S. cerevisiae. Survival was also maximal at pH 4-5. Analysis of the methylated DNA after treatment with labeled NMG indicated that incorporation of methyl groups was maximal from pH 5-6. Moreover, in studies of methionine prototrophic revertants, Loprieno and Clarke (1965) found that NMG induced primarily suppressor mutants in methionine-requiring strains of Schizosaccharomyces pombe. Additionally, in studies of selected revertants at three different loci of Neurospora, Brink, Kariya and Stadler (1969) reported that NMG and other mutagens exerted highly specific effects at each locus, depending upon the type of mutagen used. These mutagens included ultraviolet irradiation and chemical mutagens such as the alkylating agent, EMS, and the intercalating agent, ICR-70. NMG definitely could not induce frame-shift mutations, whereas all the other mutagens tested could.

To summarize, the studies of NMG-induced mutagenesis indicated a specific induction of mutation in both bacteria and fungi, probably through methylation of DNA or by single strand breakage of

the polynucleotide strands in DNA. In bacteria it was shown that NMG was highly specific for active replication regions of the genome. In addition, experimental data for diploid *S. cerevisiae* indicated that NMG caused primarily mitotic gene conversion in heteroallelic loci.

MATERIALS AND METHODS

Cultures

S. cerevisiae strain XS-380, a heteroallelic diploid yeast, requiring leucine, tryptophan, methonine, tryosine, lysine and adenine was used in most of the experiments. This strain was obtained from Dr. R. K. Mortimer, University of California, Berkeley. The genotype of this strain is shown below. All six loci were centromere linked in the left arm of chromosome VII. The abbreviations were standard for the genetic mapping of yeast (Von Borstel, 1963). The diploid strain was cultured on amino acid and adenine supplemented YCM agar slants. All cultures were kept at 4° after initial growth and transferred every week.

$$0 \; \frac{LE_{1-13} \quad TRP_{5-A} \quad MET_{13-3} \quad TY_{3-3} \quad LY_{5-2} \quad AD_{5,7-1}}{LE_{1-1} \quad TRP_{5-B} \quad MET_{13-1} \quad TY_{3-1} \quad LY_{5-1} \quad AD_{5-1}}$$

Growth Conditions

For asynchronous cultures, XS-380 was grown aerobically at 25° or 30° with shaking in a modified Wickerham's complete synthetic medium (Wickerham, 1946). For strain XS-380, the supplements to the Wickerham's minimal medium were modified in concentration according to Esposito (1968). Inoculation of the synthetic media was accomplished by transfer of washed cells that had been in balanced

logarithmic growth in YCM+ medium (2% glucose, 2% tryptone, 1% yeast extract) supplemented with required amino acids and adenine (Esposito, 1968). The cells in complete synthetic medium were again allowed to reach balanced growth before they were used for the experiments. Yeast cultures were subjected to amino acid starvation in Wickerham's minimal medium plus adenine (Wehr and Parks, 1969) and also Wickerham's complete synthetic medium lacking leucine, tryptophan, methionine, tryosine, and lysine. Transfer of cultures to different media was accomplished by collecting cells on washed 90 mm Bac-T-Flex membrane filters (Carl Schleicher and Schuell Co., Keene, N.H.) washing the cells with 500 to 1000 ml of prewarmed medium and resuspending in the new medium.

For synchronous growth, up to four liters of YCM+ broth medium were inoculated with a culture of XS-380 in exponential phase. The cells were then incubated for five to six generations in 400-500 ml aliquots at 30° on a New Brunswick gyrotory shaker (Model R-25) until the cells reached mid-log phase. A total of 2 x 10^{10} cells were harvested by either centrifugation at 4° or by filtration at 25° on washed 90 mm B-6 filters. The concentrated cells were then resuspended and adjusted to concentrations ranging from 2.5-4.0 x 10^{9} cells/ml in distilled water at 4° or 15°. Aliquots of the concentrated cell suspension ranging from 0.5-0.7 ml were then layered onto 10-40% linear 40 ml sucrose gradients kept at 4-18°. These gradients

were centrifuged in a swinging bucket rotor for eight to nine minutes at 400 x G in an International Centrifuge (Model EXD) in a procedure adapted from Mitchison and Vincent (1965). The uppermost cell layer in the band containing primarily single non-budded cells was removed by gentle aspiration. This fraction represented about 2-4% of the total cell population applied to the gradient. The sized cells were then washed with distilled water at 4-18° and counted with an electronic particle counter. Then the cells were resuspended in 200-300 ml of prewarmed fresh or conditioned YCM+ medium in concentrations ranging from 1-2 x 10^{6} cells/ml and incubated with agitation at 30°. Conditioned medium was made by growing cells to early log phase and then removing them by filtration.

Measurement of Growth and Total DNA Synthesis

Growth of the cultures was followed by the determination of total cell numbers by using a model F Coulter counter with a 100 micron aperture. Aggregated cells were separated into single or budded cells by sonic treatment of samples for ten seconds with a Branson cell disruptor (Model W-140-C) at 100 watts output. Cell samples were then suitably diluted with filtered 0.9% saline solution containing 0.2% formalin.

For the determination of total DNA, a modified version of the Kissane and Robins (1958) fluorometric procedure was used (Wehr,

1970). For DNA determinations, the reagent 3, 5-diaminobenzoic acid (commercial sources) was prepared by dissolving 0. 3 g of diaminobenzoic acid per 1. 0 ml 4N HCl; this solution was decolorized with Norit in three to five steps until the reagent reached a pale yellow color. In later experiments, a purified grade of 3, 5-diaminobenzoic acid dihydrochloride was used; 0. 44 g of the dihydrochloride was dissolved per ml of distilled water and used without further purification.

Preparation of NMG and Induction of Allelic Recombination

NMG (N-methyl-N'-nitro-N-nitrosoguanidine) was freshly prepared for each experiment by dissolving 0. 5 mg/ml in 95% ethanol. The solution was kept at 4° and in the dark until diluted into acetate buffer to suitable concentrations (Adelberg, Mandel and Chen, 1965).

Induction of allelic recombination by NMG was carried out using a modified version of the procedure also developed by Ebbs (1967) for heteroallelic yeast strains. For induction, the cells were transferred by filtration to dilute NMG solution in 0. 2M acetate buffer at pH 5. 5 in concentrations ranging from 25-50 μg/ml for 30 minutes at 25° or 30° depending upon growth temperature of the culture. After initial suspension, the cells were neither aerated nor stirred during mutagen treatment. The cells were washed before and

after mutagen treatment with acetate buffer. The same number of cells was used for each treatment and control. The control cells were suspended in acetate buffer without the mutagen; otherwise conditions were the same as above. Viable counts were determined after two days of growth on half-strength YCM medium at 30°. The prototrophs were scored on selective media containing Wickerham's complete medium modified after Esposito (1968) lacking the required amino acid or adenine after four days of growth at 30°. The tyr marker was scored on plates lacking both tyrosine and phenylalanine. The tryp marker was scored on media lacking tryptophan but supplemented with indole in equivalent concentration.

The mutagen dose response of cells during active growth at very low concentrations of NMG was also investigated. For these experiments, cells in logarithmic growth in YCM+ medium were treated with various concentrations of NMG dissolved in acetate buffer. NMG mutagenesis during active growth in broth medium was also used to induce allelic recombination in samples from a synchronized cell culture. For each sample one ml of concentrated NMG dissolved in acetate buffer was added to nine ml of culture in YCM+ medium to give a final concentration of two μg/ml. The mutagen-treated cell samples were incubated without aeration for 15 minutes before removal of the NMG by filtration and washing.

RESULTS

The graphs and tables described the results of several different procedures. Each DNA measurement was the average of determinations of two 5-ml samples taken at the given time. Each total cell count with the electronic cell counter represented the average of three consecutive readings of the same sample. In addition, measurements of the number of spontaneous or NMG-induced recombinants were expressed as prototrophs per 10^6 survivors or as prototrophs per 10^6 total cells plated. These two ratios were equivalent because no drastic decreases in viability occurred at the concentrations of NMG used for mutagenesis. For the measurement of the number of prototrophic recombinants, the average number of prototrophs from two or more plates was used. Total cell concentrations from 3×10^5 to 2×10^6 cells were spread on each plate used for the prototroph counts; total cell concentrations per plate were even higher for some of the samples obtained in the synchronous growth experiments using a constant sample volume. Moreover, viable counts were taken as the average of two or more plates containing approximately 100-150 colonies per plate after suitable dilution.

The initial experiments were designed to determine whether active DNA replication had an effect upon the mutagen response (allelic recombination) elicited by low concentrations of NMG. A

Figure 1. Mutagen dose response of five heteroallelic loci. Figure 1A - logarithmic growth; Figure 1B - 4.5 hours of amino acid starvation; Figure 1C - ten hours of amino acid starvation.
○ leu; ● ad; △ met; ▲ lys; □ tyr.

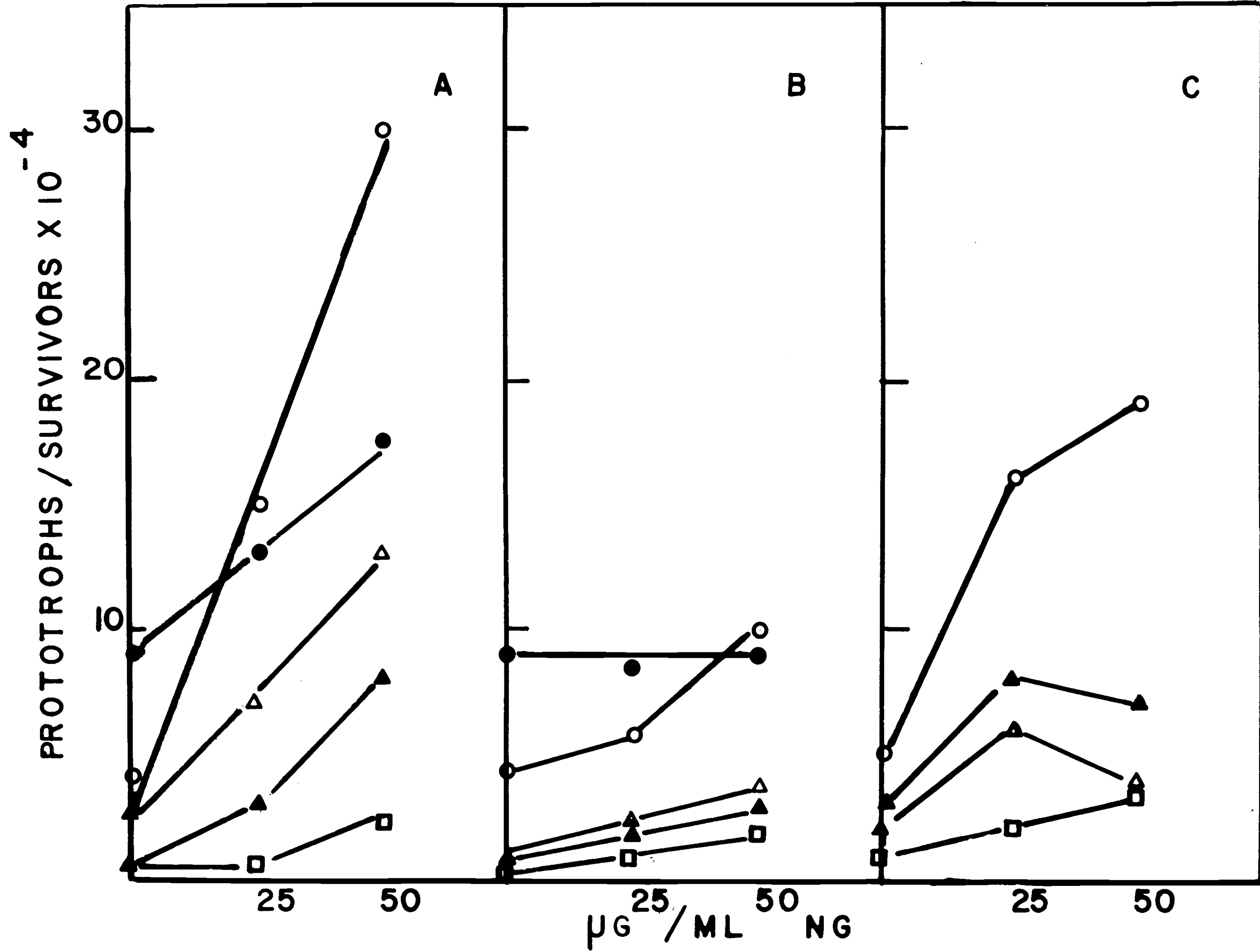

A
B
C
PROTOTROPHS / SURVIVORS X 10^{-4}
30
20
10
25
50
μG / ML NG

Figure 2. Cell number, DNA synthesis and viable count during 4.3 hours of amino acid starvation. Vertical line: time of transfer to Wickerham's minimal medium supplemented with adenine (amino acid starvation medium) from WC medium. Symbols: ● cell number, stationary phase without amino acid starvation; stationary phase: amino acid starvation: ○ cell number; △ viable cells; □ total DNA.

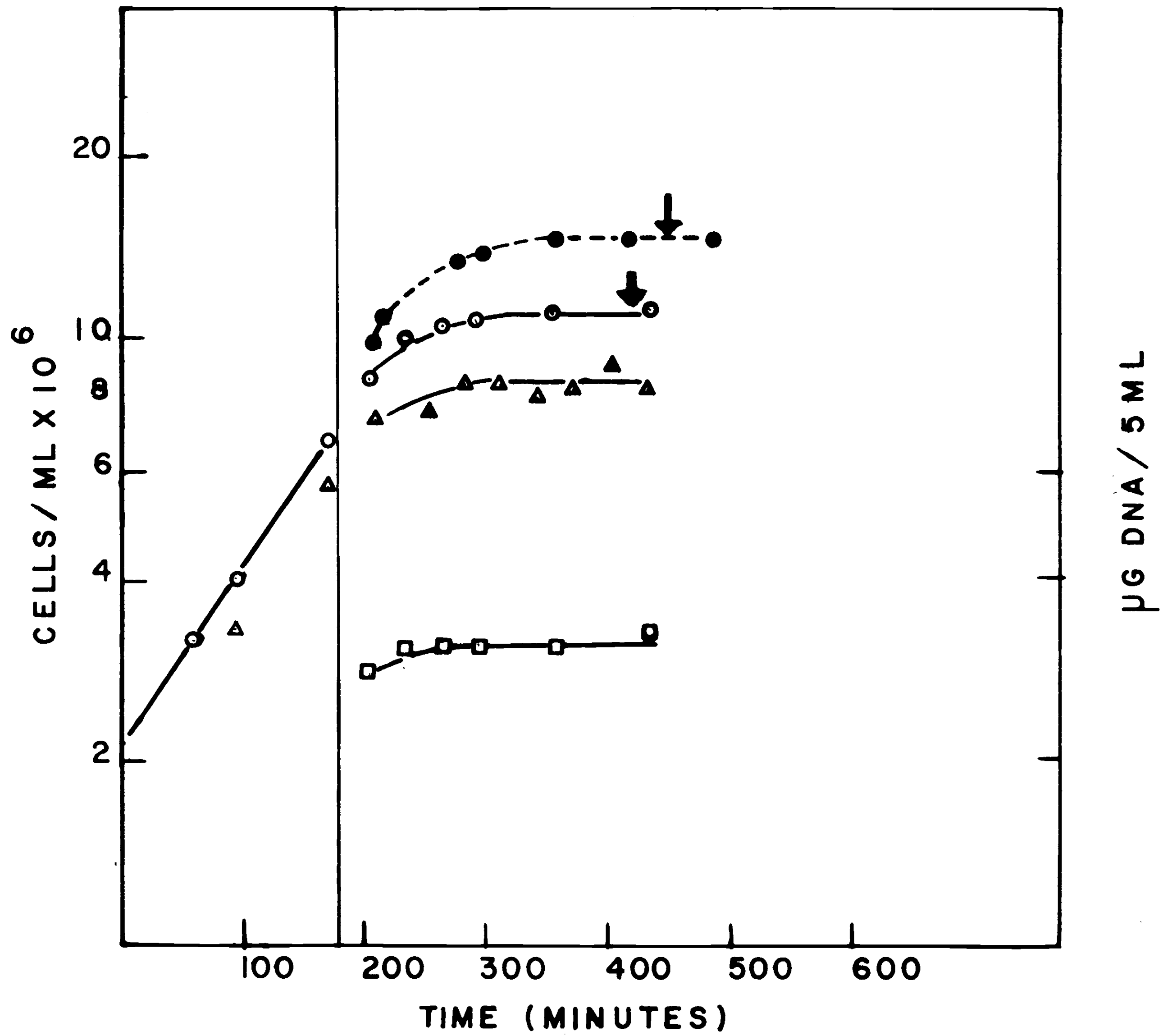

20
10
8
6
4
2
CELLS / ML X 10^6
μG DNA / 5 ML
100
200
300
400
500
600
TIME (MINUTES)

Figure 3. Parallel experiments with NMG-induced recombination using the same culture during logarithmic growth, early stationary phase, and four hour amino acid starvation. Figure 3A - Control; Figure 3B - 25 μg/ml NMG; Figure 3C- 50 μg/ml. ▭ logarithmic culture; ▨ four hour amino acid starvation; ■ early stationary phase.

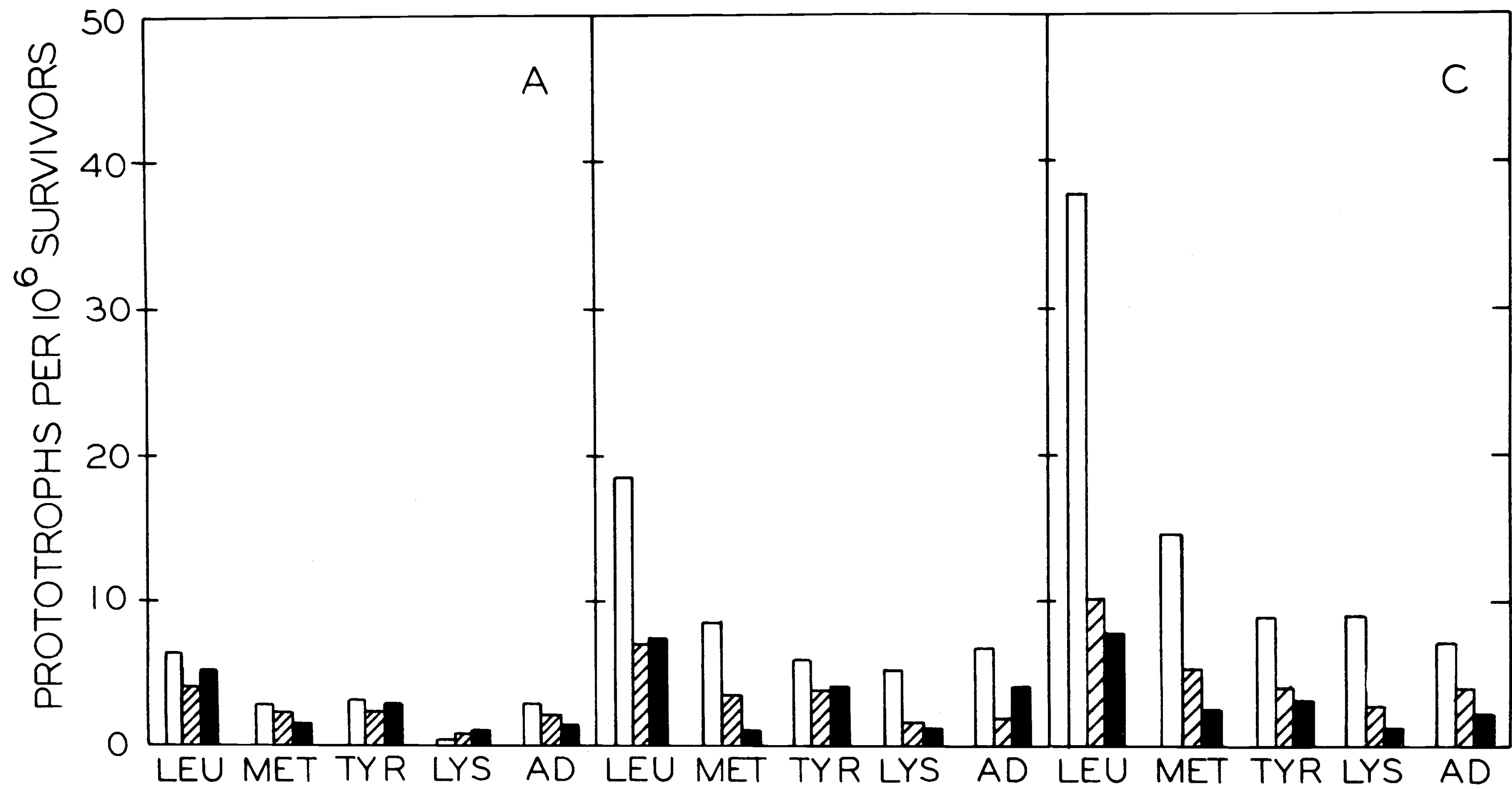

A
C
PROTOTROPHS PER 10^6 SURVIVORS
50
40
30
20
10
0
LEU MET TYR LYS AD
LEU MET TYR LYS AD
LEU MET TYR LYS AD

Table 1. Mutagen dose response to NMG of XS-380 during growth and amino acid starvation.

Condition	LEU^+	MET^+	TYR^+	LYS^+	AD^+	% Survival
Logarithmic growth[a, b]	112.5	44.4	0.7	28.2	39.4	100
Early stationary[c]						
Experiment 1	16.8	9.3	17.1	15.9	14.2	74
Experiment 2	20.0	6.6	11.0	5.6	26.5	89
Amino acid starvation[d]						
3.5 hours	10.7	11.3	7.2	16.0	15.4	94
4.0 hours	25.0	12.0	17.4	8.6	2.1	96
4.5 hours	13.7	15.2	5.4	7.5	0.5	91
10.0 hours	11.3	37.3	9.4	40.7	--	60

[a]For all experiments: NMG dose = 25 µg/ml, 30 minutes at 30°.
Cells plated = 6×10^5 for each determination.
Values expressed as the difference between the control values (spontaneous allelic recombination) and induced values in terms of prototrophs / survivors.

[b]For logarithmic growth: Values are the average of two experiments.

[c]For the early stationary phase values were obtained three hours after the initial decrease in growth rate.

[d]For amino acid starvation: medium was Wickerham's minimal medium + adenine.

Table 2. Survival of XS-380 in Figure 3.

Condition	Concentration of NG (μG/ml)	Percent survival[a]
Logarithmic growth	0	100
	25	94
	50	74
Amino acid starvation for four hours	0	100
	25	96
	50	76
Early stationary[b]	0	100
	25	89
	50	101

[a]Survival was calculated by taking the viable count of cells suspended in 0.2M acetate buffer pH 5.5 as 100%.

[b]The early stationary values for survival were obtained about three hours after the initial decrease in growth rate leading to stationary phase.

series of experiments was performed with asychronous cultures of yeast strain XS-380 in which NMG-induced recombination was measured as a function of dose and different physiological conditions. Figure 1 shows the mutagen dose response for the cells during logarithmic growth and amino acid starvation. The cells were grown four to five generations overnight in synthetic complete medium (WC) for the experiment in Figure 1A. For amino acid starvation (Figure 1B and 1C) overnight cultures of XS-380 in logarithmic growth were transferred from WC medium to equivalent volumes of synthetic minimal medium supplemented with adenine.

These independent experiments show that the mutagen dose response varies for five out of the six loci under different conditions of amino acid starvation and logarithmic growth. The largest response is obtained during logarithmic growth. After 4.5 hours of amino acid starvation, the response has declined. Figures 1B and 1C show a proportional response to mutagen except in the adenine locus. Ten hours of amino acid starvation also produced non-proportional responses. The absence of proportional response after ten hours of amino acid starvation, along with a 50% decrease in cell viability before mutagen treatment, led to experimenting primarily with cultures subjected to three to four hours of amino acid starvation.

Figure 2 compares cell division, DNA synthesis, and viable counts during 4.3 hours of amino acid starvation. For comparison,

the dotted line shows the rate of cell division of a culture allowed to reach early stationary phase.

For the experiments shown in Figures 2 and 3 and Tables 1 and 2, the cells were grown aerobically at 30° in WC medium. For amino acid starvation, the cells were transferred by filtration from balanced growth in WC to minimal medium containing adenine. The time of transfer to starvation medium is shown by the vertical line. The time of sampling for induced allelic recombination experiments is shown by the arrows in Figure 2. Total DNA was determined by a fluorometric method. After a ten-second period of sonication to separate large buds from the parent cells, total cell counts were determined with an electronic cell counter. Using this method, 16% more cells were observed than in the viable cell counts.

The results shown in Figure 2 indicated that during amino acid starvation: 1) total DNA increased 14% and cell numbers increased 35% during the first 60 minutes of starvation; 2) no further changes in cell number, total DNA, or viable count was observed at the time that samples were taken for the experiment shown in Figure 3 and Table 1. Figure 3 shows the results of three parallel experiments performed with the same culture. Induced allelic recombination frequencies were measured during: 1) balanced logarithmic growth; 2) 4.0 hours of amino acid starvation after balanced growth; and 3) the early stationary phase of the culture cycle. The physiological

state is indicated by the type of bar graph. Figures 3A-3C show the allelic recombination frequencies for each locus at concentrations of 0, 25, and 50 μg/ml NMG.

The control experiments (Figure 3A) show a significant level of allelic recombination, but the three different physiological states do not seem to change the level of this recombination. In contrast, Figures 3B and 3C show that NMG will increase and change the recombination frequencies under all three conditions. Also, no shifts are observed in the patterns of allelic recombination for all six markers, in comparing logarithmic cultures to either stationary or amino acid starved cultures.

The most marked increase in frequency occurs in the actively growing cells. Less metabolically active cells show a less sensitive response during both early stationary phase and amino acid starvation. The figures also show that each locus has a characteristic conversion frequency. The data in Table 2 indicate that the low concentrations of NMG had little effect on cell viability. Also, the physiological state do not affect the sensitivity of the cells to the mutagen because similar levels of survival are obtained under all three conditions.

The events leading to decreased induced recombination frequencies during the early phases of amino acid starvation were investigated further. These experiments were designed to observe the changes in induced allelic recombination at a time when RNA and

protein synthesis presumably had stopped, while DNA synthesis temporarily continued to increase before finally it also ceased (Wehr, 1970). At regular time intervals, early in amino acid starvation, DNA synthesis, viability, rates of cell division, and induced allelic recombination frequencies were measured. Log phase cultures of strain XS-380 grown in YCM+ medium were washed and transferred to 300 ml of WC medium. After four to five generations of growth at 25°, cells in logarithmic growth were transferred by filtration to 300 ml of prewarmed WC medium lacking only leucine and tryptophan. Before transfer, the cells were washed on the filter with prewarmed starvation medium. Similar procedures were used in the other experiments that follow. Figure 4 shows that growth had stopped by 170-180 minutes. An exponential decrease in viability was also observed. Figure 5 indicates that cell count increases ended about 100 minutes. In contrast, DNA synthesis did not stop until approximately 150 minutes. The recombination frequencies of the leucine (leu) and methionine (met) markers declined during the first 200 minutes of amino acid starvation. The frequencies for the ad locus did not show any significant decline.

In order to avoid large viability changes before NMG treatment in induced recombination frequencies as shown in Figure 4, strain XS-380 was starved for all required amino acids in WC medium at 25°. Figure 6 shows that there were no further increases in cell

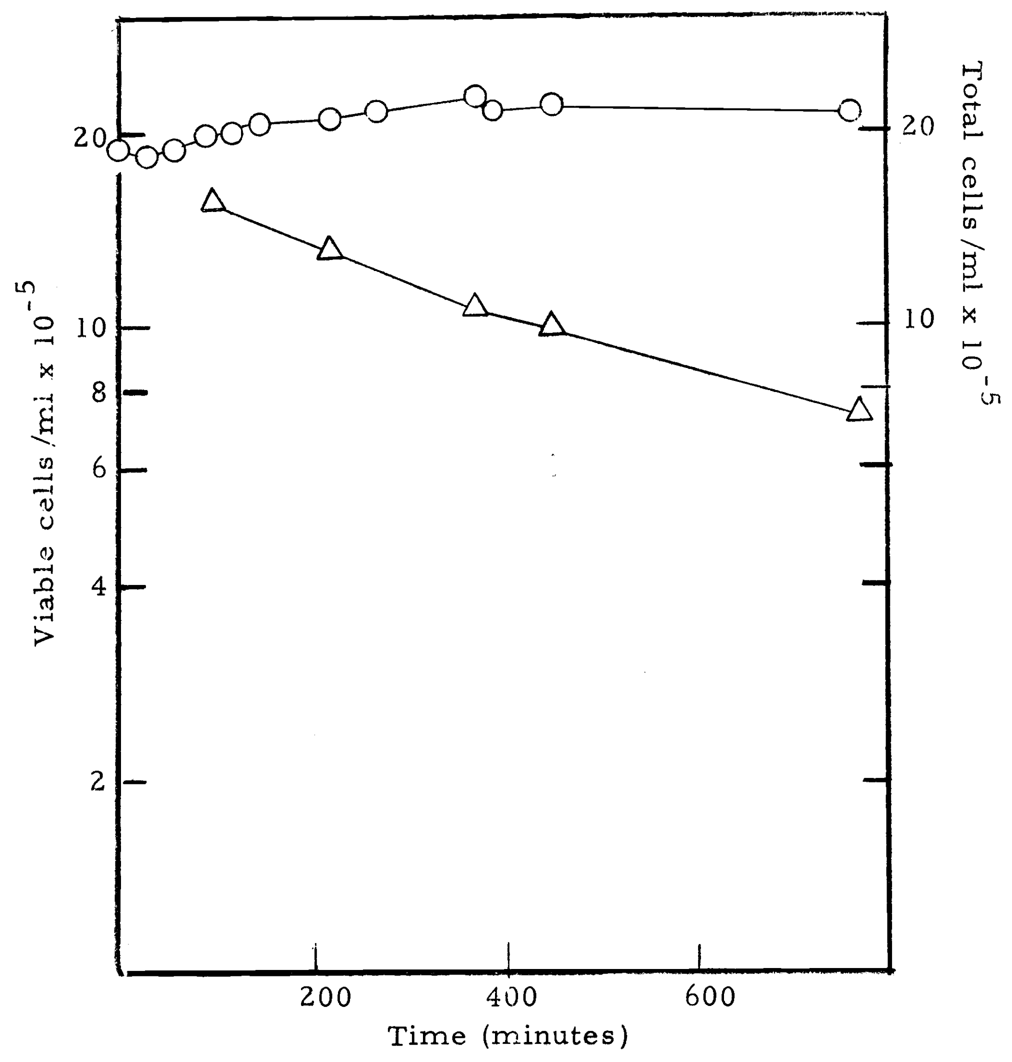
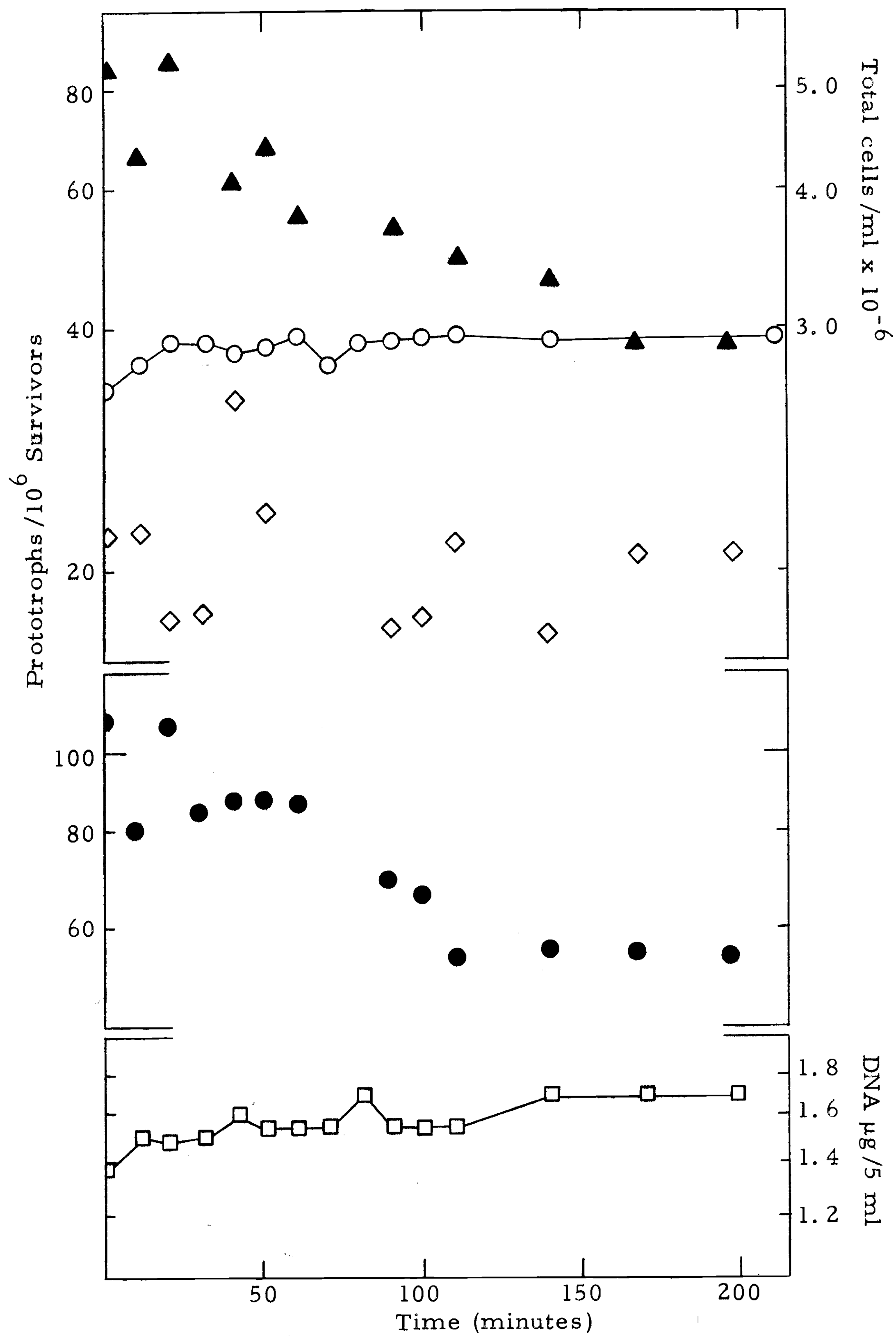


Figure 4. Viability and cell number after transfer to WC medium lacking leu and tryp.
O cell number; Δ viable cell count

Figure 5. DNA synthesis, cell number, and NMG-induced recombination frequencies during starvation for leu and tryp at 25°. NMG dose: 25 μg/ml.
△ met; ◇ ad; ● leu; □ total DNA



Prototrophs /10^6 Survivors
80
60
40
20
100
80
60
Total cells/ml x 10^-6
5.0
4.0
3.0
DNA µg/5 ml
1.8
1.6
1.4
1.2
50
100
150
200
Time (minutes)

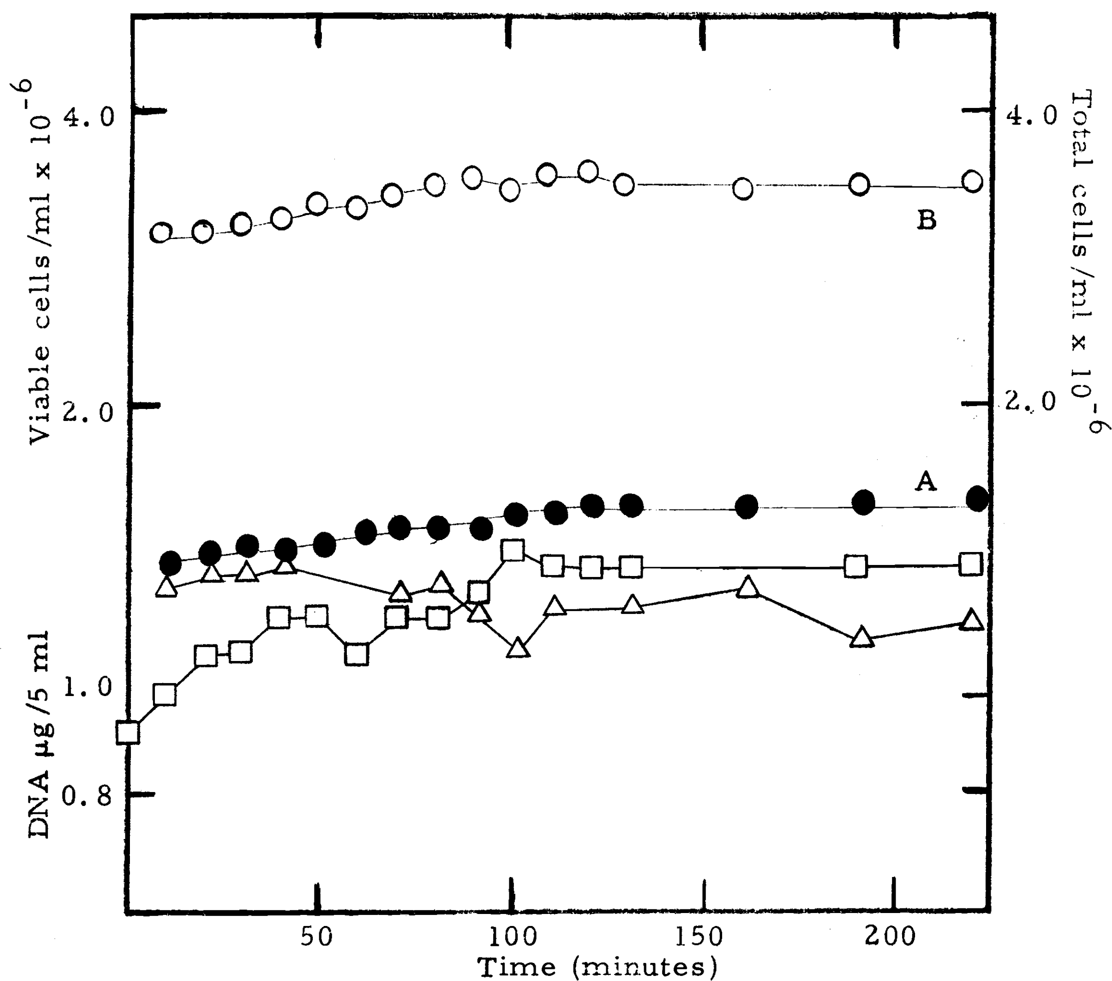
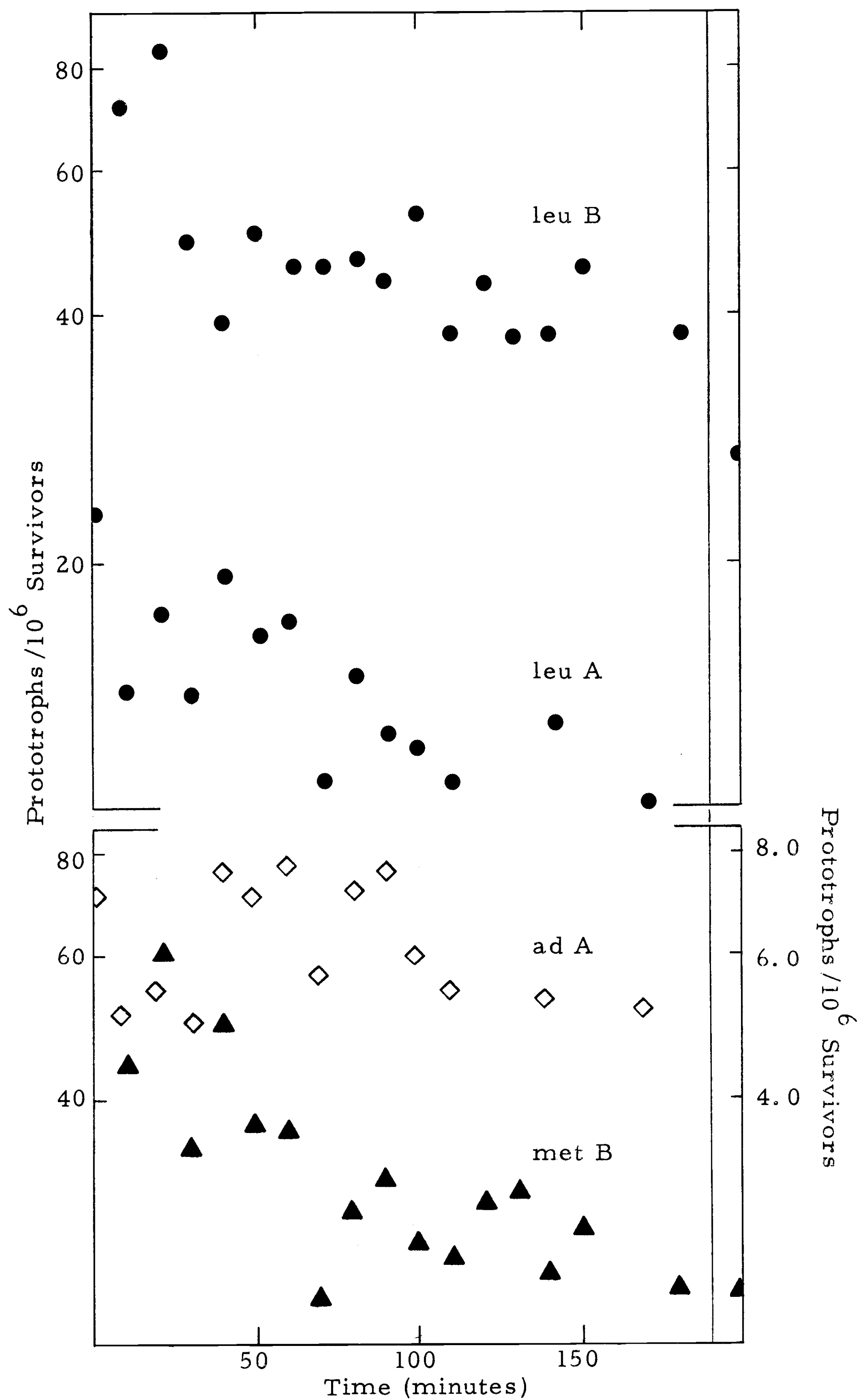


Figure 6. Cell number, viable cells, and DNA synthesis after amino acid starvation in WC medium lacking leu, tryp, tyr, phe, lys, and met. NMG dose: 25 µg/ml.
△ viable count; □ DNA synthesis; ○ cell number
A. Cell count for the DNA analysis.
B. Cell count for experiment in Figure 8.

Figure 7. Induced allelic recombination frequencies during starvation for leu, tryp, tyr, phe, lys, and met at 25°. NMG dose: 25 μg/ml. A and B indicate two different experiments. Vertical line indicates spontaneous allelic recombination. ◇ ad; △ met; ○ leu


80
60
40
20
leu B
leu A
Prototrophs /10^6 Survivors
80
60
40
ad A
met B
8.0
6.0
4.0
Prototrophs /10^6 Survivors
50
100
150
Time (minutes)

number after 100 minutes of starvation. Total DNA synthesis also ceased after 100 minutes. In contrast to the results shown in Figure 4, starvation for all required amino acids produced little or no changes in cell viability up to 165 minutes of amino acid starvation. As shown in Figure 7, the induced recombination frequencies declined at rapid rates during the first 100 minutes of starvation, followed by a shift to lower rates. In the second set of experiments, the met recombination frequencies declined to spontaneous levels by 150 minutes. However, the leu recombination frequencies had not reached spontaneous levels by 180 minutes of amino acid starvation. The adenine (ad) frequencies showed little change or decrease.

Moreover, I investigated the effect of phasing the cells in DNA replication before subjecting the cells to amino acid starvation. In the previous starvation experiments probably only about one-third of the cells were actively synthesizing DNA (Williamson, 1965). An experiment was devised to check whether the effects of amino acid starvation upon induced allelic recombination were more pronounced when almost all cells were in the phase of DNA synthesis. The cells were synchronized by sizing on sucrose density gradients. Then the cells were allowed to undergo one-half doubling in total DNA in YCM+ medium before the cells were shifted to WC medium lacking all the required amino acids at 25°. During starvation, samples were taken at various time intervals and treated with NMG. A parallel

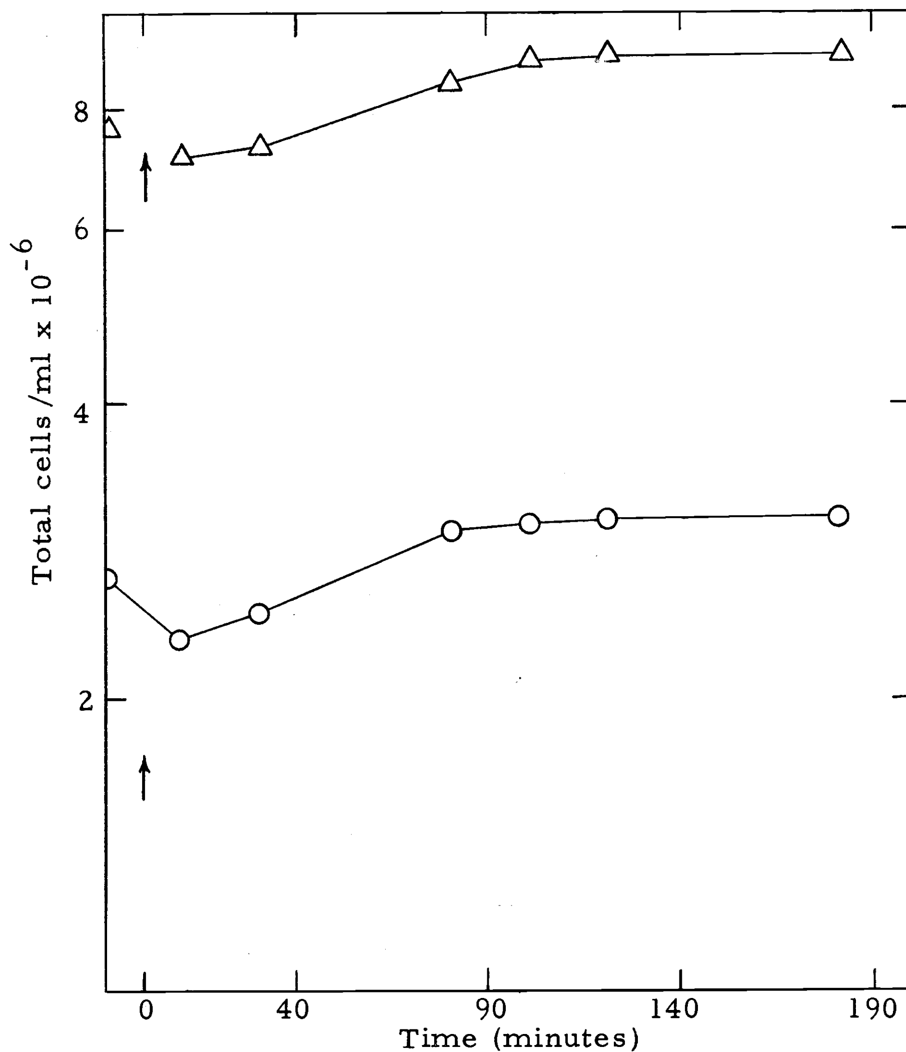


Figure 8. Cell number during growth and amino acid starvation in synchronous and asynchronous cultures. The arrow indicates the time of transfer from broth medium to WC medium lacking leu, lys, tryp, tyr, phe, and met.
△ synchronous culture; ○ asynchronous culture

Table 3. Frequencies of induced allelic recombination of synchronous and asynchronous cultures before and after amino acid starvation.

	LEU^{+}[a]	
	Asynchronous culture	Synchronous culture
Before amino acid starvation[b]	67.0	60.4
Duration of amino acid starvation[c]		
10 minutes	49.6	63.3
30 minutes	41.4	35.4
80 minutes	42.2	32.1
100 minutes	45.2	40.9
120 minutes	47.9	39.1
160 minutes	42.2	35.2

[a] Prototrophs /10^6 survivors.

[b] For all experiments: Conditions of mutagenesis = NMG dose 25 µg/ml, 30 minutes at 30°.

[c] Amino acid starvation medium: WC medium lacking leu, lys, tryp, tyr, phe, and met.

asynchronous culture was sized, but then the gradient was mixed to give a random population of cells before amino acid starvation. The same number of total cells were used for NMG mutagenesis in each sample for either culture. Figure 8 shows that no increases in cell number were observed in the synchronized or the asynchronous control cultures after 100 minutes of amino acid starvation. The frequency of induced allelic recombination was measured at the leu locus. Table 3 shows that in the asynchronous and synchronous cultures, the frequencies of induced recombination decreased ten minutes and 30 minutes after amino acid starvation respectively. Both cultures showed higher recombination frequencies during the first ten minutes of starvation. In the synchronous culture, maintenance of recombination values at the active growth level for ten minutes could be attributed to a drastic decrease in viability after NMG treatment, rather than a change in recombination frequencies, because the absolute numbers of leu prototrophs were similar for both cultures. Beyond 80 minutes of amino acid starvation, other changes in recombination frequencies did not seem to be significant.

A possible dependence of induced allelic recombination during amino acid starvation upon glucose as the carbon and energy source was also investigated. In this experiment, a culture in logarithmic growth in WC medium at 25° was divided and transferred to amino acid starvation medium with and without glucose. The cells were

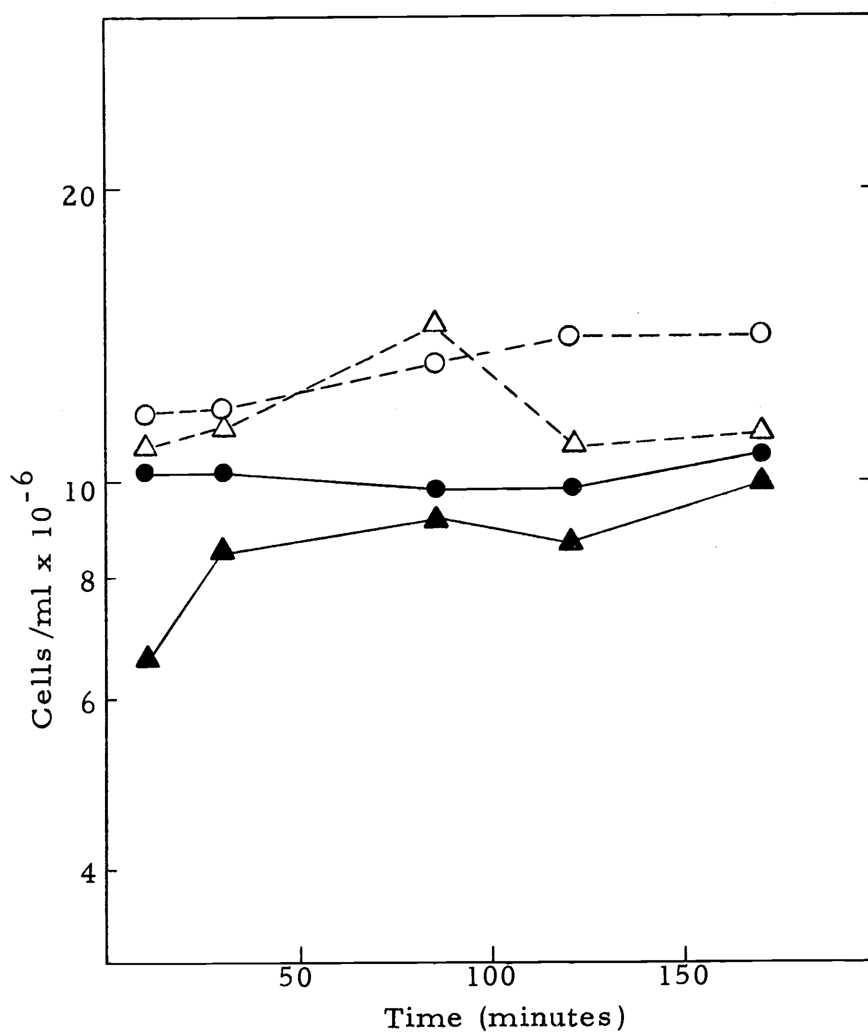


Figure 9. Viability and cell number during amino acid starvation with and without glucose in WC medium at 25°. NMG dose: 25 μg/ml.
- - - + glucose - amino acids; △ viability; ○ cell number
——— - glucose - amino acids; ▲ viability; ● cell number

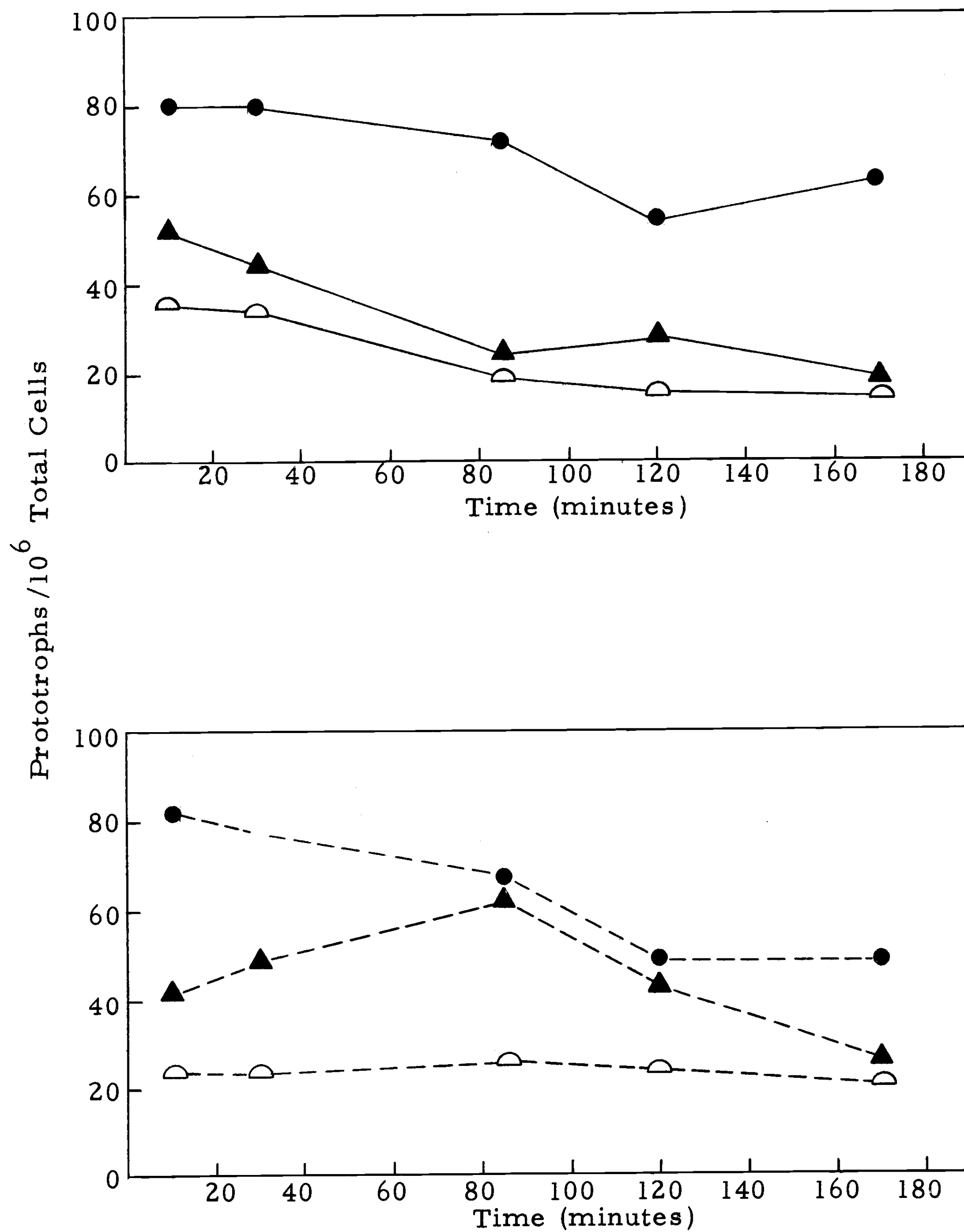


Figure 10. Induced allelic recombination during amino acid starvation with and without glucose in WC medium with and without glucose at 25°. NMG dose: 25 µg/ml. ● leu; ▲ met; ⌓ lys; —— with glucose; ---- without glucose

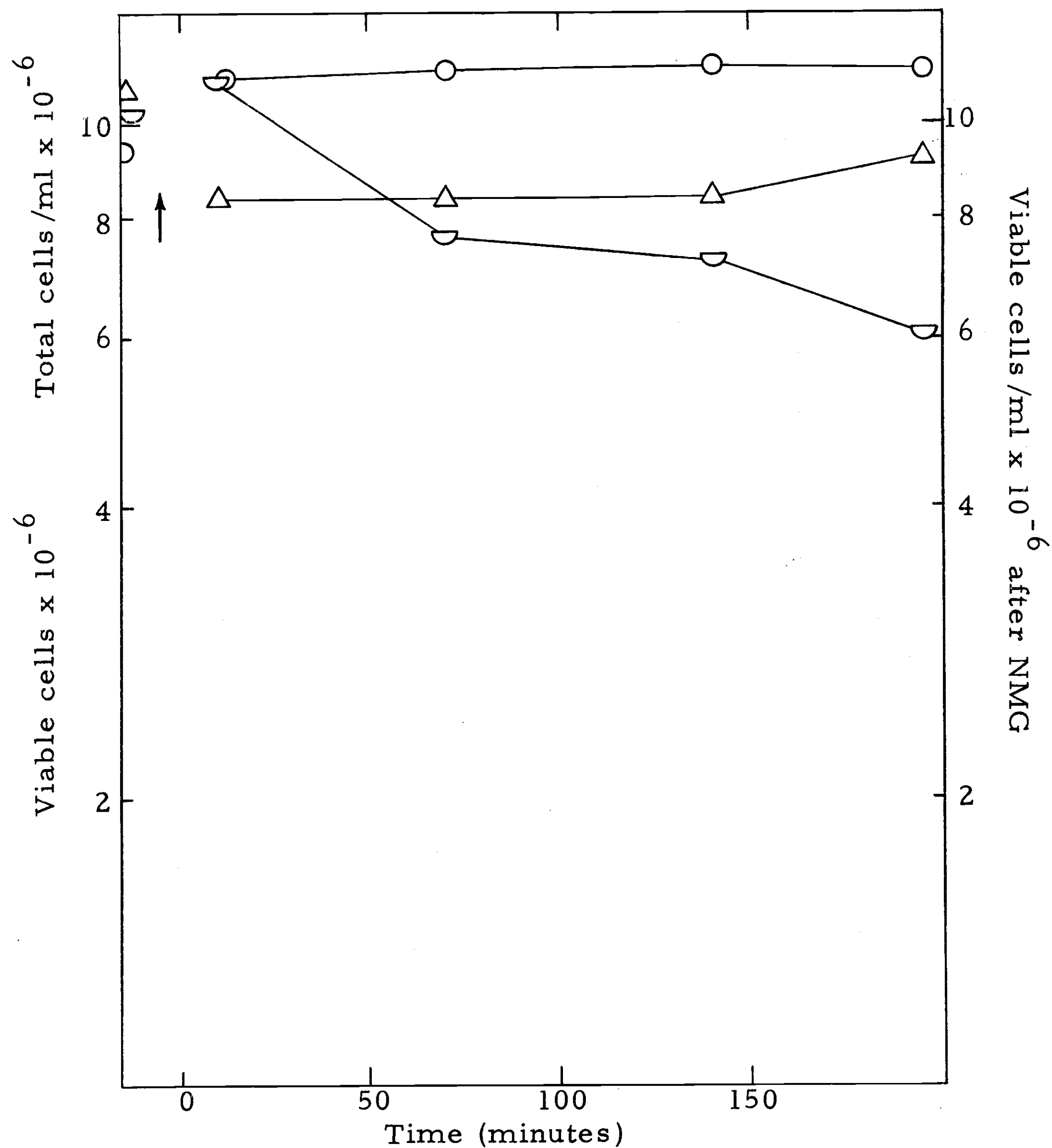


Figure 11. Cell number and viability during cycloheximide (CH) treatment; viability after CH and NMG treatment at 30°. The arrow indicates the time of CH addition to the WC medium. CH concentration: 20 μg/ml; NMG dose 25 μg/ml. ○ cell number; ◡ viability after CH treatment; △ viability after CH and NMG treatment

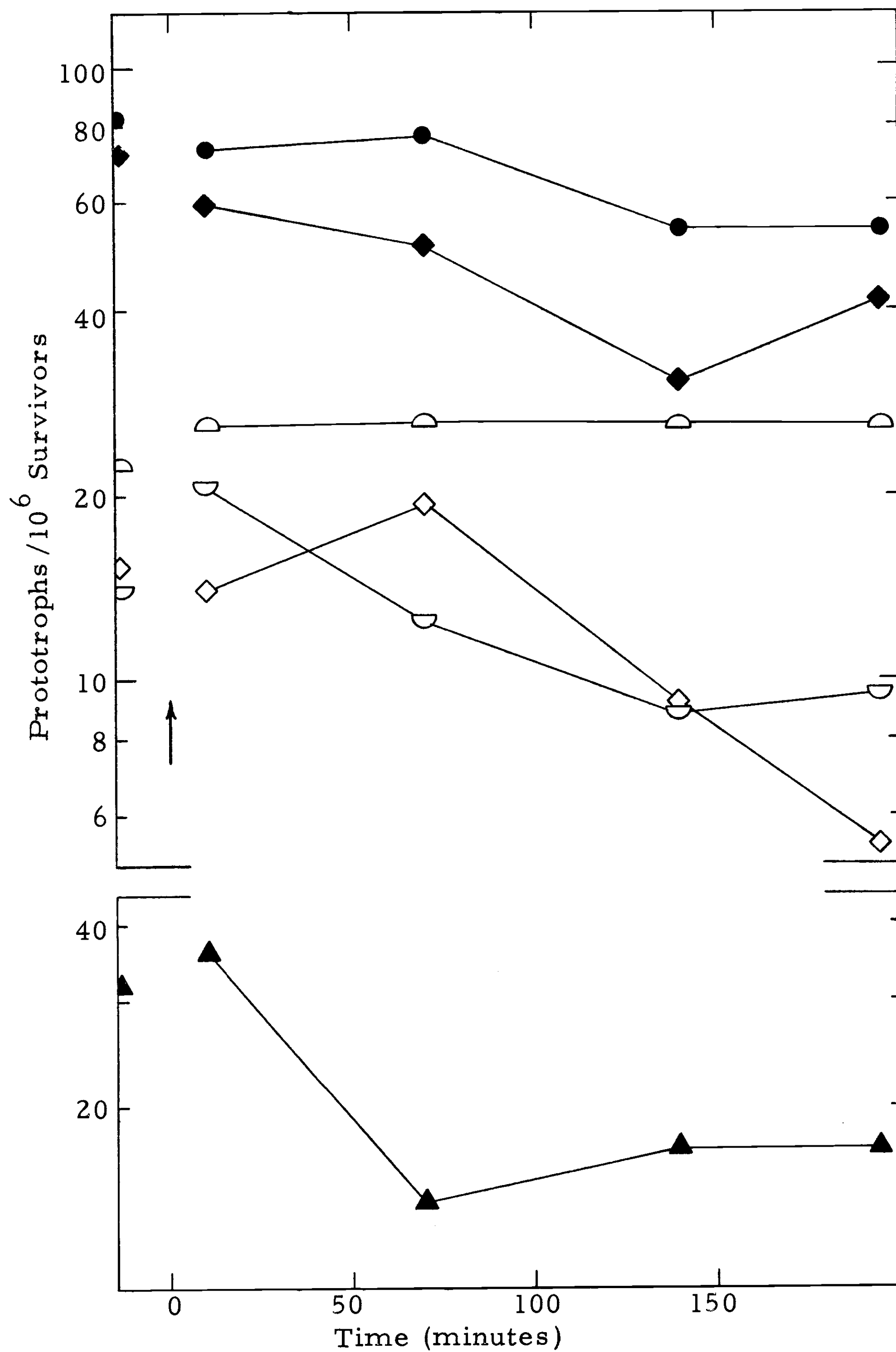


Figure 12. NMG-induced allelic recombination after cycloheximide treatment (20 μg/ml) in WC medium at 30°. NMG dose: 25 μg/ml. The arrow indicates the time of addition for cycloheximide. ● leu; ◆ tryp; ⌓ lys; ◡ tyr; ◇ ad; ▲ met

Table 4. Frequencies of induced allelic recombination during balanced logarithmic growth.

Sample	AD^+		MET^+		LYS^+	
	YCM+ Medium (3 x 10^5 cells/plate)					
1 Induced	26.2	22.0	53.4	56.1	18.2	13.2
2 Induced	32.2	21.6	70.0	51.8	16.0	15.1
3 Induced	48.4		57.0		15.3	
Mean	30.0 ± 6.2		57.7 ± 5.1		19.4 ± 6.1	
1 Spontaneous	17.2		27.9		7.38	
2 Spontaneous	17.2	17.3	27.9	27.4	7.38	9.47
Mean	17.2 ± 0.1		27.7 ± 0.3		8.08 ± 0.9	
	Wickerham's Minimal Medium + All Required Acids + Adenine (4 x 10^6 cells/plate)					
1 Induced	15.0		46.0		19.5	
2 Induced	13.0	15.0	32.0	41.0	16.5	20.4
3 Induced	17.0	16.0	37.0	36.0	19.1	17.5
Mean	15.2 ± 1.0		38.4 ± 4.0		18.2 ± 1.4	
	Wickerham's Complete Medium (2 x 10^6 cells/plate)					
1 Induced	7.71				16.3	
2 Induced	9.94				14.6	
3 Induced	9.95				12.3	
Mean	9.18 ± 1.0				14.4 ± 1.4	

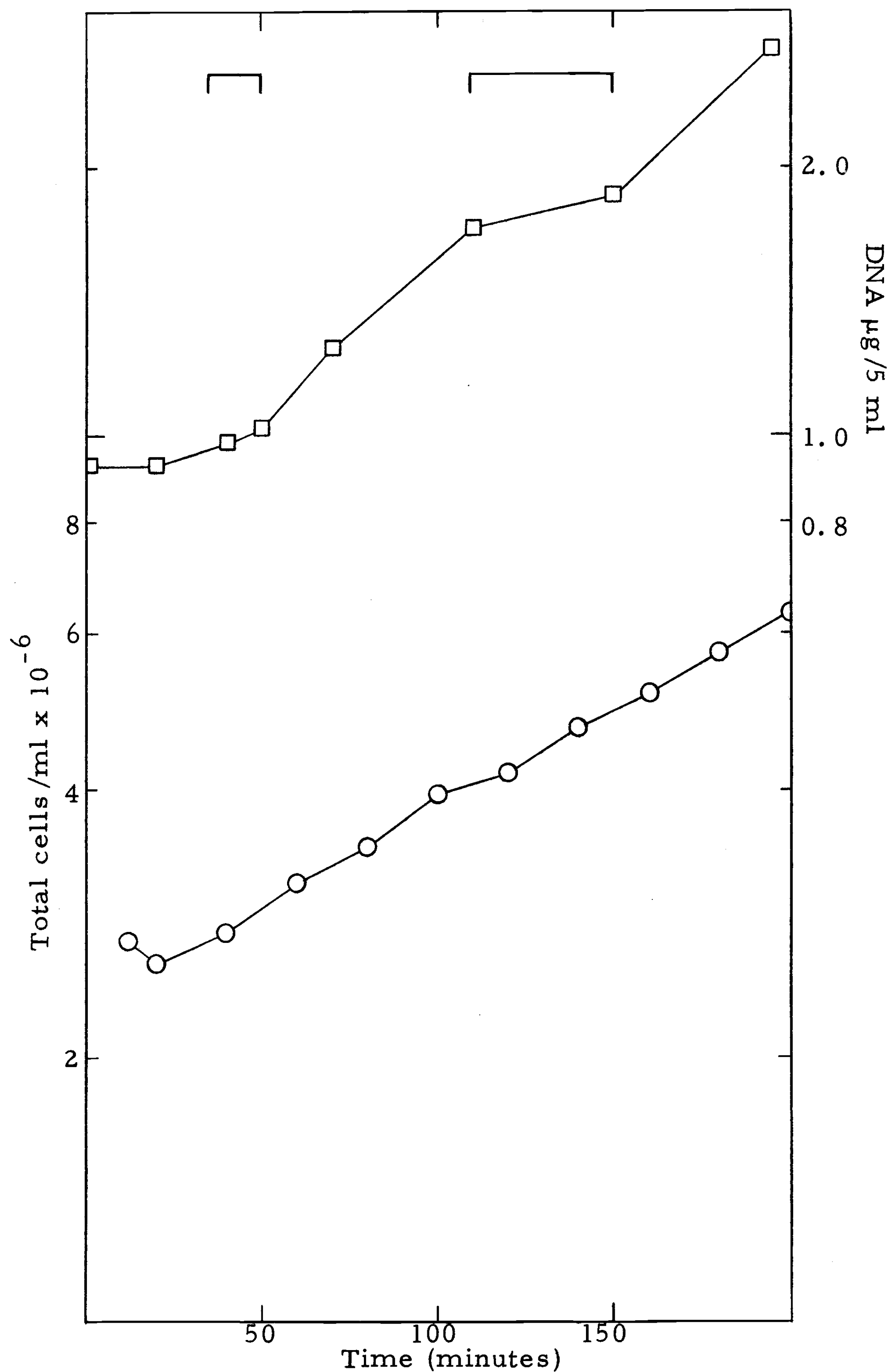


Figure 13. Cell number and total DNA synthesis in amino-acid starved cells during growth in WC medium at 25°. The bars indicate respectively initiation and termination of DNA synthesis. □ total DNA synthesis; ○ cell number

Figure 14. NMG-induced recombination after two hours amino acid starvation in WC medium lacking leu and tryp at 25°. The bars indicate respectively initiation and termination of DNA synthesis. NMG dose: 25 μg/ml. ● leu; ▲ met; ◇ ad; ○ cell number

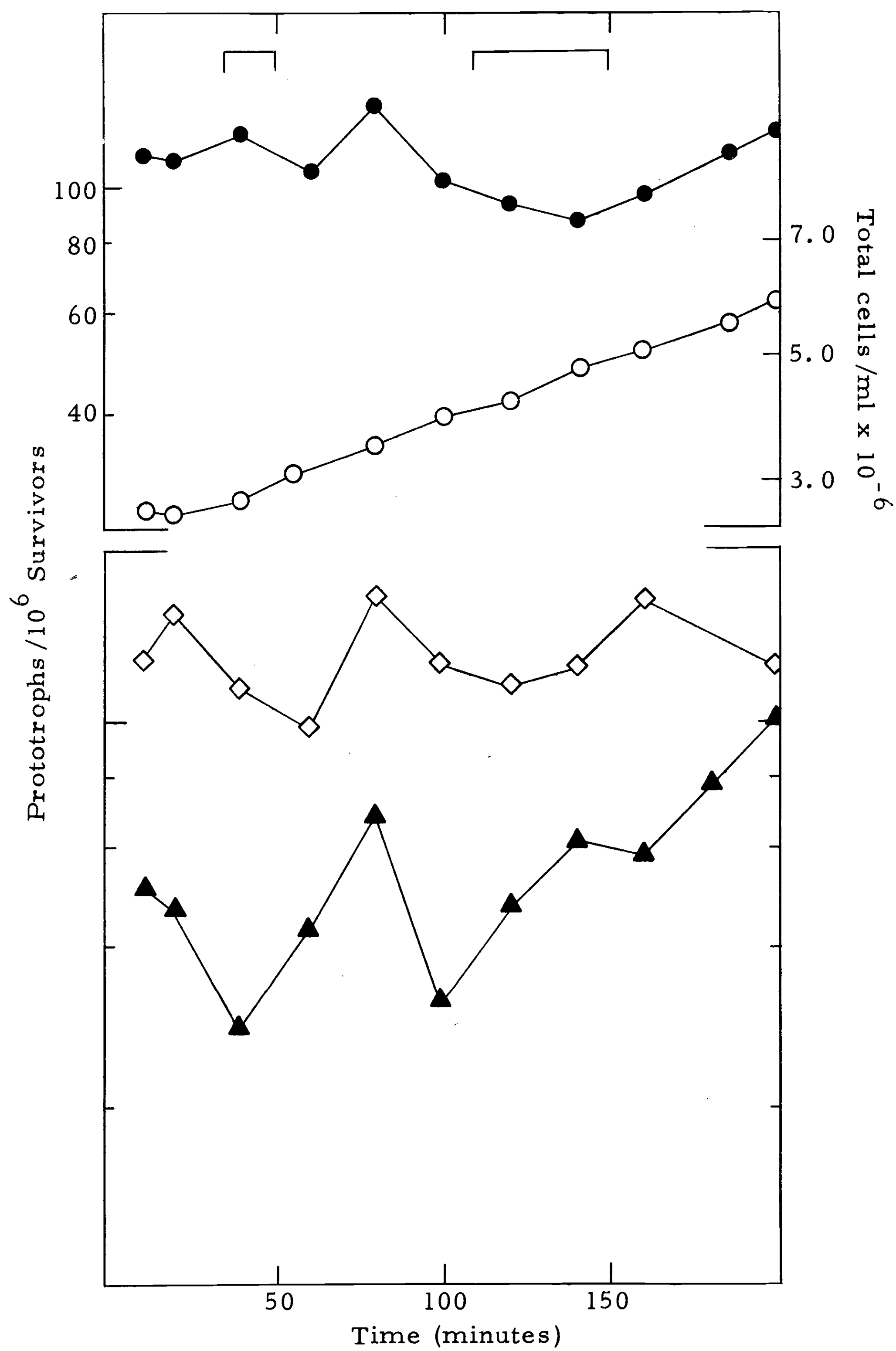

100
80
60
40
Prototrophs/10^6 Survivors
7.0
5.0
3.0
Total cells/ml x 10^{-6}
50
100
150
Time (minutes)

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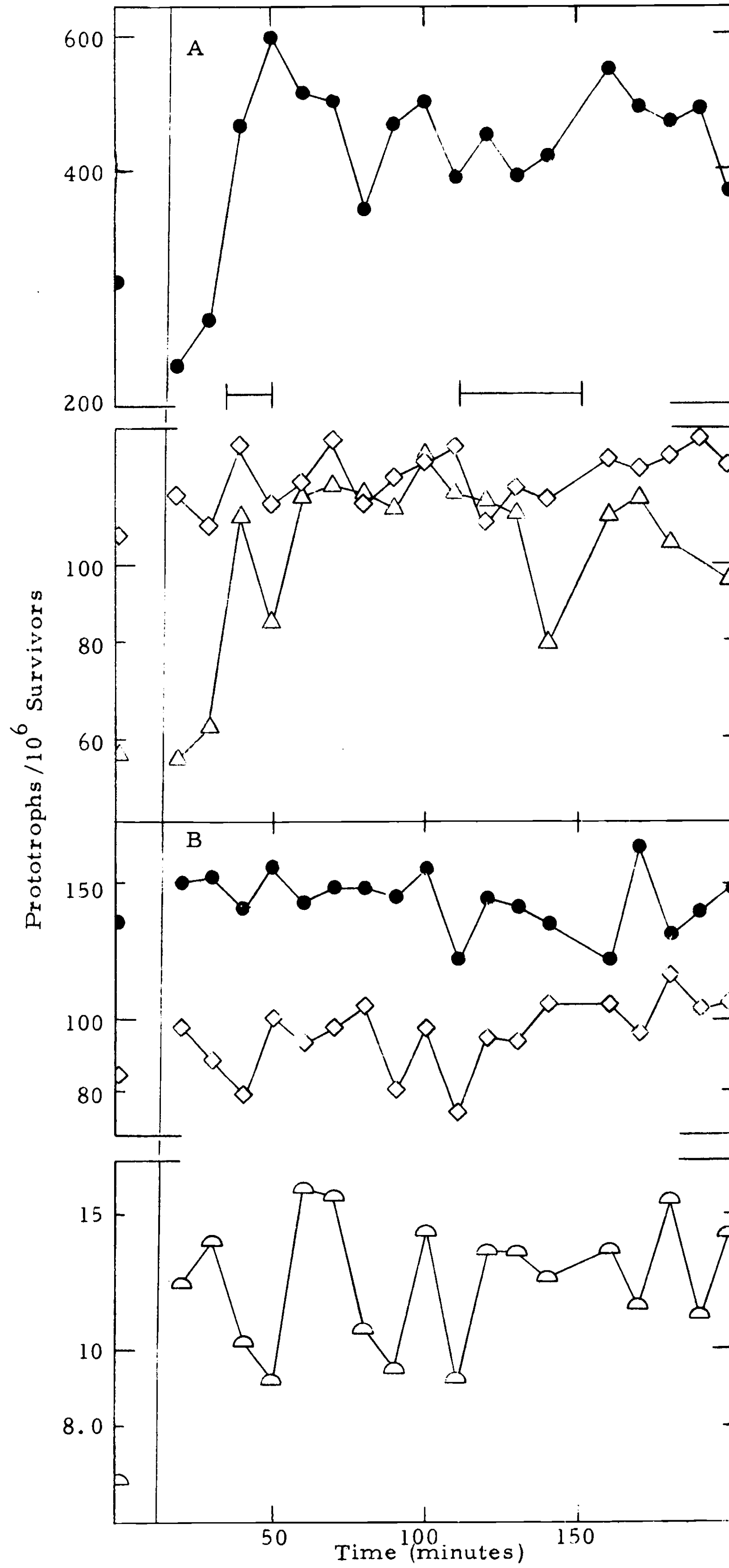
Figure 16. Induced allelic recombination frequencies during growth in WC medium after 2.5 hours of amino acid starvation for all required amino acids (leu, lys, met, try, phe, tryp) at 25°. The bars indicate time of initiation and termination of DNA synthesis. Left side of graph indicates recombination during starvation.

A. NMG dose: 50 μg/ml

B. NMG dose: 0 μg/ml

● leu; ◇ ad; △ lys (A); ⌓ lys (B)



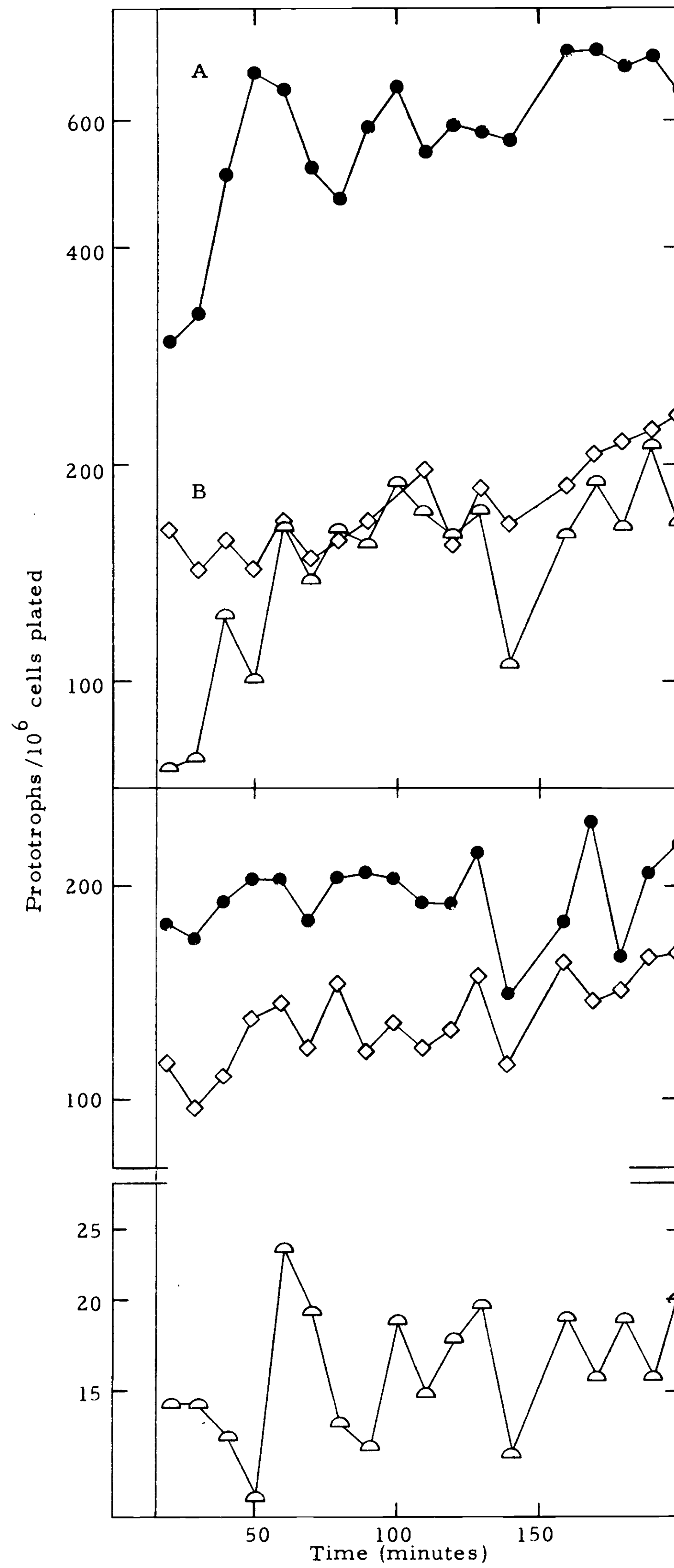
A
B
600
400
200
100
80
60
150
100
80
15
10
8.0
Prototrophs /10^6 Survivors
50
100
150
Time (minutes)

Figure 17. Spontaneous and induced allelic recombination frequencies (prototrophs/10^6 cells plated) under the same conditions as shown in Figure 16.

A. NMG dose: 50 µg/ml

B. NMG dose: 0 µg/ml

● leu; ◇ ad; ⌓ lys


A
B
600
400
200
100
200
100
25
20
15
Prototrophs/10^6 cells plated
50
100
150
Time (minutes)

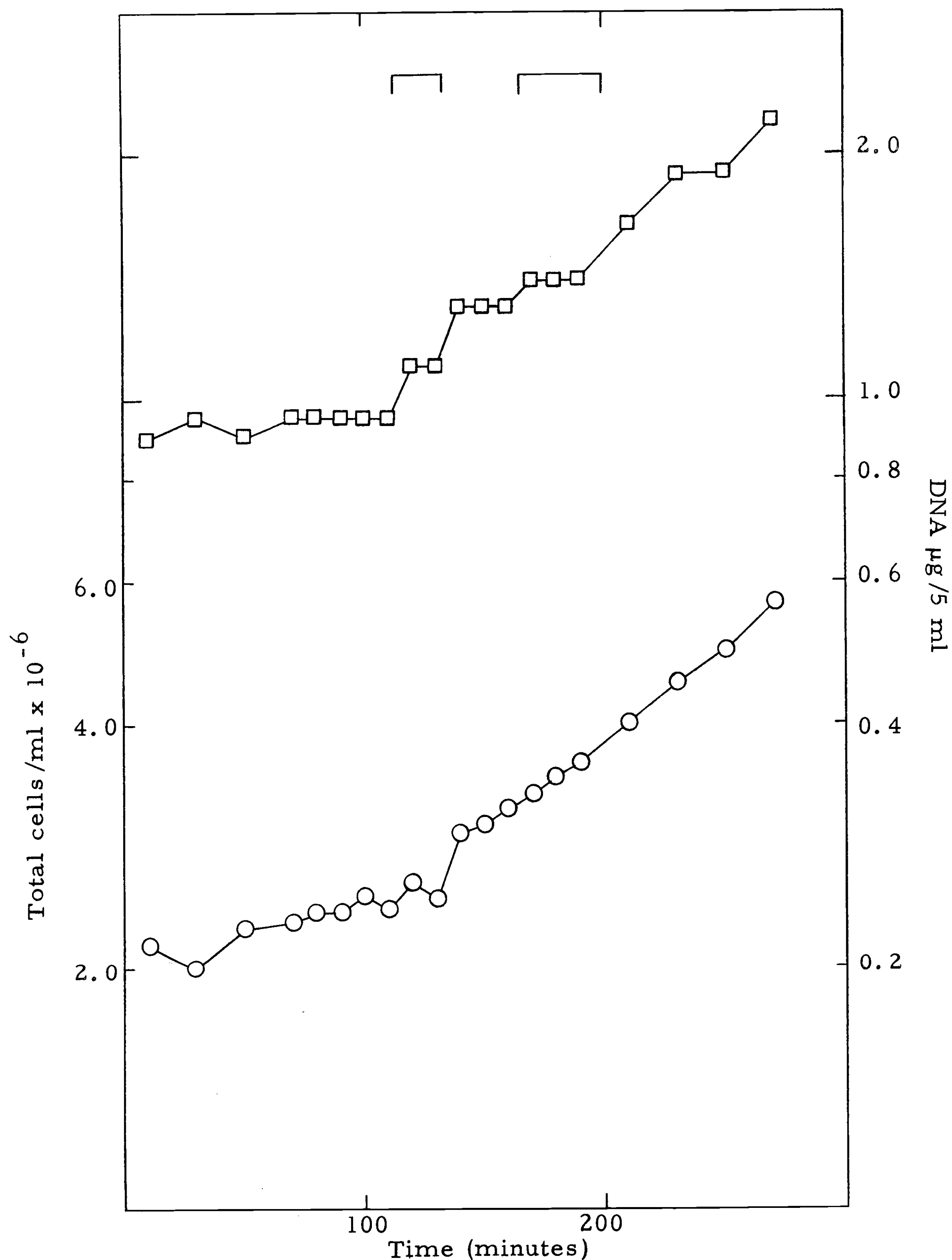


Figure 18. Total DNA synthesis and cell number during growth of amino-acid starved cells in WC medium at 25°. (Starvation: 3.5 hours in WC medium lacking leu, lys, tryp, tyr, phe, and met.) The bars indicate initiation and termination of DNA synthesis. □ total DNA synthesis; ○ cell number

Figure 19. Induced allelic recombination during growth of amino acid starved cells in WC medium at 25°. The bars respectively indicate initiation and termination of DNA synthesis. Cells were starved for 3.5 hours in WC medium lacking leu, lys, tryp, tyr, phe, met. NMG dose: 50 μg/ml. ● leu; ▲ met; ▽ tyr; ⌓ lys; ◇ ad

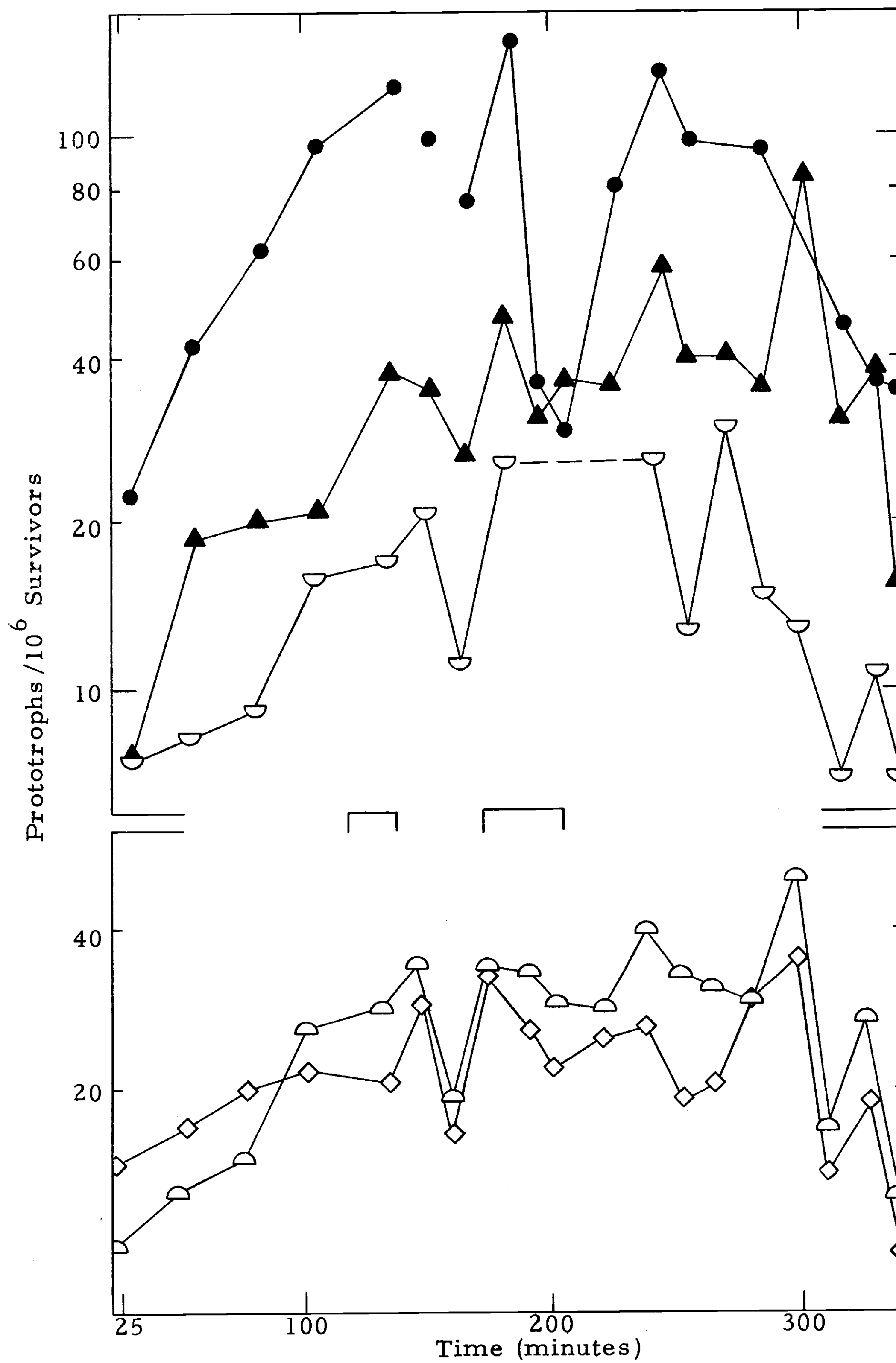
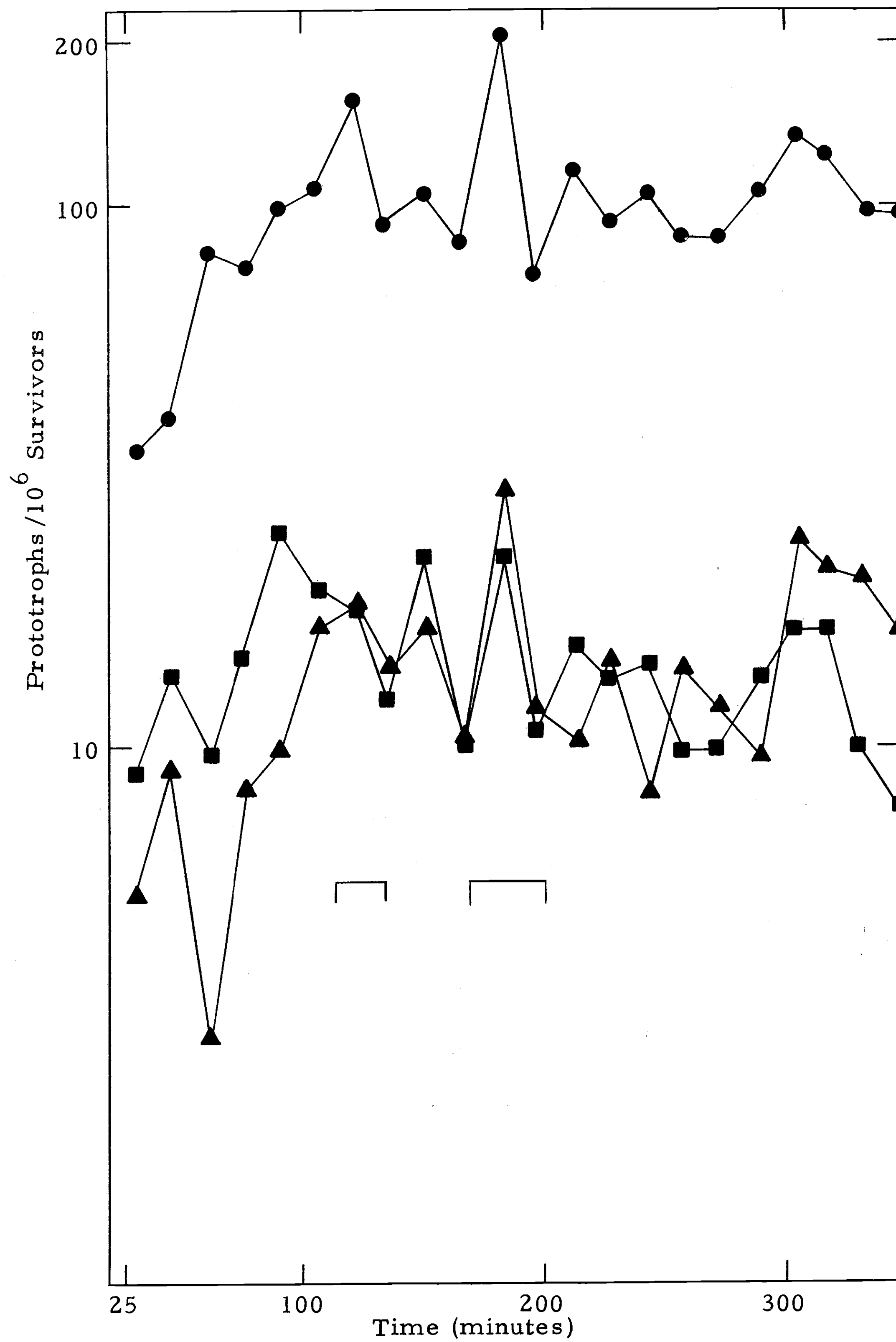

100
80
60
40
20
10
Prototrophs/10^6 Survivors
40
20
25
100
200
300
Time (minutes)

Figure 20A. Induced allelic recombination during growth of amino acid starved cells in WC medium at 25°. The bars indicate initiation and termination of amino acid starvation. NMG dose: 50 μg/ml.
● leu; ▲ met; ■ tyr; ⌓ lys; ◇ ad



200
100
10
Prototrophs/10^6 Survivors
25
100
200
300
Time (minutes)

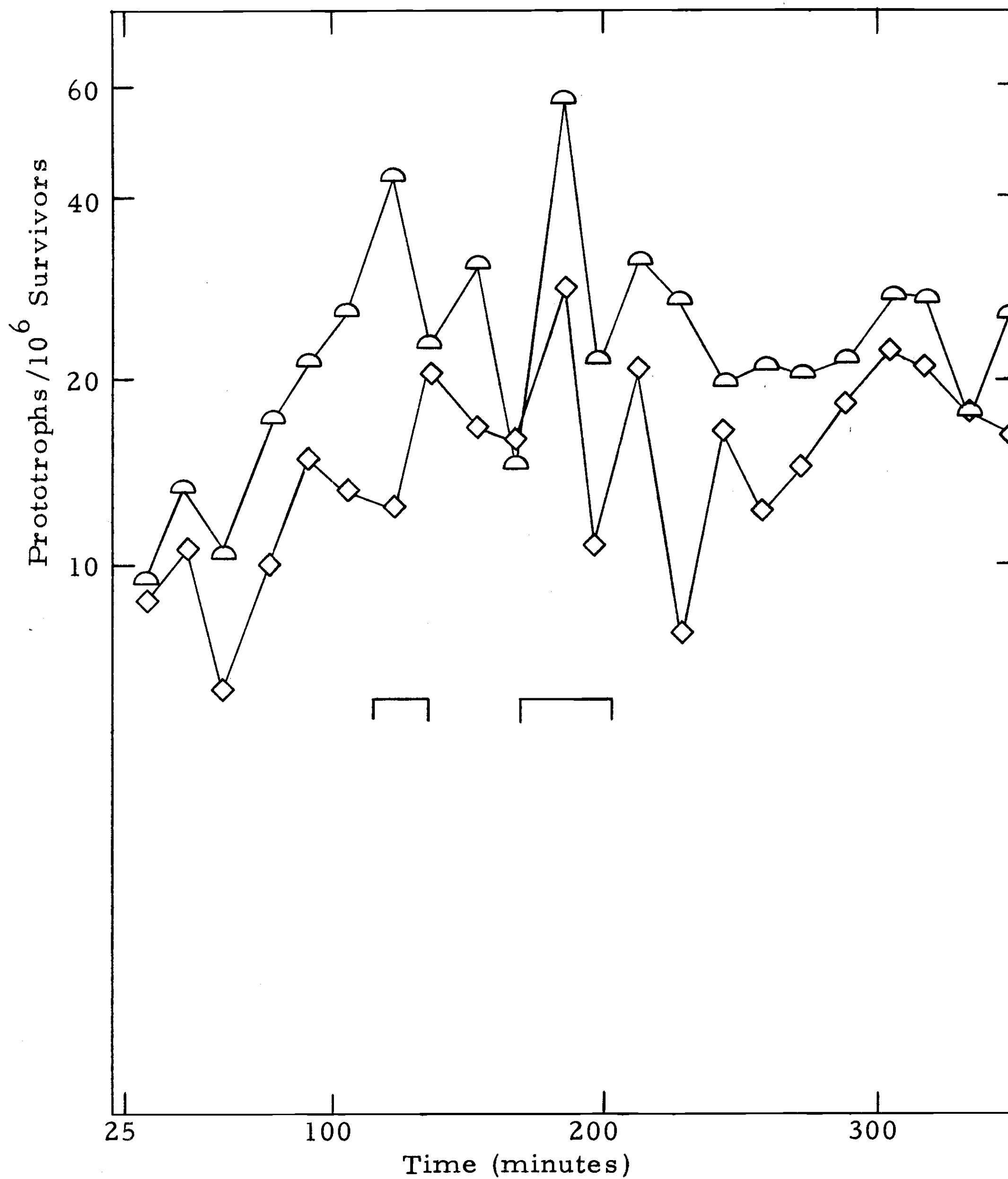


Figure 20B. Induced allelic recombination during growth of amino acid starved cells in WC medium at 25°. The bars indicate initiation and termination of DNA synthesis. NMG dose: 50 µg/ml. ● leu; ▲ met; ■ try; ⌓ lys; ◇ ad

induced for allelic recombination at the leu, met, and lys loci at various time intervals after the beginning of amino acid starvation. As shown by the glucose-supplemented amino acid-starved culture (Figure 9), there were increases in total cell number along with a slight decrease in viability after mutagen treatment by 100 minutes of amino acid starvation. In contrast, the amino acid-starved culture without glucose showed no further increase in total cell number immediately after amino acid starvation began. Viability after mutagen treatment increased in the culture deprived of both glucose and amino acids. Figure 10 shows recombination frequencies for the leu, met, and lys loci for both conditions. These graphs did not indicate more than the rapid decreases of recombination frequencies with time observed in previous experiments. However, in medium lacking glucose, the lysine marker remained unchanged in levels of induced allelic recombination.

Specific inhibition of macromolecular synthesis can also be used to investigate the possible dependence of induced allelic recombination on the metabolism of the cell. So far, specific chemical inhibition of nuclear DNA has not been observed in yeast (Wehr, 1970). However, specific inhibition of cytoplasmic protein synthesis by cycloheximide (CH) has been obtained in yeast both _in vitro_ and _in vivo_ (Lamb, Clark-Walker and Linnane, 1968; Siegel and Sisler, 1964).

It was decided to investigate whether the decrease in induced allelic recombination observed during amino acid starvation could also be observed in cultures treated with CH. Strain XS-380 was grown five generations in logarithmic culture in WC medium before treatment with 20 μg/ml CH. Measurements of viability during CH treatment, of viability after CH and NMG treatment, of total cell number, and of induced allelic recombination at all of the hetero-allelic loci were obtained. The same number of cells was used for each sample. Figure 11 indicates that CH treatment caused immediate blocking of cell division as well as progressive losses in viability; NMG, however, does not seem to have additional lethal effects on the cells treated with CH. Figure 12 shows induced allelic recombination frequencies for the same experiment. All recombination frequencies except those of the lys locus decreased in comparison to cells without CH after about 70 minutes of treatment. Except for the ad marker, allelic recombination frequencies had reached minimal values by 150 minutes.

Table 4 shows experiments with induced allelic recombination at 25 μg/ml during balanced logarithmic growth in the different media used for all of the experiments with asynchronous cultures. In time course experiments, similar values can be obtained at various sampling times. These results indicate that the frequencies of induced mitotic allelic recombination during balanced growth

remain relatively constant as long as the culture is not subjected to physiological changes.

In auxotrophic bacteria requiring amino acids, rounds of DNA replication were completed during amino acid starvation. The completion of rounds of DNA replication implied that synchronous growth could be obtained when the amino acid-starved cells were transferred to complete media (Maaloe and Kjeldgaard, 1966). However, upon readdition of amino acids, the cells were only partially synchronized for DNA replication, because rates of initiation differed among the cells. In haploid yeast Wehr (1970) observed stepwise increases in protein synthesis, total DNA synthesis, and cell number in shift-up experiments using a transfer from synthetic minimal to broth medium. These increases suggested a partial synchrony during shift-up. Synchrony was not observed for the haploid strains in complete medium after ten hours of amino acid starvation.

To determine whether partial synchrony can be observed in the diploid strain, XS-380 was starved for all required amino acids in WC medium for only two hours at 25°. The generation time of XS-380 in synthetic complete medium was 120 minutes at 25°. As shown in Figure 13, a doubling in total DNA synthesis was observed in contrast to a continuous cell division after an initial lag of 40-50 minutes. A similar stepwise increase occurred in total DNA synthesis in analogous experiments using cells starved for 3.5 hours

for amino acids. NMG-induced allelic recombination frequencies were also measured during growth of the starved cells in WC medium. Preliminary experiments (Figures 14 and 15) indicated that a peak common to all markers could be obtained with maxima between 75-100 minutes of growth after different doses of NMG and different amino acid starvation conditions. These experiments also demonstrated that the response of the ad and met loci to the mutagen was reduced at 50 μg/ml NMG. Furthermore, Figures 16 and 17 respectively show spontaneous and NMG-induced conversion frequencies plotted as prototrophs/survivors or as prototrophs/cells plated for more detailed experiments than those shown in Figures 14 and 15. The cells were subjected to 2.5 hours of amino acid starvation in WC medium lacking all required amino acids before adding the required amino acids back into the medium. Both induced and spontaneous allelic recombination frequencies were observed in corresponding experiments. As shown in Figures 13 and 18, the first peaks were observed in both leu and lys markers just prior or at the beginning of DNA synthesis (peak 1). Allelic recombination frequencies remained at a high level during the first doubling in total DNA, with a decline in frequencies occurring at 110-150 minutes of amino acid starvation.

Additional experiments were performed using 3.5 hours of amino acid starvation for all required amino acids before growth in complete medium. Figure 18 shows that DNA synthesis began 110 minutes after

the required amino acids were added. A much smaller increase in DNA was observed than in the milder starvation conditions shown in Figure 13. Growth began after a 130 minute lag period. The initial low rate of cell division was not associated with corresponding increases in total DNA synthesis. As shown in Figures 17, 19 and 20, periodic changes in recombination frequencies could also be observed. The first maximum in recombination frequencies occurred before initiation of DNA synthesis. At 200 minutes, an analogous decrease in frequencies had taken place as in Figure 16 at 150 minutes. However, total DNA had not doubled by 200 minutes, in contrast to the previous results displayed in Figures 13 and 18.

Another series of experiments was performed with synchronous cell cultures to check whether periodic increases of allelic recombination frequencies could also be observed as in the amino acid starvation experiments. For the synchrony experiments, cells were grown in balanced growth for four to six generations in broth medium supplemented with the required amino acids and adenine at 30°. The cells were then harvested during midlog phase and concentrated. The single unbudded cells were separated after centrifugation on sucrose density gradients by the procedure of Mitchison and Vincent (1965). The collected cells were then suspended on the same prewarmed broth medium after washing with distilled water to remove the sucrose. The sizing procedure presented several problems which affected the

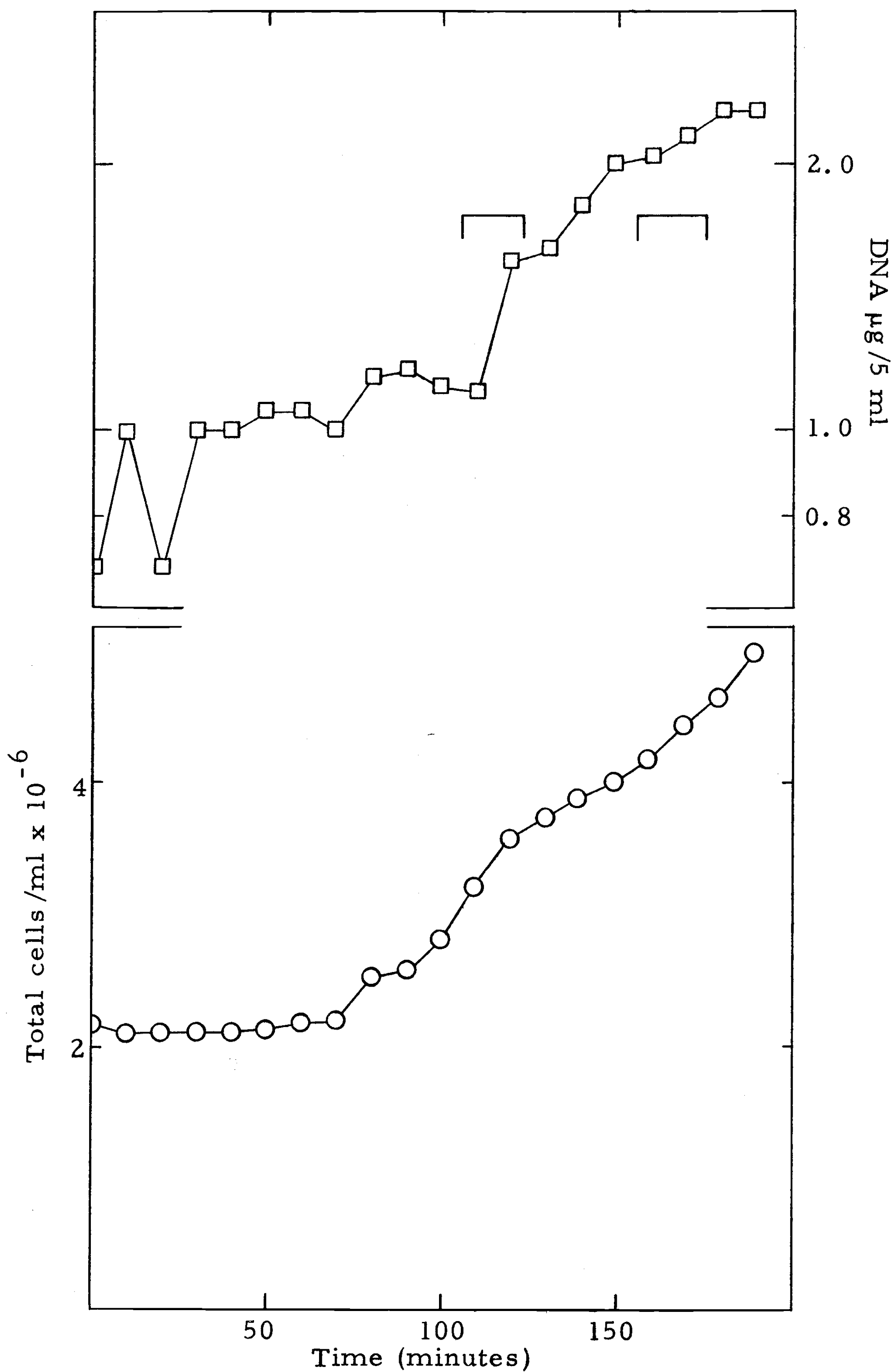


Figure 21. Total DNA synthesis and cell number in a culture synchronized by sizing on sucrose density gradients. Growth in YCM+ at 30°. The bars indicate initiation and termination region of DNA synthesis. □ total DNA synthesis; ○ cell number

Figure 22. Cell division in synchronous culture in YCM+ medium at 30°.

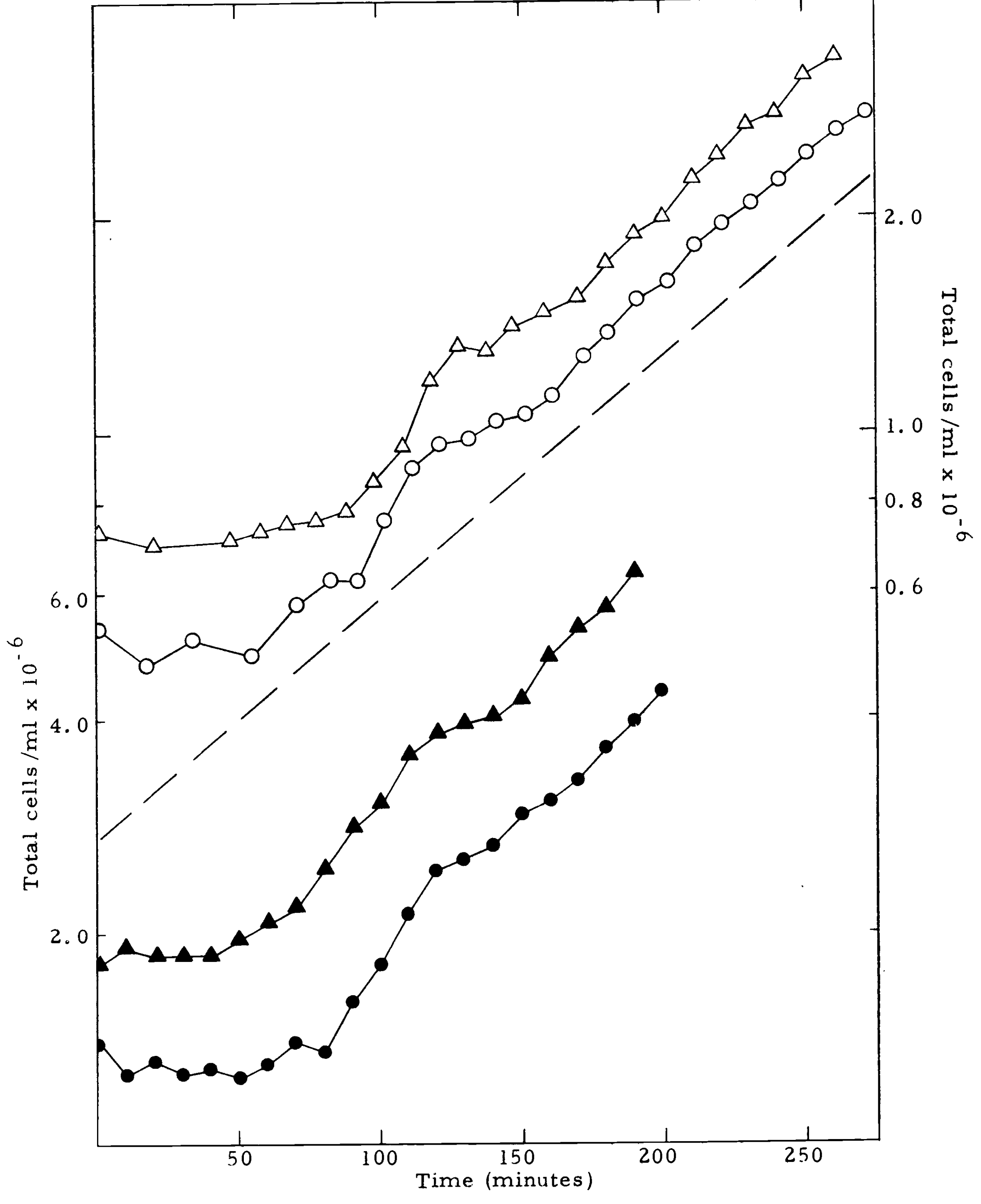
△ cell division after sizing at 18°

○ cell division after sizing at 21° in conditioned medium

▲ cell division after sizing at 15°

● cell division after sizing at 4°

- - - asynchronous growth at 30°



Total cells /ml x 10⁻⁶
6.0
4.0
2.0
Total cells/ml x 10⁻⁶
2.0
1.0
0.8
0.6
50
100
150
200
250
Time (minutes)

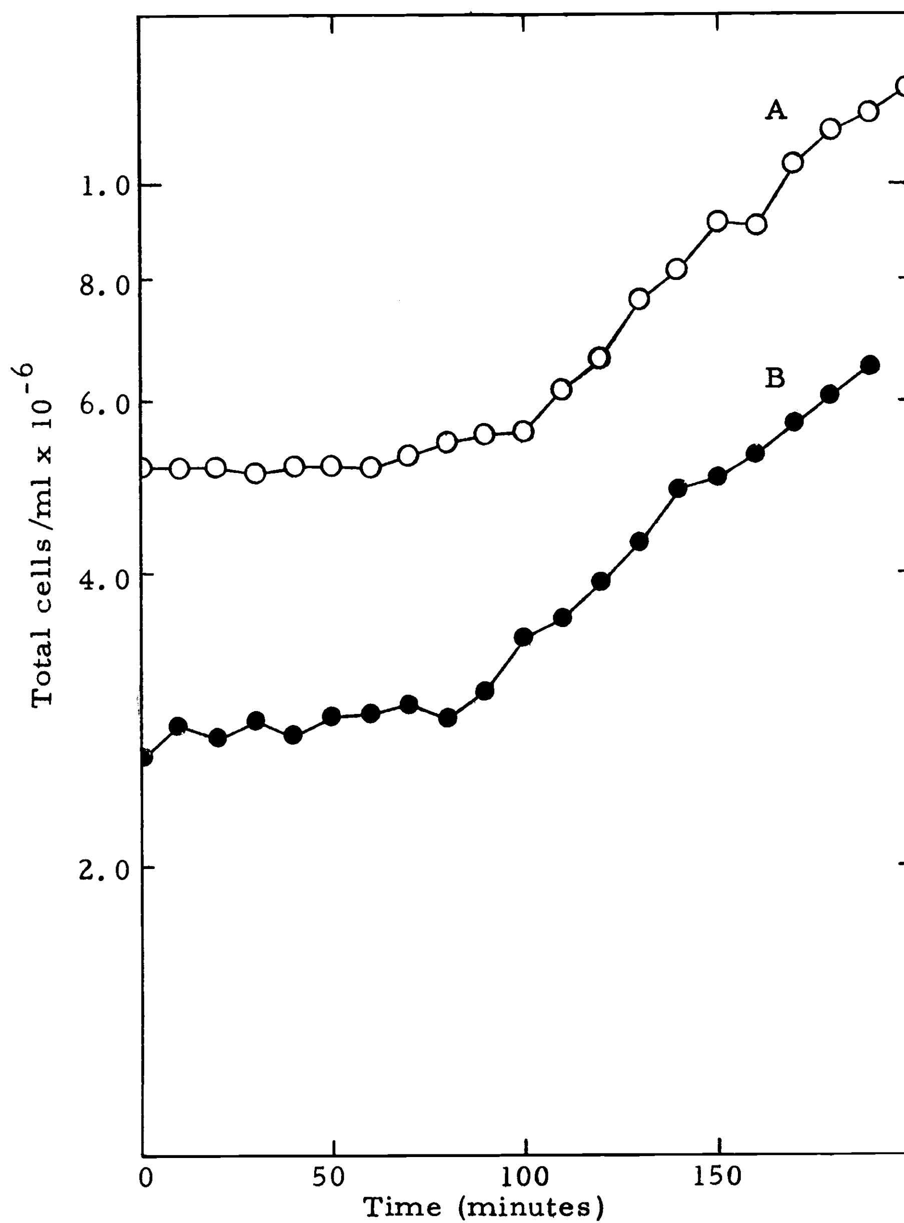


Figure 23. Effect of combined amino acid starvation and sizing procedures on cell division.
○ cell number (A); ● cell number (B)

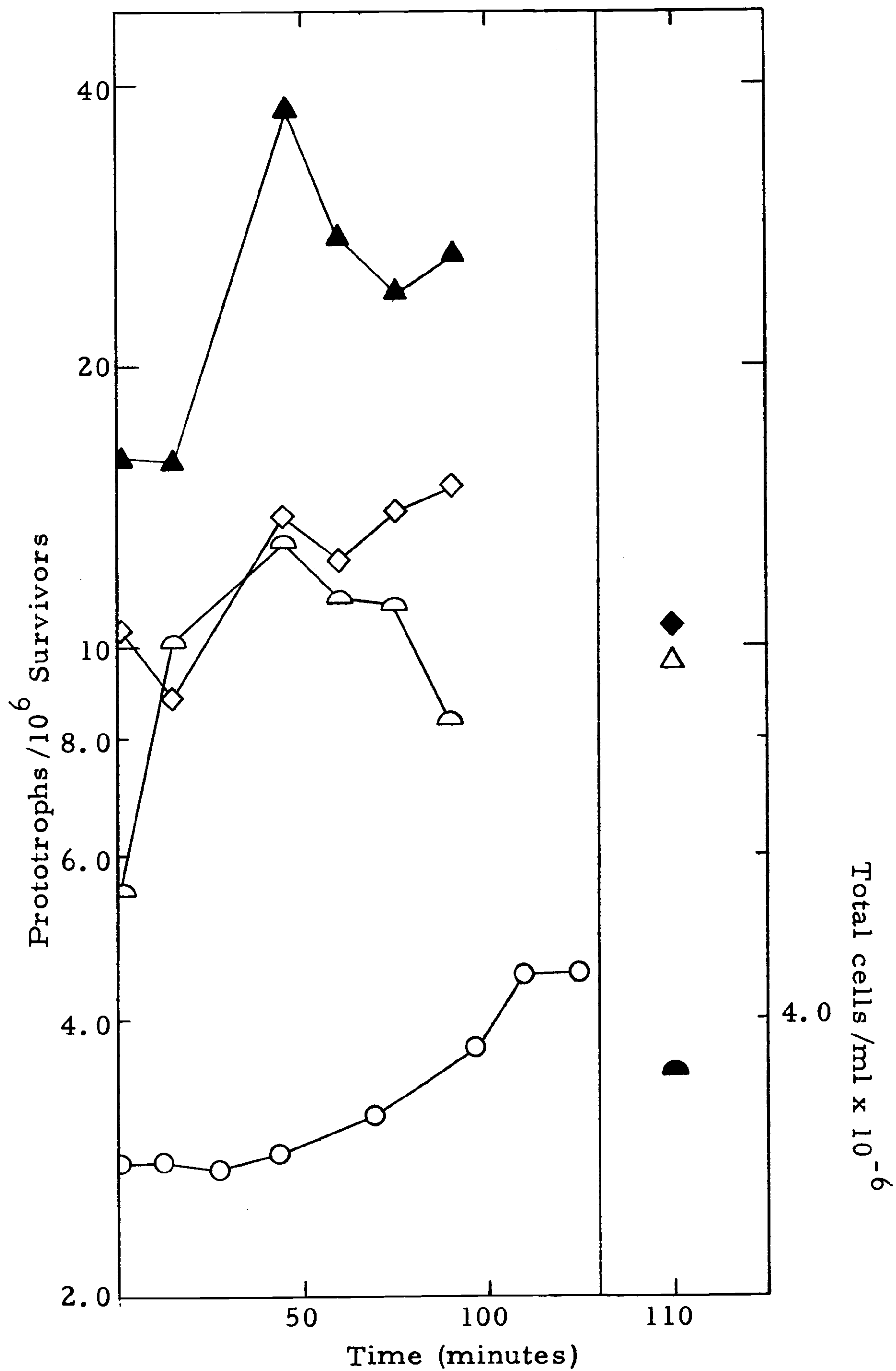


Figure 24. NMG-induced recombination in an asynchronous culture previously subjected to the sucrose density gradient sizing procedure. NMG dose: 25 μg/ml. ▲ met, induced; △ met, spontaneous; ◇ ad, induced; ◆ ad, spontaneous; ⌓ lys, induced; ◖ lys, spontaneous; ○ cell number

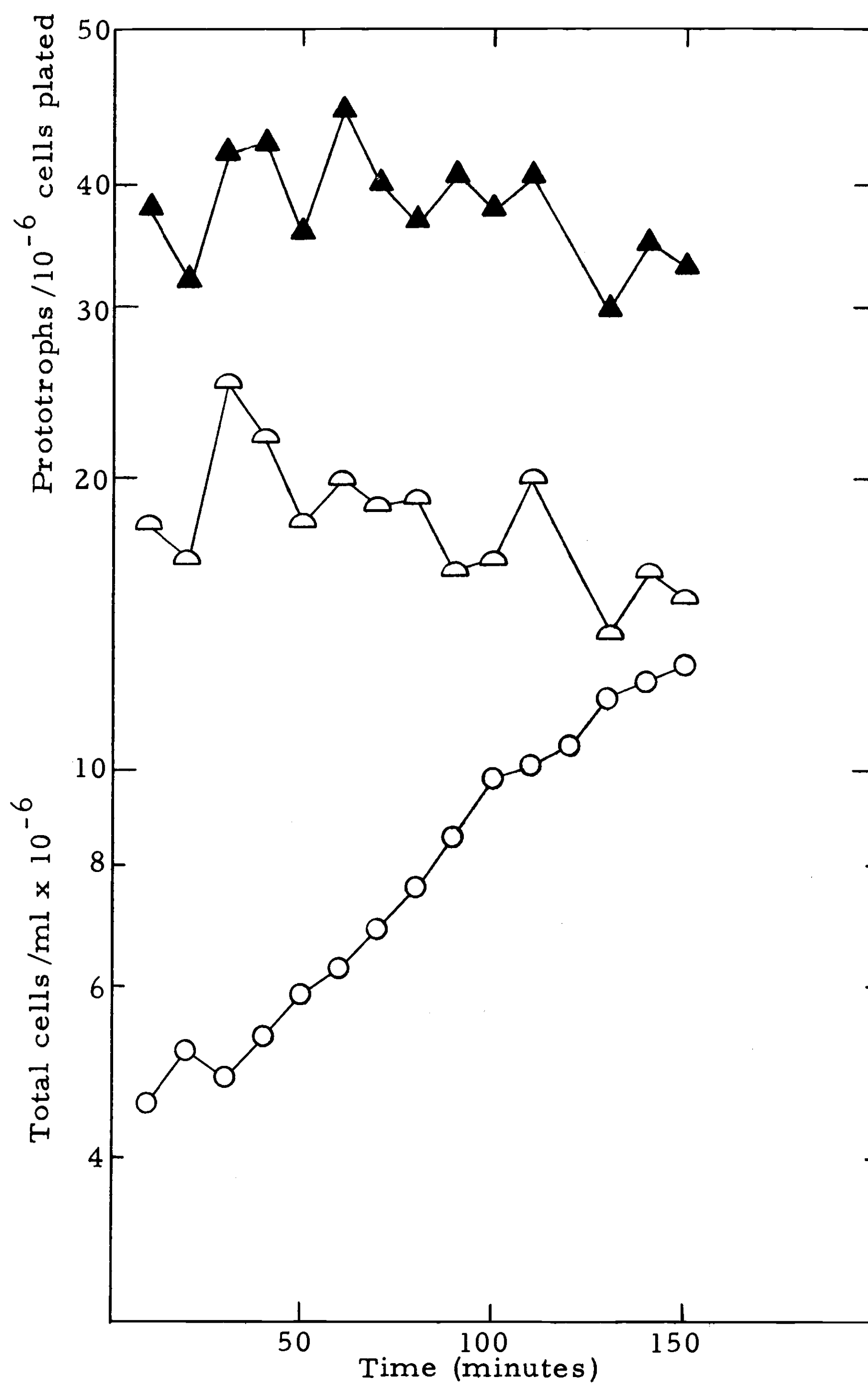


Figure 25A. NMG-induced recombination in an asynchronous culture in YCM+ medium at 30° previously subjected to the sucrose gradient procedure. NMG dose: 35 μg/ml. ▲ met; ⌓ lys; ○ cell number

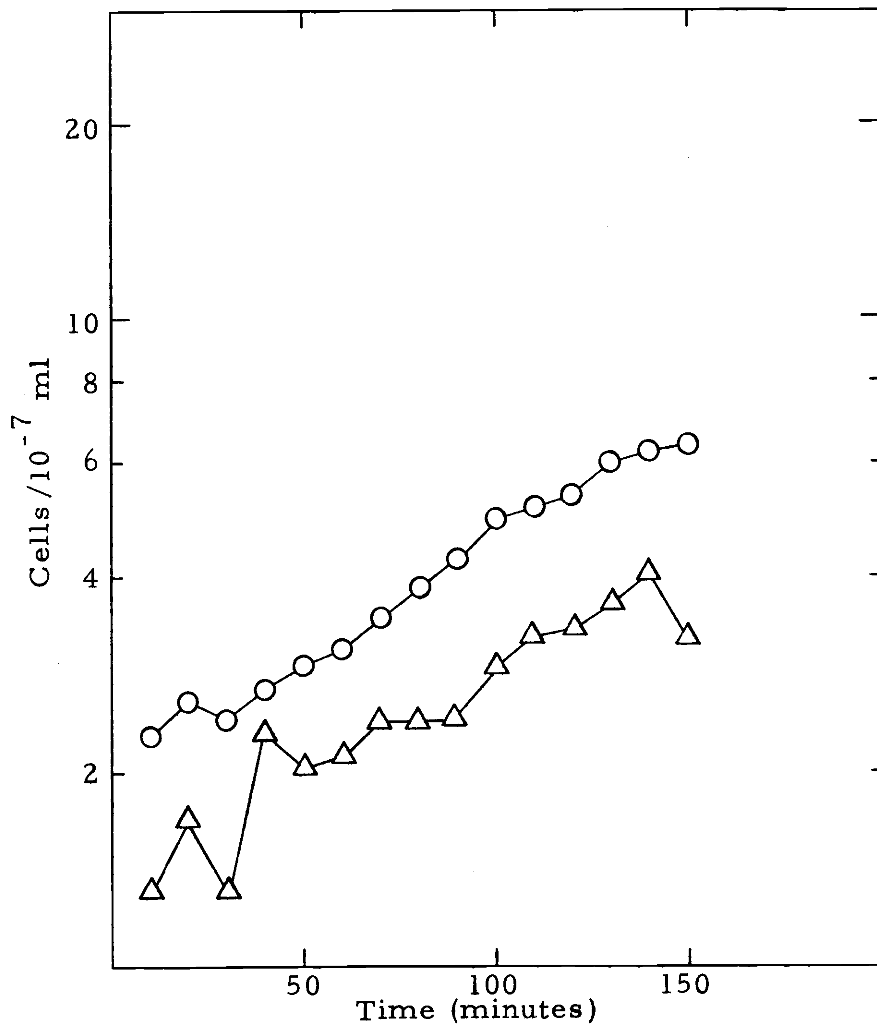


Figure 25B. Cell number and viability for the experiment shown in Figure 25A. NMG dose: 35 μg/ml.
○ cell number; △ viability

degree of synchrony and the duration of the experiments with synchronous cell cultures. At temperatures greater than 15°, the cells apparently assimilated the sucrose to produce gas which agitated the sized cells to some extent. It would have been better to use gradients made up materials that were not utilized by the cells. However, Dextran, Ficoll, Renograffin, and glycerol gradients all failed to either give adequate sizing or enough cell yield for the experiments.

Figure 21 indicates that only one cycle of synchronous total DNA synthesis and cell division can be obtained with the Mitchison and Vincent procedure. The initial variation in points represents values at the limits of resolution of the fluorometric assay. Apparently the DNA synthesis necessary for the first cell division has already taken place. A small increase in total DNA synthesis is observed from 75-100 minutes of growth which probably represents asynchronous DNA synthesis. A doubling in total DNA synthesis is observed before the second cycle of cell division started at 150 minutes. In several growth experiments, Figure 22 shows that a similar degree of synchrony is obtained during the first division cycle using: 1) fresh broth medium; 2) conditioned medium in which cells had been grown to early log phase; 3) different temperatures during sizing treatment. As shown in Figure 23, starvation for amino acids combined with the sizing procedure did not result in any greater degree of synchrony. Curve A, Figure 23, represents an experiment in which cells in

logarithmic growth in synthetic complete medium were harvested, suspended for two hours in synthetic minimal medium plus adenine, and sized before growth in complete synthetic medium. Curve B, Figure 23, represents an experiment in which cells were grown to mid-log phase in supplemented broth medium and then were harvested and sized. The single unbudded cell fraction was then starved for two hours in synthetic minimal plus adenine before reinoculation into YCM+ medium.

In order to show that variation in induced allelic recombination frequencies was due to the synchronous growth of the culture, several control experiments were performed. In these experiments, the cells were subjected to the sizing procedure as shown for the synchrony experiments. However, after sizing the gradients were thoroughly mixed to again produce an asynchronous population. The same procedures were then followed as in the synchrony experiments. Figures 24 and 25A show that induced allelic recombination frequencies vary significantly during the first 50-70 minutes of growth. Figure 24 shows that frequencies of allelic recombination induced by 25 μg/ml NMG are significantly higher than several control samples of spontaneous allelic recombination frequencies taken at 110 minutes of growth. Figure 25A shows allelic recombination frequencies induced with 35 μg/ml NMG after allowing growth of an asynchronous culture. The data display a general decline in recombination frequencies after

100 minutes of growth. Measurements of increases in total cell number in both Figures 24 and 25B indicate that a balanced logarithmic growth rate had not been established by 200 minutes of growth. Moreover, Figure 25B demonstrates that the variability in asynchronous recombination frequencies could not be due to large changes in viability after mutagen treatment.

Figures 26A and 27A show plots of spontaneous recombination frequencies during 200 minutes growth after sizing. Although different methods of sampling were used, both experiments indicated that a similar degree of variation existed in recombination frequencies for different markers. The experiment shown in Figure 26B used a constant number of cells per sample (6×10^5 cells/plate). The experiment shown in Figure 27 used a constant volume of culture, which meant that a constantly increasing number of cells was plated in each sample. The starting concentration of cell samples shown in Figure 27 was 6×10^5 cells/plate. The levels of spontaneous allelic recombination frequencies were similar for the met and lys markers used in both experiments. Figure 27B shows that no significant changes in viability occur during spontaneous recombination after suspending the cells in acetate buffer without the mutagen, using the previously described conditions for mutagenesis.

Figures 28-30 show the variation of induced allelic recombination frequencies of several markers during synchronous growth using

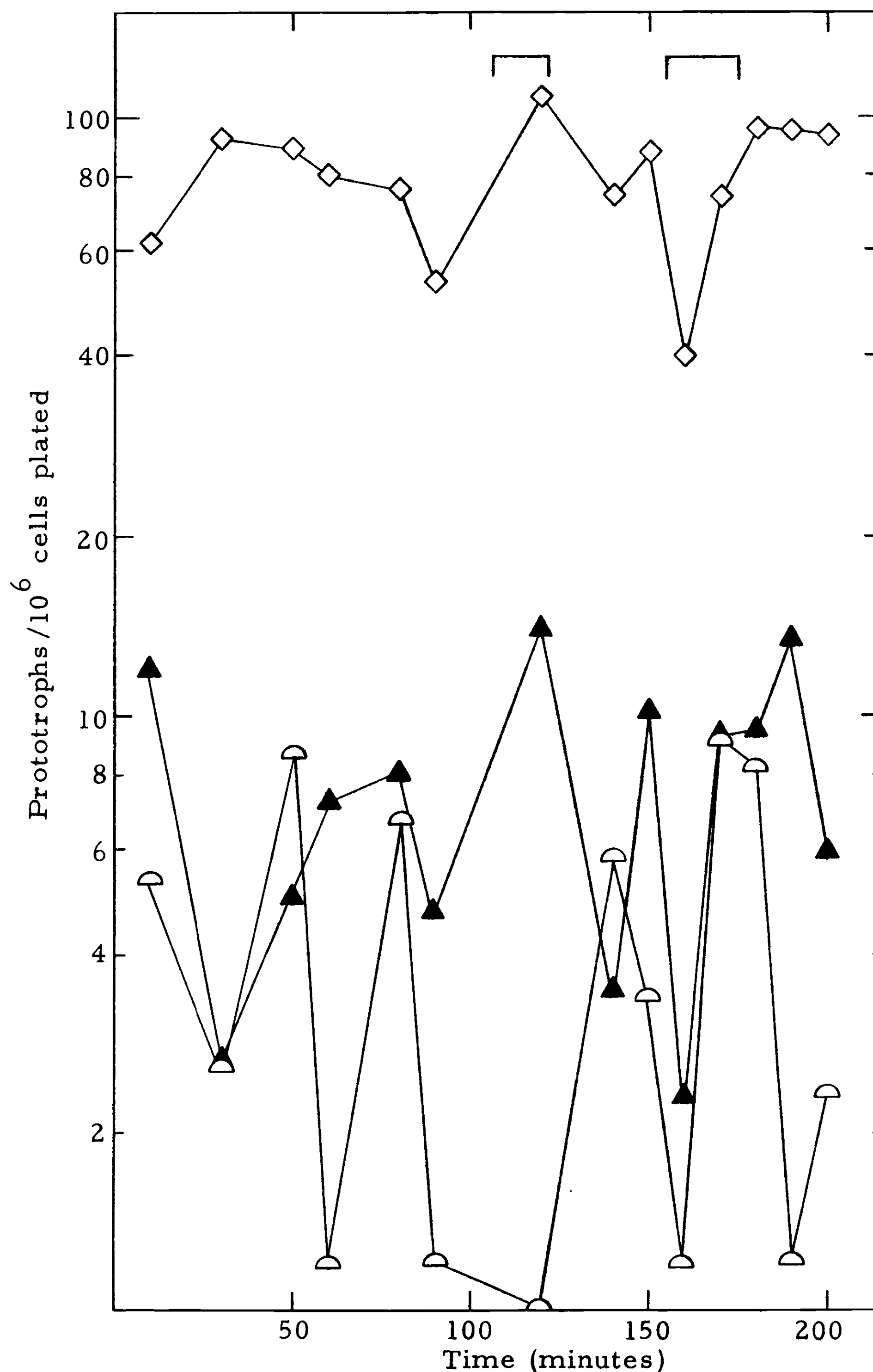


Figure 26A. Spontaneous allelic recombination during synchronous growth in YCM+ medium at 30°. The bars respectively indicate initiation and termination of the first round of DNA synthesis. NMG dose: 0 μg/ml. ◇ ad; ▲ met; ⌓ lys

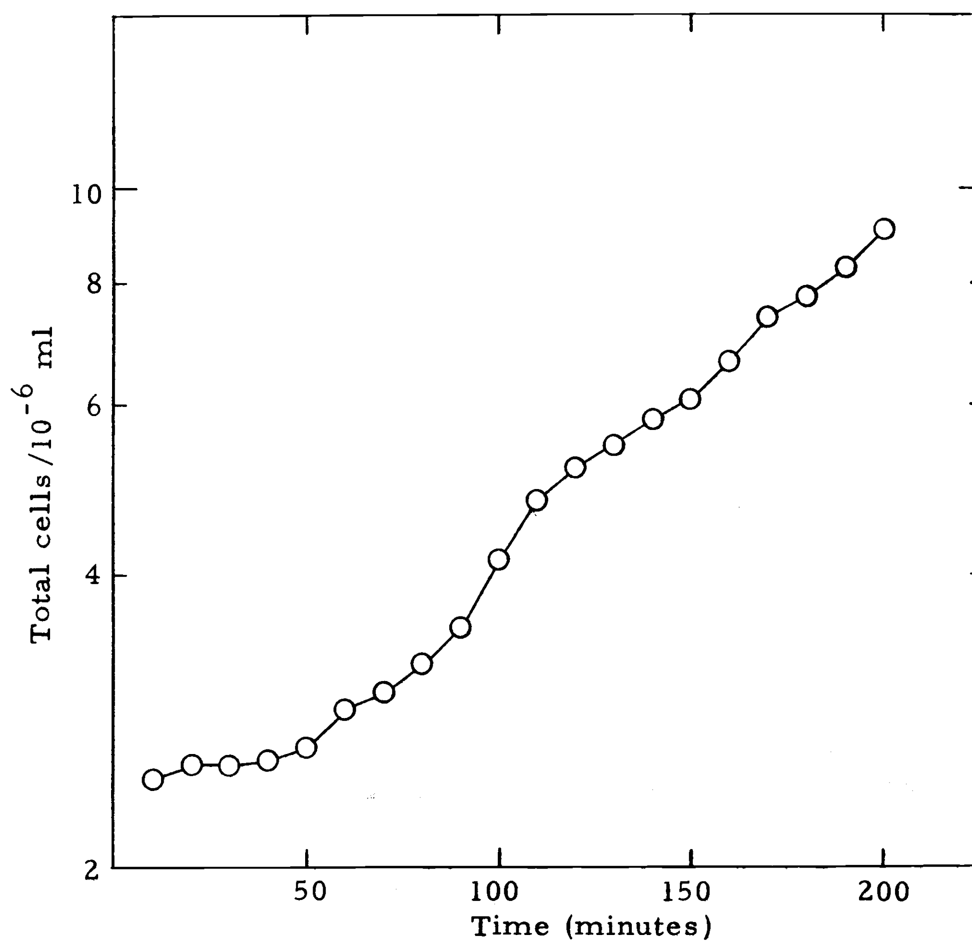
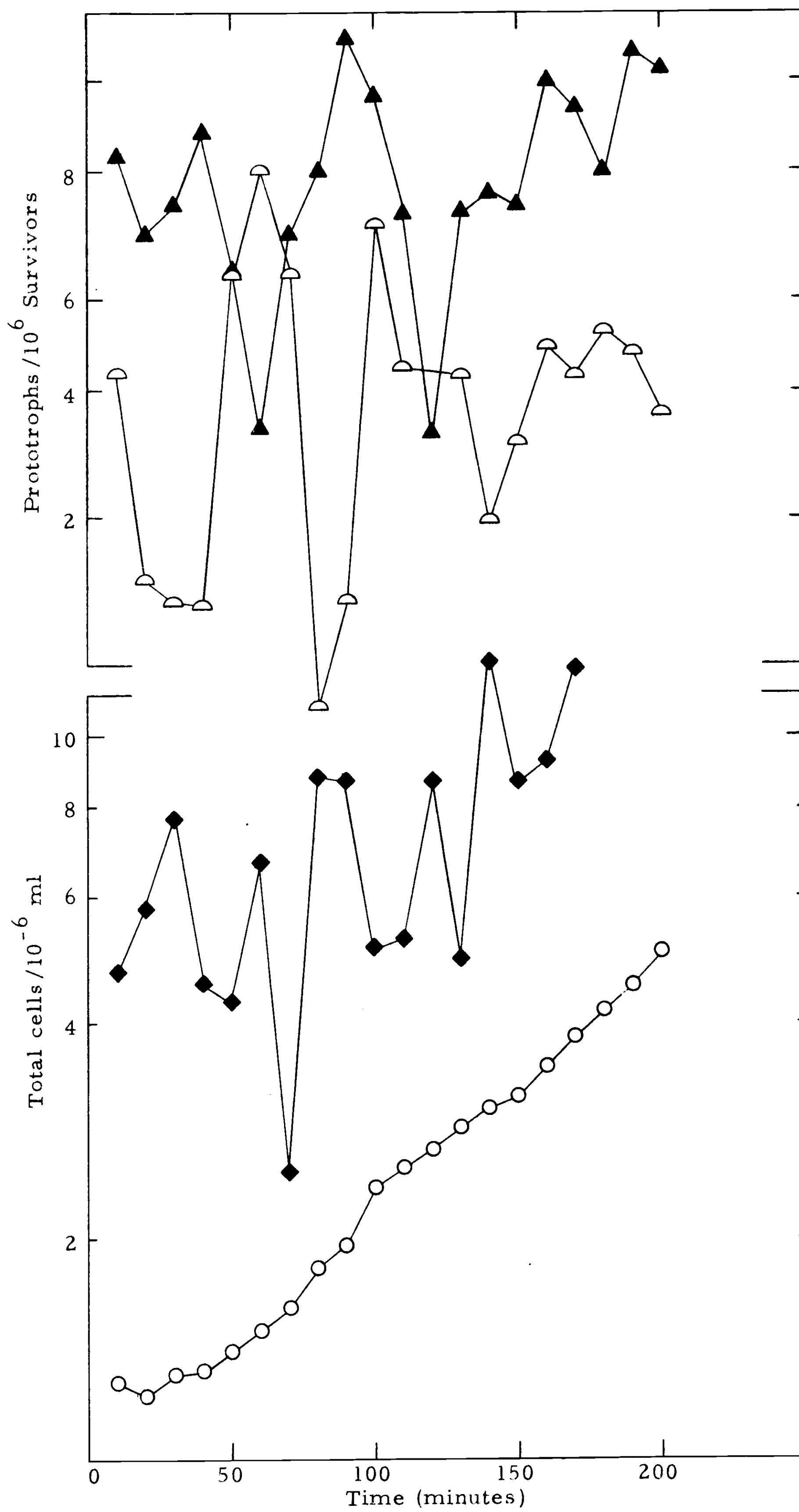


Figure 26B. Cell numbers for the experiment shown in Figure 26A.
○ cell number

Figure 27A. Spontaneous allelic recombination during synchronous growth in YCM+ medium at 30°. NMG dose: 0 μg/ml.
▲ met; ⌓ lys; ◆ tryp; O cell number


Prototrophs /10^6 Survivors
8
6
4
2
Total cells /10^-6 ml
10
8
6
4
2
0
50
100
150
200
Time (minutes)

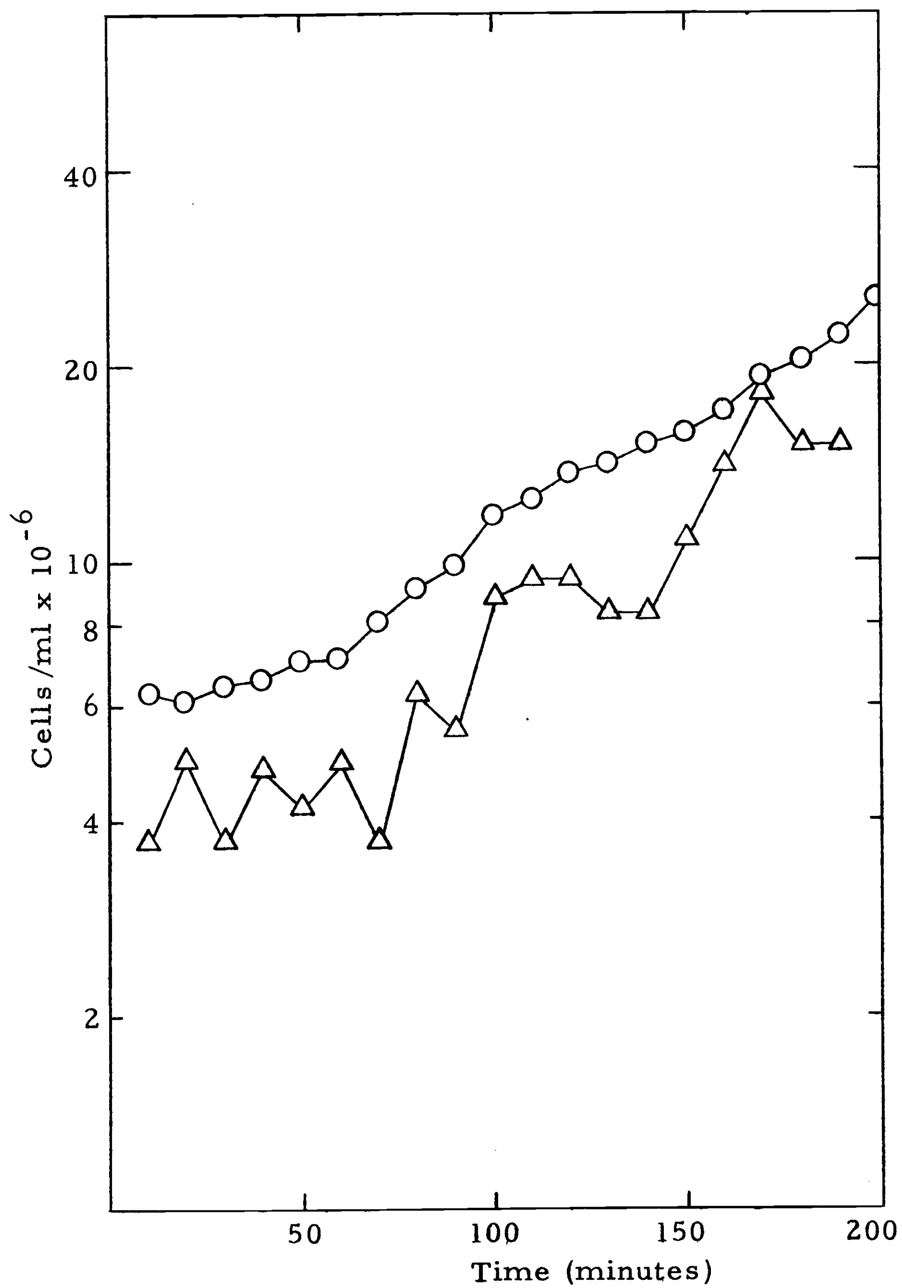
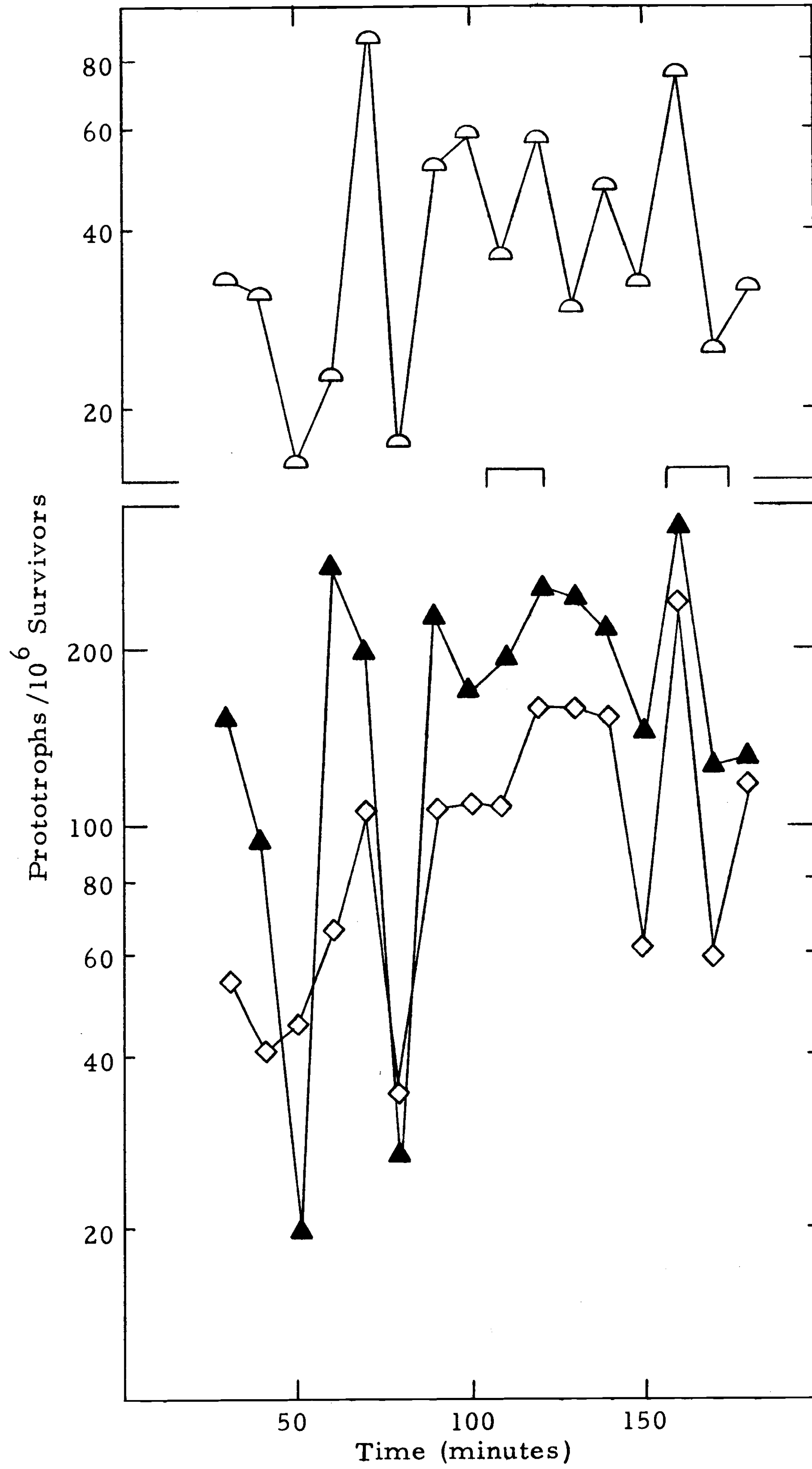


Figure 27B. Cell number and viability for the experiment shown in Figure 27A.
○ cell number; △ viability

Figure 28A. Induced allelic recombination during synchronous growth in YCM+ medium. The two bars respectively indicate initiation and termination of DNA synthesis. NMG dose: 25 μg/ml.
⌓ lys; ▲ met; ◇ ad



80
60
40
20
200
100
80
60
40
20
Prototrophs /10^6 Survivors
50
100
150
Time (minutes)

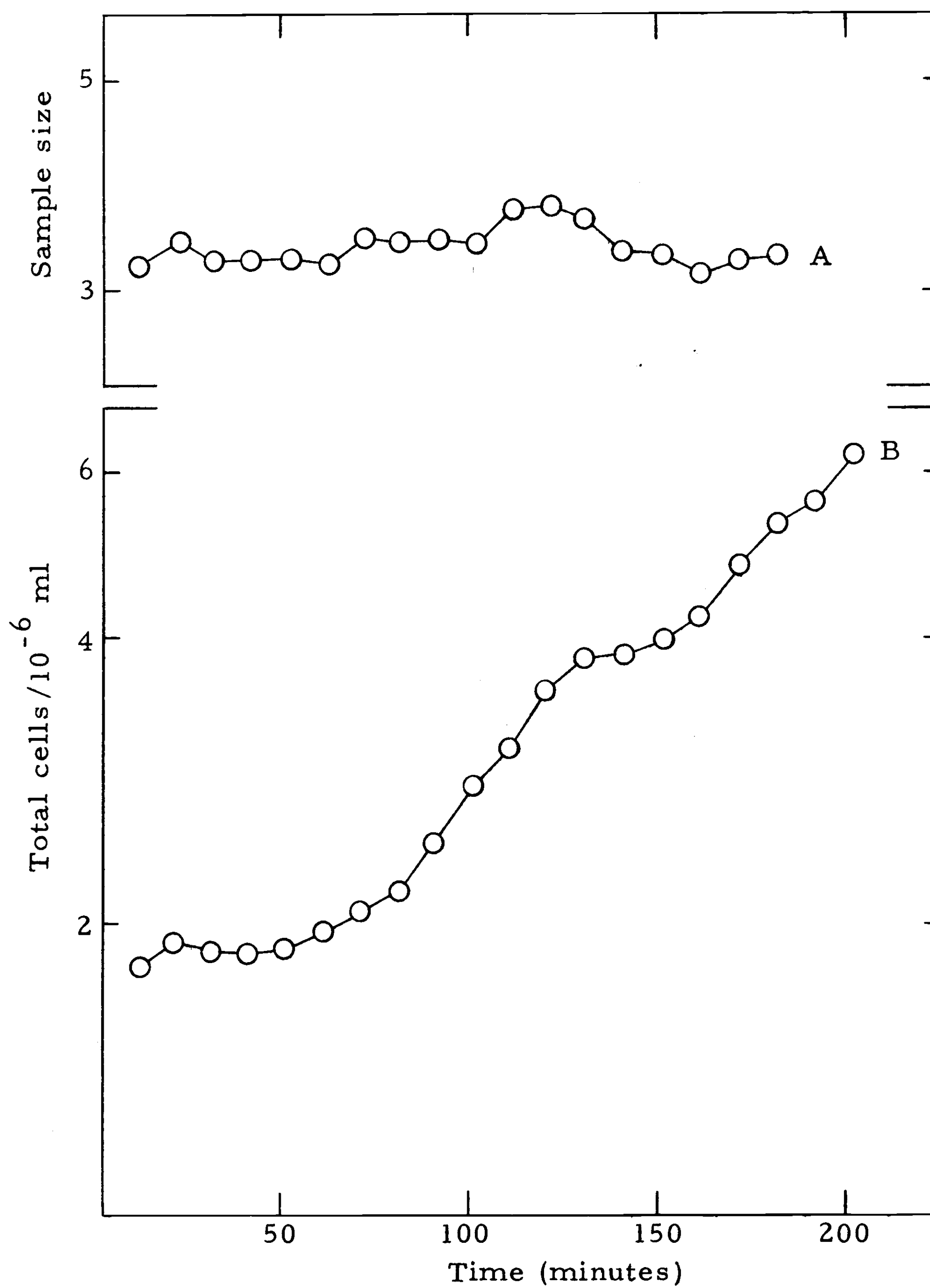


Figure 28B. Cell number for the experiment shown in Figure 28A. A. Sample size; B. Cell number during growth.

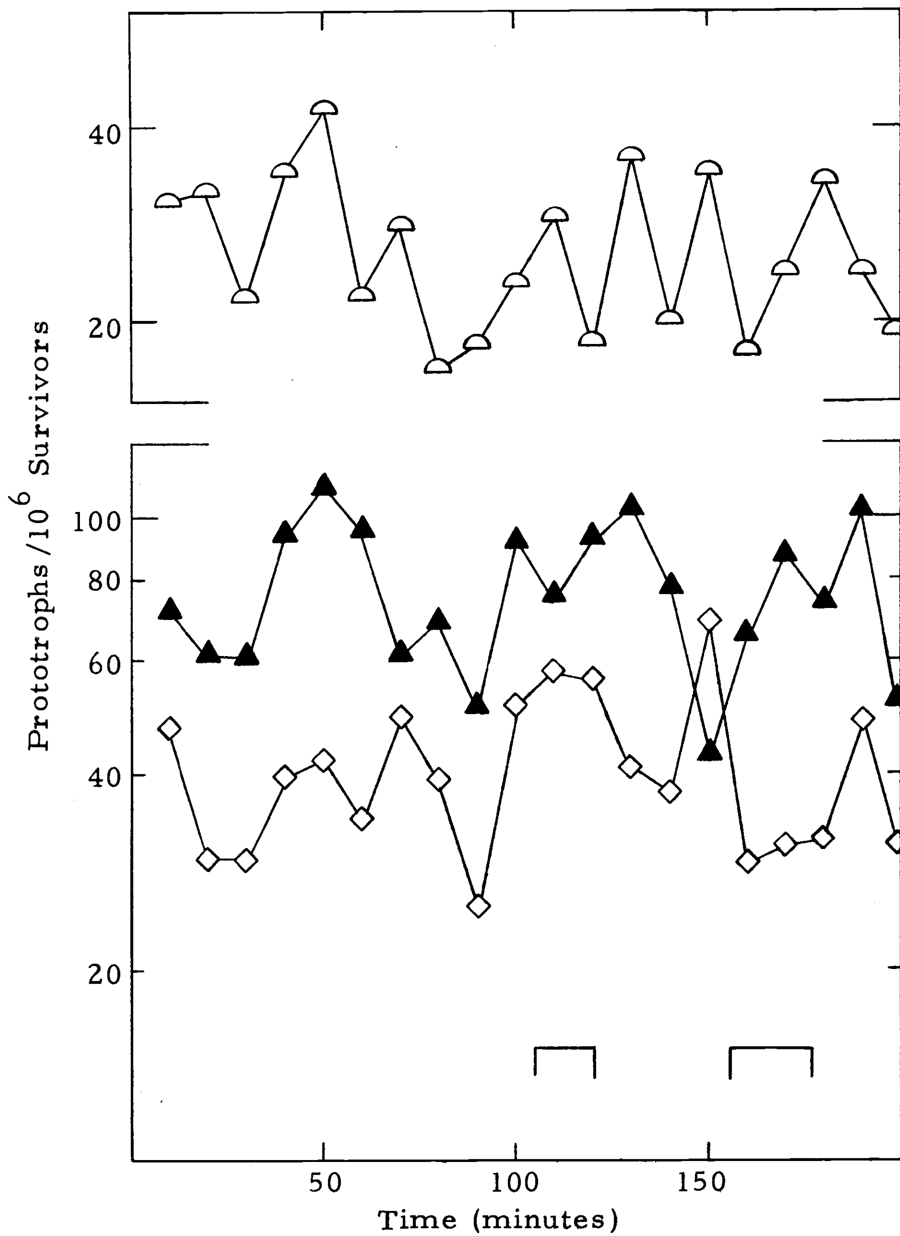


Figure 29A. Induced allelic recombination during synchronous growth in YCM+ medium at 30°. NMG dose: 35 μg/ml. The two bars indicate respectively initiation and termination of first round of DNA synthesis.
⌓ lys; ▲ met; ◇ ad

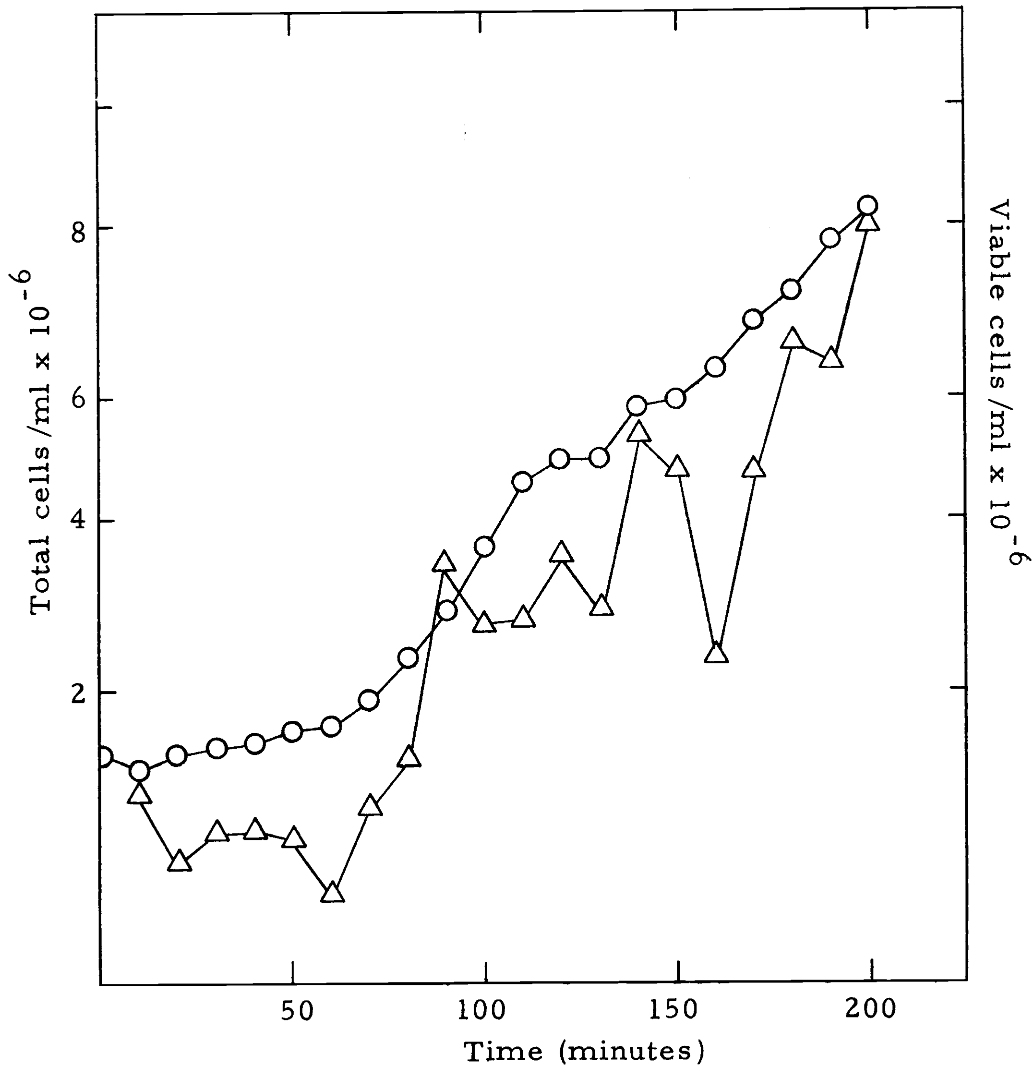


Figure 29B. Cell number and viability for the experiment shown in Figure 29A.
○ cell number; △ viability

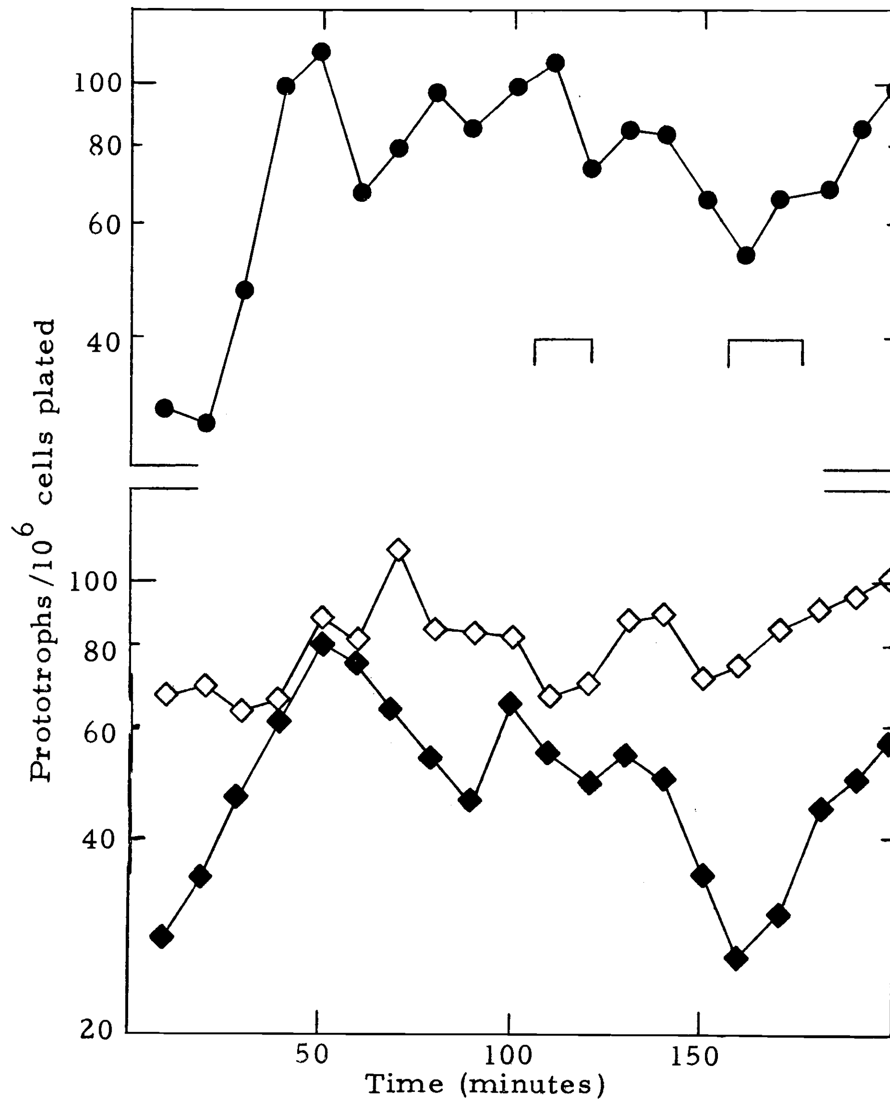


Figure 30A. Induced allelic recombination during synchronous growth in YCM+ medium at 30°. The bars indicate respectively initiation and termination of the first round of DNA synthesis. NMG dose: 40 μg/ml.
● leu; ◇ ad; ◆ tryp

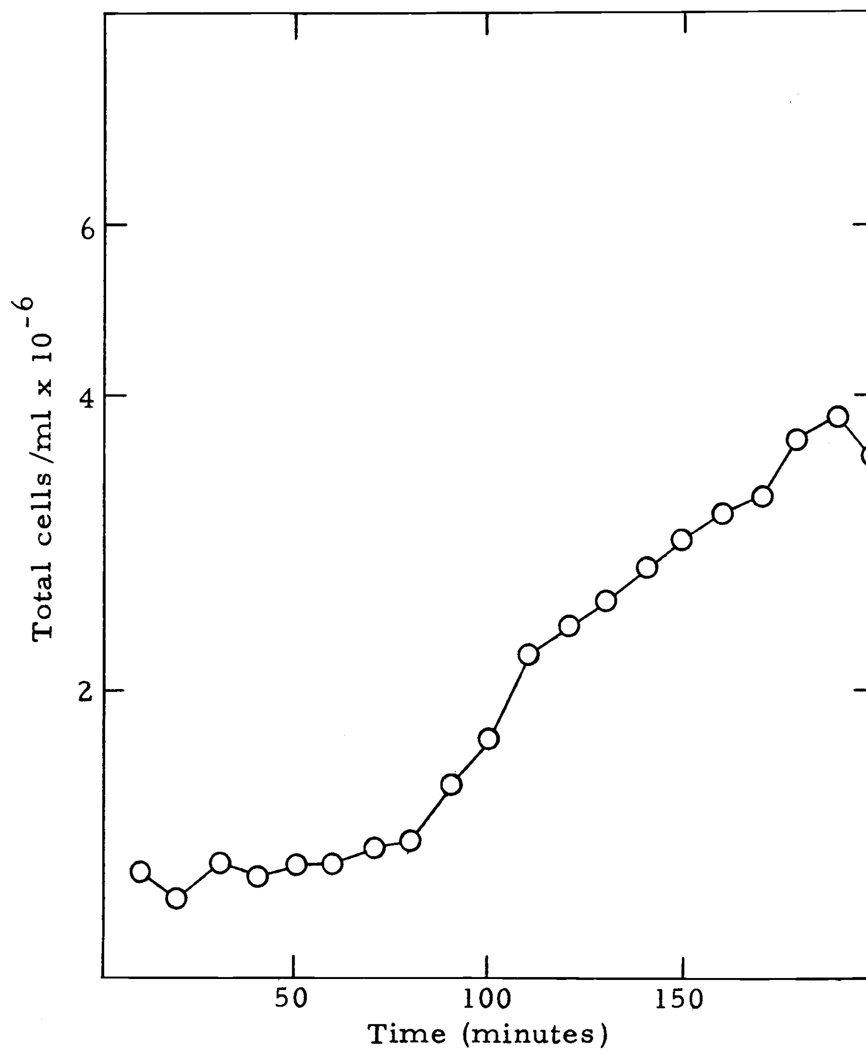


Figure 30B. Cell number for the experiment shown in Figure 30A.

20-40 μg/ml NMG to induce allelic recombination. The experiments shown in Figures 28 and 29 were performed using constant numbers of cells per sample as described above for Figure 26A. The experiment shown in Figure 30 used constant volumes of cells per sample. The time of initiation of total DNA synthesis (Figure 21) and the approximate time of doubling is shown by the bars on the graphs. Peak 1 definitely occurred before total DNA synthesis started. Another general increase in induced recombination frequencies occurred at the time of initiation of DNA synthesis. A general decline in recombination frequencies then occurred to a minimal value after 140-160 minutes of growth. This coincided with the termination of DNA synthesis. The decline to a minimum at 150-160 minutes was especially noticeable in Figure 30 where very large numbers of cells were used for each sample.

Comparisons between the synchronous control experiments showing spontaneous recombination frequencies (Figures 26A and 27A) indicate that the ad marker does not show a proportional response to the mutagen dose used. However, the lys , met, and tryp markers do show increased recombination frequencies when the induced allelic recombination frequencies are compared to the spontaneous recombination frequencies. As shown in Figure 29B, the higher doses of mutagen do not seem to produce sudden changes in viability after treatment with NMG.

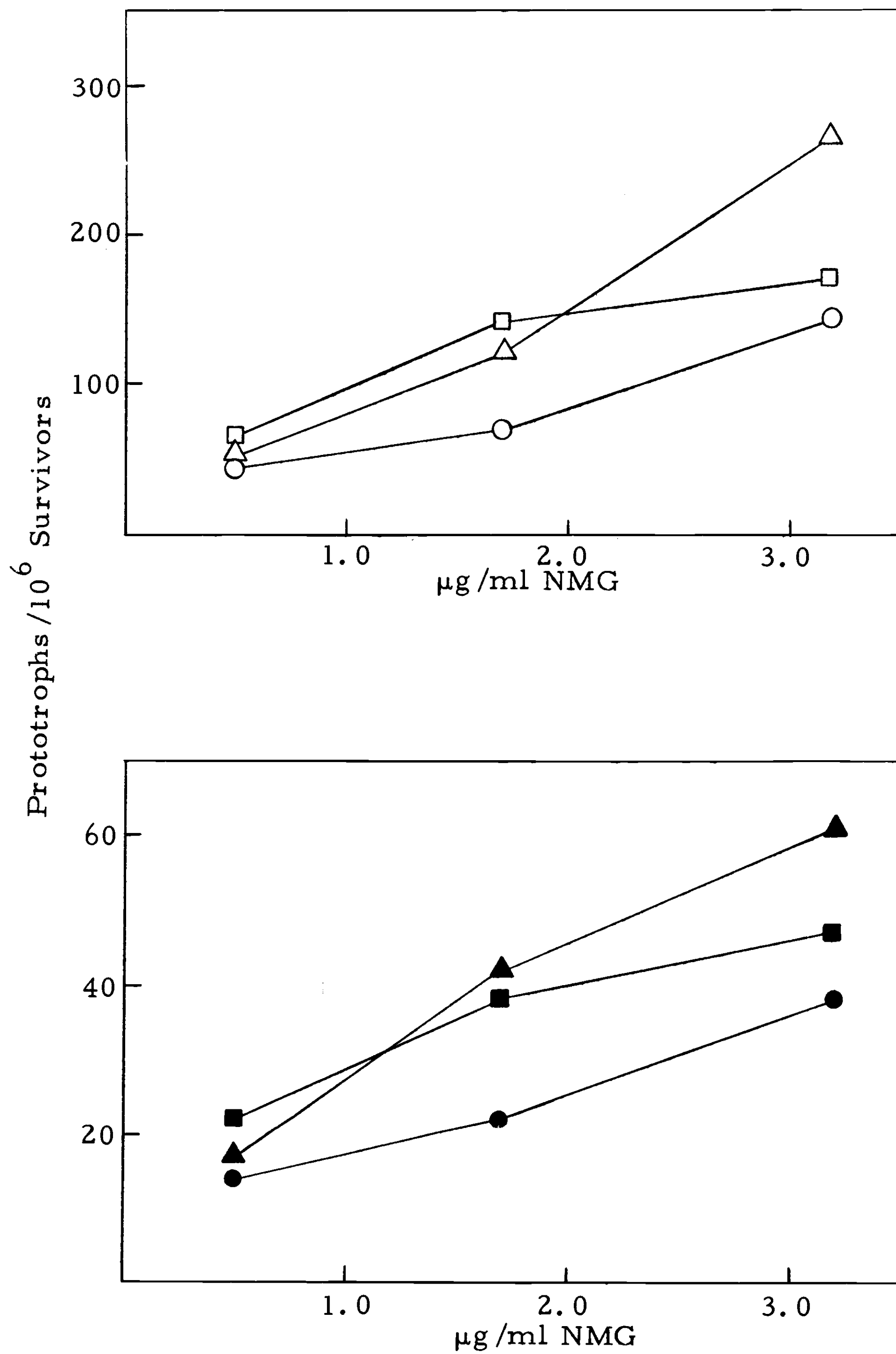


Figure 31. Mutagen dose response of the leu and lys loci during active growth as a function of NMG concentration in YCM+ at 30°.
leu: ○ 15 minutes; △ 33 minutes; □ 45 minutes
lys: ● 15 minutes; ▲ 33 minutes; ■ 45 minutes

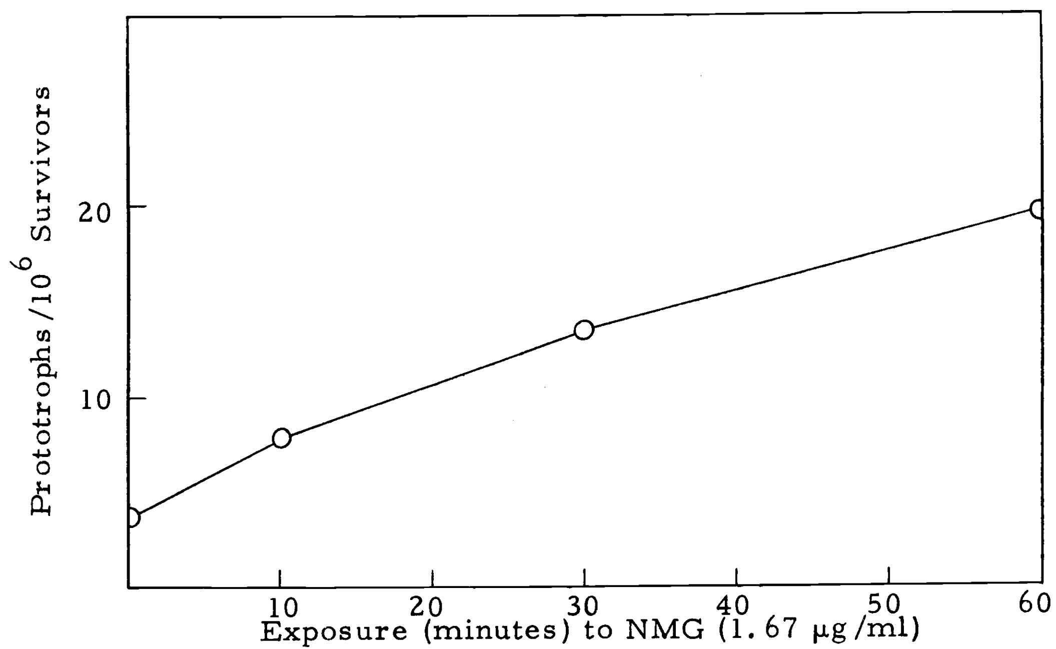


Figure 32. NMG-induced recombination at the tyr locus as a function of time during active growth in YCM+ medium at 30°. NMG dose: 1.67 μg/ml.
O tyr

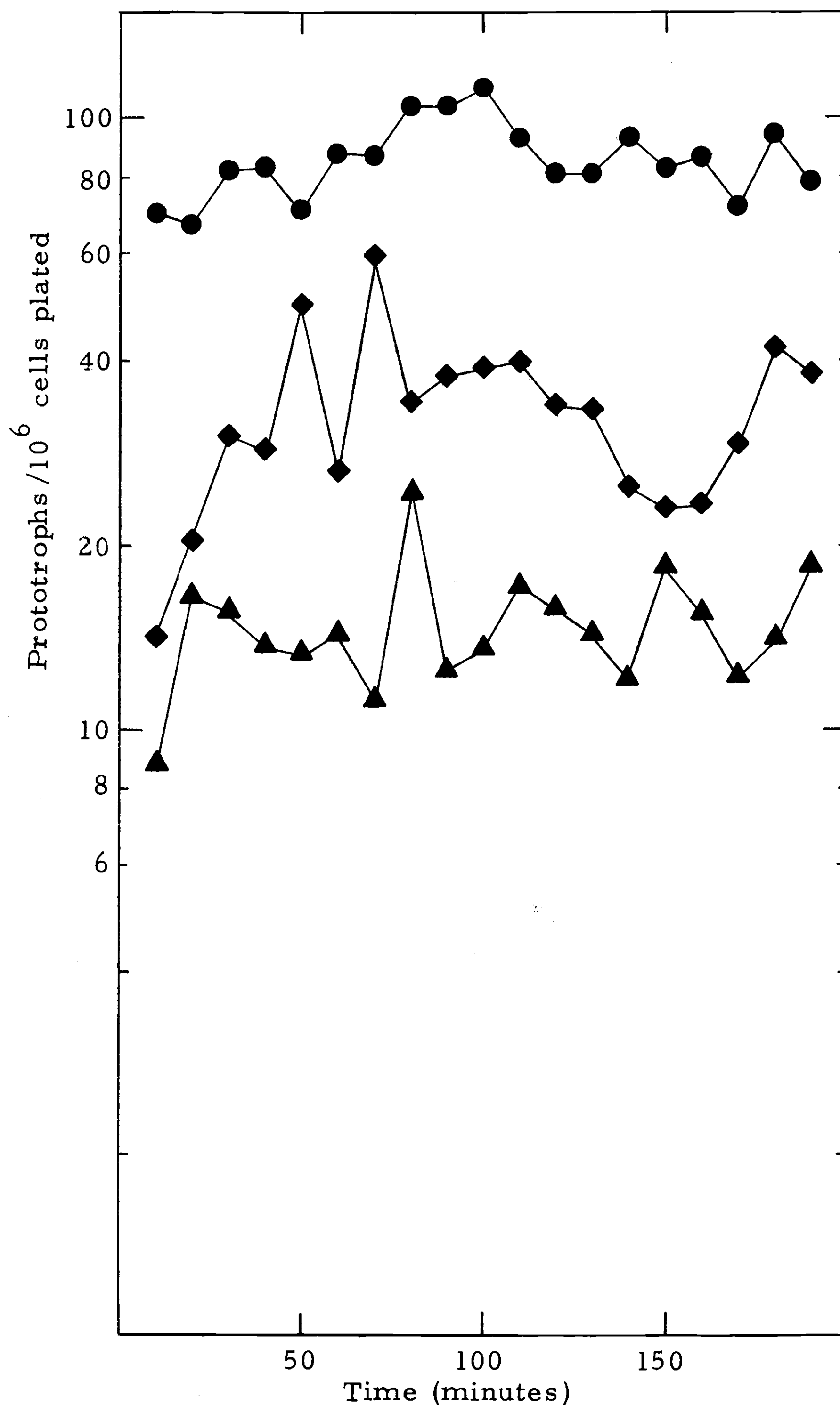


Figure 33. NMG-induced allelic recombination during synchronous growth when cells were treated during active growth in YCM+ medium at 30°. NMG dose: 2 μg/ml, 15 minutes. ● leu; ◆ tryp; ▲ met

Table 5. Viable counts for NMG treatment of actively growing cultures in the experiment shown in Figure 31.

Time (min)	μg/ml NMG		
	0.5	1.6	3.1
15	1.58[a]	1.99	1.84
33	1.14	1.52	1.37
45	1.87	1.85	1.92

[a]All values x 10^7 cells/ml.

Table 6. Viable count after addition of NMG to actively growing cells in the experiment shown in Figure 32.

Time (min)	Total cell counts	Viable cell counts
15	1.23[a]	1.11
30	1.65	9.9[b]
60	1.89	1.37

[a]All values x 10^7 cells/ml except as indicated.

[b]Value x 10^6 cells/ml.

The mutagen dose response of cells during active growth at very low concentrations was also investigated. As shown in Figure 31, the mutagen dose response curves indicate that the dose response up to 3 μg/ml is proportional for 15 minutes. Longer periods of treatment produce a declining response to mutagen concentration. Table 5 shows the viability data for the experiments presented in Figure 31. Pronounced changes in viability after treatment are not observed at these mutagen concentrations. Under the same conditions as above, Figure 32 shows that allelic recombination frequencies for the tyr locus also give a nonlinear response with time at a given concentration of NMG. For the same experiment as in Figure 32, Table 6 shows decreasing survival with time of treatment.

DISCUSSION

Experiments with amino acid starved and early stationary phase cells (Figures 1-3, Tables 1-2) indicated that definite decreases in induced recombination frequencies occurred under those conditions in comparison to frequencies induced during balanced logarithmic growth. These experiments suggested a possible role for active DNA replication during NMG-mediated mutagenesis (mitotic allelic recombination).

Further experiments with induced allelic recombination during the early phases of amino acid starvation indicated a possible involvement of DNA synthesis in mutagenesis by NMG. After initial difficulties with viability losses due to starvation for leucine and tryptophan (see Figure 4), the results shown in Figure 5 indicated that the most drastic decreases (up to 50%) in induced allelic recombination were observed for the leu and met markers before the time that DNA synthesis stopped after starvation treatment. Furthermore, when the cells were starved for all required amino acids, only a 10% loss in viability was observed by 200 minutes of amino acid starvation No further increases in DNA and cell number occurred by 100 minutes of starvation. Figure 7 shows that the met and leu markers also decreased 50% in induced allelic recombination by 100 minutes. The adenine marker did not show any pronounced decreases either in the mutagen dose response experiments (Figures 1-3) or in

the experiments dealing with the early phases of amino acid starvation (Figures 5 and 6). These experiments did not eliminate the possibility that further changes in NMG-induced allelic recombination could develop after DNA synthesis stopped. Rather, a major decrease for two out of three markers studied could definitely be observed during the first 100 minutes of amino acid starvation coinciding with the limited period of DNA synthesis.

To explain the major decreases in recombination frequencies during the early phases of amino acid starvation, it is possible that as the rounds of DNA replication finish during starvation, there are corresponding decreases in induced allelic recombination frequencies caused by the specific action of NMG on regions of active DNA replication. The remaining active replication points would provide fewer targets for the action of NMG.

The effect of amino acid starvation was also investigated during growth in complete medium after amino acid starvation to see if partial synchrony could be obtained as in experiments with bacteria. Moreover, it was of interest to measure the effect for a discrete component, amino acid deprivation, on induced allelic recombination frequencies because of previous experiments. In these experiments, allelic recombination was induced in cells synchronized by alternate feeding and starvation techniques. In contrast to amino acid starvation, these starvation treatments consisted of suspending the cells in

buffer for extended periods of time. As shown by Figures 13 and 18, partial synchrony was evident in both DNA synthesis and cell division. In Figure 18, initiation of synthesis of total DNA occurred approximately 20 minutes before an increase in the rate of cell division. Initial DNA synthesis was not associated with an initial 20% increase in cell number. After the initiation of DNA synthesis, rates of cell division of 0.43-0.45 were obtained. These values indicated that the observed rate of growth was not that of the balanced growth rate of 0.5 at 25° in WC medium. Figure 13 indicates a 44% increase in total DNA before the steady growth rate is established; Figure 18 shows an increase of 37% for total DNA before a steady growth rate is established. A doubling in DNA before steady cell division was not observed as was observed previously in haploid strains (Wehr, 1970). The results seemed to show a partial synchrony in the initial round of DNA replication.

Figures 14-17 and 19-20 show that periodic changes in NMG-induced recombination frequencies can be obtained after amino acid starvation, in contrast to the unchanging levels of recombination during balanced logarithmic growth in WC medium (Table 4). The data seemed to show that a partial synchrony of DNA replication had taken place. Although induced allelic recombination was not necessarily a function of DNA synthesis, it was decided to analyze the variations in recombination using termination and initiation of DNA synthesis as

reference regions. For each experiment, the initiation of DNA synthesis was determined from the initial increase in cell numbers and was related to the experiments showing DNA synthesis. Several observations emerged from analysis of all of these figures: 1) a peak of recombination frequency occurred before initiation of DNA synthesis; 2) in Figures 14-17, a trough of frequencies at 110-150 minutes was associated with a leveling out of the initially rapid rate of total DNA synthesis; 3) a similar trough could be found at a later time at 200 minutes in Figures 19 and 20; 4) and apparently this trough was associated with a similar shift in DNA synthesis depending on the initial lag period. It seemed that the variations in recombination frequencies were in some way related to the cell cycle. Furthermore, the lag period before growth could be prolonged by the longer periods of amino acid starvation. When growth and DNA synthesis began after a long lag in the 3.5 hour starvation experiment, there was also an analogous change to later times of the periodic increases and decreases in recombination frequencies. It could be that the different periods of amino acid starvation were showing a definite pattern of events connected with the cell cycle, although not necessarily DNA synthesis, which was primarily used as a reference point for this analysis.

Since mild amino acid starvation had pronounced effects in producing variation of induced allelic recombination, attempts were made to obtain synchronous cultures by relatively mild procedures

and observe whether variations in induced allelic recombination could be related to those encountered in growth after amino acid starvation. Figures 21 and 22 show that one cycle of synchronous division could be obtained, although the degree of synchrony was again quite limited.

In Figures 24 and 25A, control experiments employed induced allelic recombination during asynchronous growth. These experiments indicated that peaks occurring before the initiation of DNA synthesis could be partly or completely due to changes of induced recombination frequencies associated with the experimental manipulations of the medium. These peaks were obtained during the first 50 minutes of growth. After 50 minutes, a gradual decline in recombination frequencies was observed with no further maxima or minima. Variable rates of cell division also indicated that unbalanced growth conditions prevailed after the sucrose density gradient sizing procedure.

As shown in Figures 26 and 27, experiments with spontaneous recombination during synchronous culture indicated that periodic changes in recombination could be obtained using both methods of sampling. These methods included sampling by constant cell number and by constant volume. A comparison of the experiments above was made with experiments using NMG induction of allelic recombination during synchronous growth. As shown in Figures 28-30, the results indicated: 1) a pronounced common peak (peak 1) at 110 minutes before initiation of DNA replication; 2) a region between peaks 1 and 2

where recombination frequencies increased to a high level; 3) a decline to a trough at 140-150 minutes coinciding with termination of the first round of DNA synthesis; 4) a sharp peak, peak (2), common to most markers at the end of DNA synthesis (Figures 28A and 29). The adenine marker also did not respond to mutagen induction in the synchrony experiments, because the frequencies after mutagen treatment were well within the range of spontaneous recombination.

The experiments with synchronous cultures (Figures 28-30) indicated that maximal effects of NMG-mediated mutagenesis could be observed both before the period of DNA replication as well as during active replication, with sharp decreases associated with the beginning and the end of the first round of DNA synthesis. Further observations with two cycles of synchronous growth could not be made because of the lack of synchrony of the sized cells after one generation. However, even these rather limited observations demonstrated that the mild sizing procedures had physiological effects in terms of changes in induced allelic recombination frequencies, as seen in the experiments with asynchronous cultures in sucrose gradients (Figures 24 and 25A).

In addition to lack of a definite relationship between NMG-mediated mutagenesis and DNA replication, no displacement in time of the different genetic markers could be observed either in the synchronized cell cultures or in cultures subjected to chromosome alignment procedures by amino acid starvation. These results

indicated that a directional recombination process or a polarity in induced mitotic gene conversion had not been observed. This interpretation must be made cautiously, because a higher degree of synchrony may be necessary to observe such polarity phenomena. However, Esposito (1968) also did not find any polarity in very detailed studies of x-ray and ultraviolet irradiation-induced allelic recombination in cultures of XS-380, synchronized by alternate feeding and starvation treatments.

Nevertheless, the results from both the asynchronous and synchronous cultures indicated that a possible role for active DNA replication during allelic recombination could not be eliminated. Thus, non-reciprocal recombination could consist of two events sensitive to NMG. The first event is associated with the replication mechanism; the second event is not tied to DNA replication but is essential for completion of the conversion process.

In relation to existing models of recombination, the two-step mechanism, which includes active replication as a part of non-reciprocal recombination, favors the models of Stahl (1969) as well as Boon and Zinder (1969). In these models, an active role for DNA replication is postulated. Two other models proposed for recombination in fungi (Whitehouse, 1963; Holliday, 1964a, 1968) postulate a role for DNA replication primarily as a limited repair replication. These models argue that recombination processes occur primarily

at times other than the S phase of active DNA replication.

Although active DNA replication cannot be entirely excluded from recombination, there is some additional evidence that there is no direct relationship between DNA replication and NMG-induced mutagenesis. In the initial experiments with amino starvation and various phases of growth (Figures 1-3, Tables 1 and 2) several facts were noted: 1) no changes in overall marker distribution were observed during amino acid starvation or early stationary phase as have been shown for bacteria during NMG-mediated mutagenesis in stationary phase; 2) no direct relationship between growth rate and induced allelic recombination was observed in several experiments; 3) allelic recombination could also be induced after termination of DNA synthesis during amino acid starvation. Neither were Magni and Von Borstel (1962) able to observe any relationship between spontaneous rates of mutation in homoallelic diploid strains and growth rates. Zimmermann and Schwaier (1967) were able to observe that allelic recombination could be induced with nitrosamides in late stationary phase cultures which presumably had completed their rounds of DNA replication.

Furthermore, during amino acid starvation, the indirect role of DNA replication in NMG induction of allelic recombination was also illustrated by harvesting a synchronized culture during the S period. The harvested cells were subjected to amino acid starvation and

compared with regard to NMG-induced recombination with an asynchronous culture under similar conditions. The phased cells should all be replicating their genomes, rather than the one-fourth of the population that was observed in the asynchronous culture. The results shown in Figure 8 and Table 3 indicated that no significant changes in induced recombination were observed in either culture during the first 100 minutes of amino acid starvation. These results could be explained if: 1) DNA was not directly implicated in induced allelic recombination; 2) the shift of media was too drastic for other changes to be observed.

Wehr (1970) had also shown that in a haploid yeast strain auxotrophic for uracil, total DNA synthesis could be immediately inhibited by glucose deprivation in addition to amino acid starvation. However, in media containing glucose but lacking amino acids, continued DNA synthesis could be observed for a limited period in a strain auxotrophic for amino acids, adenine, and uracil. This suggested that the role of DNA synthesis in induced allelic recombination could also be investigated by depriving the cells of glucose during amino acid starvation. One would predict that if DNA and cell division are immediately shut off during amino acid starvation, there should be an immediate decline in induced allelic recombination frequencies. As shown in Figures 9 and 10, the results indicated that there were no increases in cell number, but rather increased resistance to the action

of NMG when cells were deprived of glucose and amino acids simultaneously. Under these conditions viability increased 56% at 175 minutes. The increasing resistance to NMG would tend to agree with the results of Adelberg et al. (1965), which indicated that bacteria were more resistant to the lethal effects of NMG when deprived of energy sources. However, Figure 10 shows that no differences in the patterns of induced allelic recombination could be observed when recombination frequencies with and without glucose were compared during amino acid starvation. The results shown in Figures 8-10 and Table 3 implied that DNA replication may have only an indirect effect on NMG-induced allelic recombination.

In addition to inhibition of protein synthesis by amino acid starvation, inhibition of cytoplasmic protein synthesis was also studied by using low concentrations of cycloheximide (CH). As shown in Figure 14, comparison of induced recombination frequencies before and after CH treatment indicated that a decrease in recombination frequencies occurred after 70 minutes of treatment, with the exception of the lys marker. The decline in NMG-induced allelic recombination frequency could be due to the observed loss in viability after CH treatment. Several observations, however, would argue against loss of viability after CH treatment as the primary causal factor of the observed decreases in induced recombination frequencies.

For example, no decrease in recombination was observed in the lys locus compared to the culture in logarithmic growth. Also, the decreases in marker frequencies for some markers, such as ad and met exceeded changes produced by the losses in viability. There was a 55% decrease in viability by 195 minutes of CH treatment, of which 16% was due to the difference in the methods used for counting total cells and viable cells. This would produce a net observable loss in viability of only 71%. But the ad marker decreased 43% and the met marker decreased 46% in induced allelic recombination frequencies after 195 minutes of CH treatment. In general, the effects of CH treatment were similar to those produced by amino acid starvation upon induced allelic recombination frequencies, but decreases in recombination frequencies did not take place until 70 minutes of treatment. By 140 minutes most markers had reached minimal frequencies. However, the tyr and met marker frequencies followed different patterns from the general pattern noted above. These experiments provided additional evidence that inhibition of protein synthesis in some fashion produced effects on induced allelic recombination frequencies, although more points would have to be taken to establish this definitely.

It is interesting that similar patterns of induced recombination can be obtained in growing cultures both after amino acid starvation and synchronous growth. Furthermore, a specific shift in initiation

patterns of DNA synthesis during growth of amino acid-starved cells could be related to shifts in the patterns of induced allelic recombination when the period of amino acid starvation was changed. Wildenberg (1969) has shown that a broad peak in x-ray-induced allelic recombination can be obtained at the his_{1-315}/his_{1-1} heteroallelic locus when a synchronous culture growing in broth was suspended in buffer supplemented with histidine. The duration of the peak was 210 minutes. Under these conditions, DNA synthesis was blocked immediately after the beginning of the starvation treatment. This experiment indicated part of the x-ray-induced allelic recombination was independent of DNA synthesis.

As suggested by Esposito (1968), another possibility for generating patterns of induced allelic recombination during synchronous growth could be the activity of cyclic dark repair enzymes. Recently, the DNA dependent DNA polymerase has been assigned dark repair functions (Kornberg, 1969; Kelly et al., 1970). In yeast, DNA polymerase activity was shown by Eckstein et al. (1967) to undergo periodic peaks in activity which were not subject to control by various effector levels. These peaks occurred before doubling of DNA during synchronous growth. If these synchronous cultures were irradiated with x-rays, cell division stopped and total DNA synthesis was inhibited, but the cyclic activity of the polymerase continued. This latter experiment indicated that the polymerase was not directly

controlled by DNA synthesis. Other periodic increases of glycolytic enzymes and other cell constituents were also observed in irradiated yeast cultures (Hilz and Eckstein, 1964; Eckstein, Paduch and Hilz, 1966). These data were interpreted to indicate that there may be unknown factors controlling the cyclic appearance of enzymes such as DNA polymerase, as well as DNA replication during the cell cycle.

Studies with synchronous cultures obtained by alternate feeding and starvation have shown that periodic changes in induced recombination can be obtained by a variety of agents. Esposito (1968) used x-ray and ultraviolet to induce both reciprocal and non-reciprocal allelic recombination in synchronous cultures of XS-380 and other strains carrying heteroallelic loci. Both intergenic (reciprocal) and intragenic (non-reciprocal) recombination were observed to have peaks of induced recombination frequencies before the first cycle of DNA doubling. In addition, specific inhibition of DNA replication by FUDR could not be taken as a conclusive demonstration that allelic recombination was directly involved in DNA synthesis because recent work (Wehr, Kudrna and Parks, 1970) showed that FUDR was a permanent inhibitor neither of growth nor of DNA synthesis in yeast.

In work with XS-380, Esposito showed that the first peak associated with both ultraviolet and x-ray-induced recombination was poorly defined. However, the maximum of the second peak of induced allelic recombination was associated with the beginning of DNA

synthesis. In relation to our experiments, this suggested that strain XS-380 was unusually sensitive to shifts in medium. The first peak could be due in part to metabolic changes as shown by the asynchronous control experiments in Figures 24 and 25A and by the initial changes in induced allelic recombination during growth after amino acid starvation as shown in Figures 16-20.

EMS, an alkylating agent, has also been used to induce allelic recombination in the heteroallelic locus trp_{5-1}/trp_{5-2} in a culture synchronized by alternate starvation and feeding (Ebbs, 1967). In these experiments, only one round of replication was studied. Maximal frequencies were induced by EMS before initiation of budding (DNA replication) with a decrease in recombination frequencies continuing throughout the period of DNA replication.

As shown in Figures 31 and 32, NMG could also be used in very low concentrations during active growth to give proportional mutagen dose responses. As shown in Figure 33, induction of allelic recombination in a synchronous culture during active growth in broth also suggested that cyclic variations in allelic recombination could be observed. Therefore mutation in broth during active growth seemed to have similar effects as the treatments in buffer at the higher concentrations of NMG.

SUMMARY

Saccharomyces cerevisiae strain XS-380, a heteroallelic diploid, carries six genetically analyzed loci on the left arm of chromosome VII. Each locus carries two different non-complementing mutant alleles in repulsion, producing growth requirements for five amino acids and adenine in the diploid strain. If one or both mutant alleles of a given locus revert by either spontaneous or mutagen-induced recombination, the diploid strain becomes prototrophic at that locus. These revertants can be selected on suitable diagnostic media.

During vegetative growth, high rates of spontaneous and mutagen-inducible reversion characterize the process that produces recombinants from such heteroallelic diploids. These prototrophs have been previously shown to be produced by mitotic non-reciprocal recombination or mitotic gene conversion, using the experimental conditions employed in our studies.

In these studies, nitrosoguanidine (NMG) at low concentrations was observed to cause allelic recombination (either mitotic recombination or gene conversion) of the heteroallelic loci with low lethality. This mutagen was very selective in its mutagenic action upon regions of active DNA replication in bacteria.

The NMG-inducible heteroallelic system was used in studies of the relationship of mutagenic action to DNA replication in both

randomly and synchronously growing yeast cultures. Studies of asynchronous cultures in balanced logarithmic growth, in the early stationary phase, and in the early phases of amino acid starvation indicated that each locus had a characteristic recombinant response. Two of the conditions, amino acid starvation and early stationary phase, produced a drastic reduction in numbers of induced recombinants. Moreoever, after onset of amino acid starvation, a timed series of samples could be individually induced by pulse mutagenesis to show that the mutagen response decreased up to 50% by the time that DNA synthesis stopped. Similar results were also obtained by substituting cycloheximide treatment for the amino acid starvation conditions in the time course experiment. However, a definite relationship of mutagenic response to DNA replication could not be demonstrated by amino acid starvation or cycloheximide inhibition, because coordinate protein synthesis may be necessary for the maintenance of DNA synthesis.

Synchronous cultures also produced ambiguous responses to NMG-mediated pulse mutagenesis. The frequency of inducible mitotic gene conversion increased rapidly before initiation of a discrete synchronous doubling of total DNA. In contrast, high levels of inducible mitotic recombination could also be obtained during the period of DNA replication with decreasing levels toward the end of the first round of doubling. Most of the markers on the chromosome arm

displayed similar cyclic variations in recombination frequency. It was suggested that the cyclic activity of DNA repair enzymes may be responsible for the variations of mutagen-inducible prototroph frequencies during synchronous growth. Also some changes in induced allelic recombination seemed to be related primarily to conditions of unbalanced growth caused by experimental manipulation of the cells. Moreover, no definite direction of allelic recombination could be observed on the chromosome.

The above experiments suggested that DNA replication may have a possible role in mediating the specific prototroph response to NMG mutagenesis. However, no direct relationship between DNA replication and mutagenic action could be demonstrated in the heteroallelic system.

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