GENETIC VARIATION IN YEAST DURING EXTENDED CULTIVATION

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C O N T E N T S

INTRODUCTION

The genetic stability of microorganisms during prolonged cultivation is important in both industrial processes and academic investigations. Genetic variation of significant proportions can have far reaching effects. The conclusions deduced from the results of research experiments and based upon the assumption that the culture has remained 'pure', may be invalidated. In industrial processes the end product may be altered, qualitatively or quantitatively, and by-products may be formed which render the end product unusable.

Spontaneous genetic mutation has been regarded as the main cause of variation in cultures of microorganisms (Winge, 1944). Any genetically controlled characteristic, morphological or physiological, can be subject to change by spontaneous mutation. However, in diploid heterozygous yeasts, variation may not be due entirely, or even primarily, to spontaneous mutation. It has been suggested (Emeis, 1961) that the frequency of mitotic segregation may exceed the frequency of spontaneous mutation for many loci in diploid heterozygous yeasts. Mitotic segregation would, therefore, seem to be an important source of variation in many strains.

Besides gene mutation and mitotic segregation a third source of variation, namely extrachromosomal mutation, must be considered. In yeast, variants which are respiratory deficient and are known as 'petites' (Ephrussi, 1953) have been observed to occur spontaneously at an average rate of about one per cent (Sherman 1959). Petites are the result of the mutation of a cytoplasmic determinant and the frequency of this mutation can be highly dependent upon environmental conditions.

Continuous cultivation in an apparatus such as the Chemostat is an extremely useful tool for the investigation of variation in microorganisms, (Novick and Szilard, 1955). Moreover, continuous cultivation is of great importance in industry since it has distinct economic and practical advantages over the more conventional batch processes. The production by continuous cultivation techniques of antibiotics, yeast, beer, and chemicals such as citric acid is either in practice or under active investigation.

The aim of this research was to investigate the variations which might arise during prolonged cultivation of certain diploid strains of yeast. Both Chemostat and serial transfer techniques were used to obtain conditions of prolonged cultivation. Variations due to mitotic segregation at four adenine loci and variations due to the effects of temperatures between $15^{\circ}C$ and $30^{\circ}C$ on a cytoplasmic determinant were studied. Before considering these particular variations, some more general aspects of mutation and mitotic segregation will be reviewed.

Aneuploidy and Gene Mutation in Yeast

Aneuploidy is a change in the chromosome number of an organism which is the result of the addition or subtraction of one or more whole chromosomes giving polysomy or monosomy. The spontaneous occurrence of aneuploidy in yeast has been reported (Hawthorne and Mortimer, 1960; Cox and Bevan, 1962). Both disomic (n + 1) and trisomic (n + 3) aneuploids have been isolated (Cox and Bevan, 1962) and it was suggested that they might be quite common in old laboratory stock cultures. The spontaneous occurrence of tetraploid yeasts has been observed (Roman et al., 1955; Johnston and Mackinnon, 1966), and aneuploids commonly arise from tetraploids following meiosis. The significance of aneuploidy in yeast is that during cultivation it provides a source of variation. As will be seen later mechanisms of mitotic recombination of genes are applicable to some aneuploid as well as diploid microorganisms. Since aneuploids usually have only one or two extra chromosomes, variation by mitotic segregation is possible of only a fraction of the genetic material. A system which allows a proportion of genetic variation in an otherwise stable genetic background may well be of at least temporary advantage to a fast-multiplying unicellular organism (Bevan, 1960). This statement could be very significant when considered in the context of continuous cultivation, since under these conditions yeast may be multiplying rapidly and if a significant level of aneuploid yeast cells were present then any variation resulting from them could have significant effects on the nature of the population. It has been suggested (Hurst and Fogel, 1964) that the events taking place during the mitotic segregation are 'parameiotic',

rather than mitotic, in nature. If this is true then aneuploids might be formed more frequently.

Aneuploidy may be important in a context other than as a source of variation during continuous cultivation. Aneuploids have been suggested as a source of aberrant ratios observed in yeast tetrads (Emerson, 1956). The significance of this will be seen when the results of the investigation of spontaneously occurring flocculation are considered.

Wild type yeast can synthesize all the amino acids, vitamins, purines and pyrimidines it requires for growth from carbon and nitrogen sources, and simple inorganic salts. If mutation of a gene which codes for an enzyme occurs and results in the enzyme becoming non functional, then a block occurs at that point in the synthetic pathway. Such mutations can easily be identified in haploid yeasts as the culture medium must be supplemented with the particular amino acid, vitamin, purine or pyrimidine before the mutant yeast can grow. Most of the studies of mutation in yeast have been concentrated on those which affect biochemical pathways because they are easily identified. Besides numerous biochemical mutants of this type, mutants involving characteristics such as sugar utilisation and resistance to antibiotics have been identified. Many of these gene mutations have been mapped so that chromosome maps for Saccharomyces are fairly extensive (Hawthorne and Mortimer, 1960; Mortimer and Hawthorne, 1966). Gene mutation of one allele in a wild type diploid yeast is not phenotypically expressed unless it is dominant or semi-dominant, and recessive mutations can only be identified by tetrad analysis. If, however, the diploid yeast is heterozygous at a

particular locus, mutation of the dominant allele will be phenotypically expressed. Such gene mutations are the most common source of variation in cultures of microorganisms and consequently are of great importance.

Respiratory Deficiency in Yeast

Saccharomyces cerevisiae is a facultative anaerobe, which means that under aerobic conditions energy is provided by oxidative breakdown of substrates, while under anaerobic conditions energy is provided by the fermentative breakdown of substrates. A spontaneous mutant Saccharomyces cerevisiae, unable to respire under aerobic conditions, has been observed (Ephrussi et al., 1949). This mutant, named 'petite', when grown on a solid medium containing fermentable substrates, forms characteristically smaller colonies than the respiratory competent parent strain. It is unable to utilise non-fermentable substrates such as glycerol, lactate, acetate, or succinate, because of the absence of cytochromes a + a_3 and b and certain dehydrogenase enzymes. The petite mutant is apparently very stable and during twenty years of investigation of petite mutants there has been only one claim of reversion to respiratory competency (Kraepelin, 1964). Ephrussi found, on analysis of this mutant, that it exhibited non-Mendelian inheritance and consequently postulated that the mutation was of cytoplasmic rather than chromosomal nature. Since the early work of Ephrussi a wide variety of petites have been investigated and it has become apparent that nuclear petites (Chen, Ephrussi and Hottinguer, 1950; Reilly and Sherman, 1965), as well as cytoplasmic petites, exist. However cytoplasmic petites are much more common and occur spontaneously with an average frequency of 1% (Sherman, 1959) but can occur at much higher rates even under normal cultural conditions.

Cytoplasmic petites

Cytoplasmic or Vegetative petites can be divided into two

main classes, 'Neutral' and 'Suppressive'.

Petites classified as 'neutral' conform to the following pattern of inheritance. When haploid 'neutral' petites are crossed with haploid normal strains, they give rise to respiratory sufficient diploids. These diploids, when sporulated and the segregants analysed, give segregation in the expected ratios for all the genetic markers, whereas all the segregants are respiratory sufficient. As a result it has been postulated that respiratory deficiency is caused by the mutation or loss of a cytoplasmic genetic factor. When the petite is crossed with the respiratory sufficient strain the factor is introduced into the diploid and into all the haploid progeny arising by sporulation. The cytoplasmic genetic factor has been called the rho (ρ) particle (Sherman, 1964). The rho particle replicates normally in the respiratory sufficient strain and is passed onto its progeny, thus the parent strain is rho positive (ρ^+). A daughter cell not inheriting a rho particle is rho minus (ρ^-), and petite. Reversion from petite to respiratory sufficiency has not been observed during vegetative growth of haploid 'neutral' petites (Raut, 1954). Complementation, which would result in a respiratory sufficient diploid, does not occur when haploid neutral petites are mated (Jakob, 1965). This indicates that all these neutral petites arise from the same basic mutational change (Roodyn and Wilkie, 1966).

'Suppressive' petites show a different pattern of inheritance to that exhibited by 'neutral' petites. When haploid 'suppressive' petites are crossed with haploid respiratory sufficient strains only a proportion of the resulting diploid progeny are respiratory

sufficient (Ephrussi et al., 1955). Effectively the respiratory sufficient parent has failed to transmit the rho particle to the progeny of the diploid zygote. It was concluded that 'suppressive' petites included a suppressive factor (SF) which inhibits the replication of the rho particle (Ephrussi et al., 1966). Moreover the degree of suppressiveness is a characteristic of each cell of the suppressive strain (Ephrussi and Grandchamp, 1965) since the proportion of petite diploids depends on the strain of petite. The suppressive factor must be transmitted through the ascospore to the haploid segregant, as ascospores of suppressed zygotes give suppressive petite colonies and ascospores of non-suppressed zygotes do not. It thus seems that SF is passively transmitted to daughter cells and, when present, eliminates all the normal rho particles. It has been suggested that the degree of suppressiveness depends on the rate of turnover of SF (Wilkie, 1968).

Nuclear petites

The P series of genes

Nuclear petites, morphologically identical with cytoplasmic petites, have been isolated (Chen, Ephrussi, and Hottinguer, 1950; Raut, 1953; Ephrussi, 1953). These were also unable to utilise non-fermentable substrates as an energy source. However, on tetrad analysis following the crossing of these nuclear petites with haploid respiratory sufficient haploid strains, Mendelian segregation with respect to respiratory deficiency is observed. In this class of petite respiratory deficiency is, therefore, the result of mutation of a nuclear gene. However, respiratory deficiency

in these mutants is not due to gene mutation alone. Of nine gene mutants of the P series analysed by crossing with haploid neutral petites (Sherman, 1963; Sherman and Slonimski, 1964), three gave diploid petite zygotes and six gave respiratory sufficient zygotes. Presumably the first group of three gene mutants lacked the cytoplasmic factor for respiratory sufficiency and were consequently ρ^-. The other six gene mutants must have been ρ^+ and transmitted the rho particle to the diploid zygote on crossing with the haploid neutral petite.

The cy mutants

It has been concluded that the formation of cytochromes $a + a_3$ and b is under the control of the cytoplasmic factor since they are absent in neutral petites. As cytochrome c is present in normal or even increased amounts in neutral petites, its formation has been assumed to be under the control of the nuclear genetic material. Petite mutants containing normal levels of cytochromes $a + a_3$ and b, but reduced levels or completely absent cytochrome c have also been isolated (Sherman, 1964). Six unlinked genes were identified as controlling the synthesis of cytochrome c and mutations of these genes block, or partially block, the synthesis of cytochrome c. These mutations also result in the non-utilisation of some non-fermentable substrates. However it was shown that cytochrome c synthesis was entirely independent of cytoplasmic genetic information (Sherman et al., 1966).

The relationship between mitochondrial DNA and the rho particle.

As a result of the observations on cytoplasmic petites the location and nature of the cytoplasmic determinant, the rho particle,

became of prime importance and many investigations have aimed at its elucidation. It had been suggested that petites arose from the loss or inactivation of autonomous self-replicating non-chromosomal particles (Ephrussi and Hottinguer, 1951; Raut, 1953). As petites can be induced by U.V. light with maximum efficiency at 2600 A^{o} it was suggested that the rho particle was nucleic acid (Raut and Simpson, 1955) and that this nucleic acid was associated with the mitochondria. Mitochondria are, of course, the site of many of the oxidative enzymes and of the cytochromes. The number of mitochondria present in a yeast cell can vary widely according to the environmental conditions, but even numbers, commonly 6 or 8, are usually found (Sugimura et al., 1966). Mitochondria have also been found to reproduce at a different rate to the cell and apparently by a process of binary fission (Lehninger, 1964). Based on these observations, mitochondria would be the logical source or site of the cytoplasmic determinant.

Mitochondrial DNA has been found to occur generally in cells from all kinds of tissues (Nass, Nass, and Afzeluis, 1965). A chemical assay indicated that DNA would be found in association with the mitochondria of yeast (Schatz, Haslbrunner and Tuppy, 1964), and mitochondrial DNA was isolated and characterised from yeast shortly afterwards (Tewari, Jayaraman and Mahler, 1965). The initial observation was later confirmed by several groups of workers (Moustacchi and Williamson, 1966; Corneo et al., 1966; Mounolou et al., 1966; Tewari et al., 1966). Analysis of total cell DNA showed a major component with a buoyant density of 1.700 g/cm^{3}, which was assumed to be nuclear DNA. A minor or satellite component with an

approximate buoyant density of 1.682 g/cm^3 was assumed to be the mitochondrial DNA, especially as it could be concentrated in the mitochondrial fractions. Another satellite component, of buoyant density 1.706 g/cm^3, was observed, but this component could not be obtained pure and its origin and relationship to other components remain unknown (Moustacchi and Williamson, 1966).

Mitochondrial DNA has been reported to be absent from respiratory deficient yeasts (Corneo et al., 1966; Moustacchi and Williamson, 1966). The design of the experiments of the above authors was, however, criticized, and the careful analysis and comparison of a variety of respiratory deficient yeast mutants revealed that mitochondrial DNA is present in cytoplasmic petites (Mounolou, Jakob and Slonimski, 1966). Their results showed that mitochondrial DNA differs according to the cytoplasmic genome. Mitochondrial DNA is lighter in cytoplasmic neutral petites and heavier in cytoplasmic suppressive petites (1.687 : 1.695), the respiratory sufficient strain having a buoyant density of 1.682 g/cm^3 for mitochondrial DNA. Based on their observations and the design of the experiments they concluded that 'the cytoplasmic petite mutation is not due to a loss of mitochondrial DNA but to a change in its buoyant density'. In essence this infers that mitochondrial DNA is altered and that petites arise as the result of this alteration. This alteration could be quite large and result in 'nonsense' DNA, which could be the reason why reversion to respiratory normal cells has been reported only once (Kraepelin, 1964). This initial report of mitochondrial DNA in cytoplasmic petites was later confirmed in both haploid and diploid strains of yeast (Avers, 1967; Avers et al., 1968; Bernardi et al., 1968). Moreover it was demonstrated that

one of the satellite DNA components in respiratory deficient cells was an adenine - thymine rich DNA, probably mitochondrial DNA. A second satellite component was found to be a guanine - rich nucleic acid, but no further light has been shed on its origin (Bernardi et al., 1968). The occurrence of a DNA - polymerase in isolated yeast mitochondria supports the evidence that mitochondrial DNA exists and that it can replicate (Wintersberger, 1966).

It is now assumed that mitochondrial DNA is the cytoplasmic determinant, the rho factor, and that it controls the formation of cytochromes a + a_3 and b in Saccharomyces. Electron microscopy has shown that the mitochondria of cytoplasmic petites are malformed, with distorted inner membranes or christae (Yotsuyanagi, 1962; Smith et al., 1969). The mitochondria of nuclear petites are completely normal in appearance (Yotsuyanagi, 1962) showing that it is alteration of the cytoplasmic determinant rather than the nuclear determinant which causes the alteration in the structure of the mitochondria. The relationship between the malformed mitochondria of petites and the absence of cytochromes a + a_3 and b, has been suggested by Prezbindowski and collaborators (1968). In this elegant work, mitochondrial christae of normal yeasts were split into two membraneous fractions by the use of detergents. This was called the 'red - green split', and cytochromes b and c, were found in the 'red' fraction and cytochromes a and a_3 were found in the 'green' fraction. Physical measurements of the cytochrome molecules and of the thickness of the membranes of the christae supported the hypothesis that the christae membranes were entirely composed of cytochrome molecules. Assuming that mitochondrial DNA carries the

information for the formation of cytochromes a + a_3 and b, it may be concluded that mutation of mitochondrial DNA could result in the absence of these cytochromes and consequently malformation of the inner membranes of the mitochondria.

The precise relationship between the amount of mitochondrial DNA and the number of mitochondria is not yeat clear. A relationship of one mitochondrial DNA unit to one mitochondrion has not been established, although target analysis has shown that the number of mitochondrial genetic units is greater in aerobic unrepressed cells, which have large numbers of mitochondria, than in repressed cells which have only a few mitochondria (Allen and MacQuillan, 1969). Studies involving U.V. irradiation (Maroudas and Wilkie, 1968) and the induction of petites by ethidium bromide (Slonimski, Perrodin and Croft, 1968) show that the number of cytoplasmic genetic determinants for respiratory sufficiency is less than the number of mitochondria in a cell. This suggests that only a fraction of the mitochondrial population carries a functional rho factor. If one assumes that rho factor and mitochondrial DNA are identical, then only a fraction of the mitochondria appear to contain functional DNA.

At present there is only indirect evidence to suggest that mitochondrial DNA has a genetic function, namely, carriage of the information for formation of cytochromes a + a_3 and b. Induction of petites by acriflavine suggests that mitochondrial DNA may also control the formation of some enzymes, such as succinie dehydrogenase (Avers, Pfeffer and Rancourt, 1965; Avers, Rancourt and Lin, 1965). However, non-chromosomal genes like these observed in

Chlamydomonas (Sager and Ramanis, 1965, 1967, 1968) have not been identified so far. Moreover, it has been suggested that mitochondrial DNA is heterogeneous (Avers, 1967; Avers et al., 1968) in which case one type of mitochondrial DNA could be controlling cytochrome formation and another type controlling the formation of enzymes. Furthermore mitochondrial DNA could have functions, as yet undetected, other than those connected with the respiratory activities of the cell. Although only one unconfirmed report of spontaneous reversion to respiratory sufficiency has been made (Kraepelin, 1964), reversion via an enzyme repair mechanism during photoreactivation following U.V. irradiation has been observed (Pittman and Pedigo, 1959). The stability of thepetite mutant has been used to support the hypothesis that 'nonsense' DNA is formed on mutation. However, in one case of entrachromosomal respiratory deficiency in Neurospora, it has been established that the cause is substitution of a single amino acid in a protein (Woodward and Munres, 1966). In this case the alteration in mitochondrial DNA is probably very small, and if a similar mechanism existed in Saccharomyces spontaneous reversion might be less unlikely.

Induction of petites.

Petites can be induced by a wide variety of agents which can be divided into two main categories:

(a) mutagens which induce respiratory deficiency of a cytoplasmic nature,

(b) agents, such as antibiotics, which block protein synthesis in the mitochondria resulting in respiratory deficient cells.

Mutagens

Mutagens

Since early investigations (Ephrussi et al., 1949; Ephrussi and Hottinguer, 1951; Marcovitch, 1951; Ephrussi, 1953), the acridine dye, acriflavine, has been widely used to induce petites in Saccharomyces. At low concentrations acriflavine induces petites at a very high frequency. Moreover, it was found that all the buds produced by a yeast cell, while in the presence of acriflavine, ultimately formed petite cells. However, if the same yeast cell was then washed and placed in a normal nutrient medium all the buds thereafter formed normal cells (Ephrussi, 1953). It has been suggested that acriflavine combines preferentially with mitochondrial DNA rather than nuclear DNA, since at higher concentrations acriflavine induces gene mutations (Tewari et al., 1966). Combination or intercalcation with mitochondrial DNA by acriflavine could result in nonsense DNA being formed on replication (Mounolou et al., 1966). Nonsense DNA, when transmitted by a bud, would result in a petite cell. Although some doubt has been cast on this mechanism (Roodyn and Wilkie, 1968), it has been shown that acriflavine reacts with mitochondrial DNA in a 'parallel fashion' with the bases (Sugimura et al., 1969). Another mutagen, pinacyol, which also induces petites, reacts in a 'perpendicular fashion' to the bases of mitochondrial DNA. Spontaneous resistance to mitochondrial mutation by acriflavine has been shown to be under the control of recessive nuclear genes (Thomas and Wilkie, 1967).

The action of acriflavine is species specific, many species of yeast being naturally resistant to acriflavine induction of petites. These strains have been designated 'petite negative', and

those which are not resistant, 'petite positive' (Bulder, 1964a, 1964b). Petite strains induced by acriflavine have been found to differ in other respects (Avers, Pfeffer and Rancourt, 1965). When a random selection of acriflavine induced petites was analysed for mitochondrial cytochrome oxidase and succinic dehydrogenase (SDH), four groups emerged:

(1) low cytochrome oxidase, normal SDH.

(2) low cytochrome oxidase, high SDH.

(3) no cytochrome oxidase, high SDH.

(4) no cytochrome oxidase, normal SDH.

The last group was the most common. Surprisingly, members of the second group could be sporulated despite having a petite phenotype.

An interesting mutagen is 2, 3, 5, triphenyltetrazoluim chloride (TTC) which is commonly used as a diagnostic means of identifying petite colonies (Ogur et al., 1957). It was also found to be an effective inducer of petites (Laskowski, 1954), although the relationship between its inductive ability and its diagnostic usefulness has not yet been fully explained.

A number of heavy metals, such as copper, nickel, cobalt, and manganese, are effective inducers of petites (Lindegren, 1958; Nagai and Nagai, 1958a, 1958b). Manganese chloride or sulphate is particularly efficient and also has very low toxicity in comparison with the other heavy metals. A large variety of chemical compounds induce cytoplasmic petites, but as their mode of action has not been elucidated, they cannot be classified.

Ultraviolet irradiation induces petites at a very high frequency

(Raut, 1953, 1954). It was suggested that this effect was due to selective killing rather than induction, but closer examination revealed differential killing of normal and mutant cells which argued against selection. Furthermore some of the petites were found to be nuclear (segregational) mutants. Double mutants of both the cytoplasmic determinant and a nuclear gene were also isolated. A more detailed examination demonstrated that there was maximum petite induction at a wavelength of 2600A°. This wavelength is also the one which is maximally absorbed by nucleic acid and therefore it was suggested that the cytoplasmic self-replicating particle was nucleic acid (Raut and Simpson, 1955). This hypothesis was further supported by results which showed a linear (single-hit) relationship in anaerobic cells between U.V. dose and mutation, and induction curves of a multiple-hit nature in aerobic cells (Wilkie, 1963). This evidence, taken in conjunction with consideration of the numbers of mitochondria in aerobic and anaerobic cells, again indicated that the mitochondria might contain nucleic acid. Photoreactivation, whereby the production of petites is reduced by exposure to visible light immediately following U.V. irradiation, has been shown to occur (Sarachek, 1958). This phenomenon was observed only in cultures of aerobically respiring cells and subsequent investigations showed that U.V. induction and photoreactivation were independent of ploidy (Pittman and Pedigo, 1959; Pittman et al., 1959).

The production of petites in increased proportions by elevated temperatures was first reported by Ycas (1954). A more detailed investigation was subsequently performed (Sherman, 1957, 1959).

Haploid and diploid yeast strains, normally cultivated at 30°C, were cultivated at 38°C and 40°C, and an increased proportion of petites, varying between 10% and 100% of the total population depending on the strain employed, was observed. In the main, haploid strains were more unstable in this respect than diploid strains. This effect was shown to be inductions and not selection by 'daughter' and 'sister' single cell pedigree analysis. It was suggested that increased temperature interferes with the replication of the cytoplasmic factor resulting in a proportion of the offspring lacking this element. 'Heat shock' induction of petites was also observed by Sherman (1959), when yeast was cultivated in a liquid medium at 30°C and then subjected to a temperature of 54°C for 30 minutes. Approximately 99% of the cells were killed by this treatment, but 40% of the survivors were petite. Selection was also ruled out in this case as petites were shown to be more temperature sensitive than normal cells.

Lower temperatures also induce petites (Ogur, Ogur and St. John, 1959). It was found that the extremes of the temperature range 15 - 38°C were effective in inducing petites in 5 strains, the degree of induction depending on both the strain and the temperature.

Antibiotics

Antibiotics have been used extensively to study the biochemistry of mitochondria, and the genetical significance of mitochondrial DNA.

High concentrations of chloramphenicol inhibit the formation of cytochromes a + a_3 and b in yeast. However, chloramphenicol has no effect on the formation of cytochrome c. Yeast cells produced under conditions of chloramphenicol inhibition are phenotypically

identical with respiratory deficient cytoplasmic petites (Huang et al., 1966; Clark-Walker and Linnane, 1967). However, there is one major difference in that the action of chloramphenicol is completely reversible. Besides being physiologically identical, both antibiotic and cytoplasmic petites are morphologically similar in that the mitochondria of both have malformed christae (Clark-Walker and Linnane, 1967; Yotsuyanagi, 1962). As all other properties of the cytoplasm remain unchanged, it was suggested that chloramphenicol specifically blocks the translation of mitochondrial genetic information into protein of the inner membrane. The g_i mutant (Negrotti and Wilkie, 1968) exhibits the induction of respiratory deficiency by glucose, and studies of this mutant supported this hypothesis of the mode of action of chloramphenicol. Whether the genetic information originates from mitochondrial DNA or from nuclear DNA via messenger RNA has not been established. The genetics of resistance to chloramphenical and exythromycin have been investigated (Wilkie et al., 1967) and the cytoplasmic inheritance of erthromycin resistance established (Thomas and Wilkie, 1968).

The use of cycloheximide (actidione) in low concentrations slows the growth rate of yeasts but does not affect the synthesis of cytochromes $a + a_3$ (Clark-Walker and Linnane, 1966).. The conclusion drawn from these results is that yeasts have two protein synthesizing systems, one of which is cytoplasmic and the other mitochondrial. These conclusions were confirmed when the mechanism of resistance was established (Cooper et al., 1967; Wilkie, 1967).

Mitotic Recombination

Nuclear and cytoplasmic mutation are major sources of genetic variation in microbial populations. However, in diploid yeast populations another potential source of genetic variation is mitotic recombination. During the cultivation of a diploid yeast, which is heterozygous for a number of gene markers, recombinants for some or all of these markers may be isolated. Assuming that most of the recombinants for one locus are recovered, then both recombinants homozygous for the wild type allele, and for the mutant allele, should be found. Mitotic recombination was first observed in Drosophila melanogaster (Stern, 1936) and in Saccharomyces cerevisiae more recently (James and Lee-Whiting, 1955). Considerable interest has been shown in the mechanism by which mitotic recombination of genes occurs, and four possible mechanisms have been postulated.

(1) Mitotic crossing-over

Mitotic crossing-over (fig. 1a) was first postulated as the result of analyses of somatic variation in Drosophila melanogaster (Stern, 1936) and used later to explain asexual variation in Aspergillus nidulans (Pontecorvo et al., 1953). Somatic pairing of chromosomes has been observed in Drosophila melanogaster but is only assumed to occur in Aspergillus nidulans. Crossing-over occurs at the four strand stage accompanied by division of the contromeres and followed by normal disjunction of the chromatids. The following conclusions may be drawn from the analysis of the model. (1) The genes distal to the point of crossing-over are homozygous in 50% of the daughter nuclei. (2) A positive correlation will exist between the distance from the centromere and

the frequency of homozygosis of a gene.

(2) Non-disjunction of centromeres at mitosis

This model (fig. 1b) was proposed as the result of observations in Aspergillus nidulans (Pontecorvo et al., 1953). The principal result is complete homozygosis for all the genes on the chromosome.

(3) Meiotic crossing-over and restitution

This model (fig. 1c) was originally proposed as a possible mechanism of mitotic segregation induced by U.V. light in Saccharomyces cerevisiae (James and Lee-Whiting, 1955) but was later rejected as unlikely (James, 1955). However, more recently, its acceptance was strongly argued on the basis of further evidence from U.V. irradiation studies (Wilkie and Lewis, 1963). In this model it is assumed that events occur, as in meiosis up to, and including, the first meiotic division. This means that pairing of the chromosomes takes place, and crossing-over occurs at the meiotic rate at the four strand stage, and the centromeres segregate but do not divide. The difference between this first meiotic division and mitosis lies in the theory that the chromosomes do not separate into four different nuclei but merely into two. The cause of this may be nondisjunction of the centromeres, or alternatively restitution of the pairs of haploid telephase nuclei into two diploid nuclei. The consequences of this model are that all the genes proximal to the point of crossover are homozygous. There will, therefore, be negative correlation between the distance from the centromere and the frequency of homozygosis of a gene.

(4) Crossing-over at the two strand stage, meiotic action of centromeres and restitution.

This model (fig. 1d) was also proposed to explain some results of U.V. induction of mitotic segregation in Saccharomyces cerevisiae (Wilkie and Lewis, 1963). As in the previous model, normal pairing of chromosomes as at meiosis is assumed. However, crossing-over occurs at the two strand stage as a result of the action of U.V. light. The restitution or nondisjunction takes place at the second division stage. The result in this model of recombination is that all the genes will be homozygous in both nuclei, also a higher than normal frequency of crossing-over will occur. No correlation between the distance from the centromere and the frequency of homozygosis of a gene will exist.

Each of these mechanisms was consistant with the observations on which it was based, but none was valid for all the observations in the different organisms. However another mechanism has been proposed on the basis of observations of U.V. irradiation of Saccharomyces cerevisiae (Hurst and Fogel, 1964) which gives good correlation for all of the observations in the different organisms. Hurst and Fogel (1964) disagreed with the terminology and description of mitotic crossing-over and preferred the term 'parameiosis' as a more accurate description of the events taking place. They defined 'parameiosis' as 'mitotic centromere disjunction preceeded by the chromosomal events associated with the first meiotic prophase.' The hypothesis assumes that during normal vegetative growth some of the cells enter into the early stages of meiosis. However, it is proposed that the process of meiosis is reversible up to a point,

and the diploid state is maintained by the occurence of mitotic centromere disjunction. This hypothesis implies that synapsis of all homologous chromosomes takes place, thus explaining the high coincidence of mitotic recombination for unlinked markers which has been observed (Hurst and Fogel, 1962; Fogel and Hurst, 1963; Wilkie and Lewis, 1963; Hurst and Fogel, 1964). This model (fig. 1e) is superficially similar to the one proposed for meiotic crossing-over and restitution (Wilkie and Lewis, 1963). However, closer examination reveals that the products are identical with the products of mitotic crossing-over. The vital difference in the first case is the behaviour of the centromeres and the assortment of chromatids. Centromere behaviour also differs from that which is proposed for mitotic crossing-over.

Induction of Mitotic Recombination

Mitotic recombination, regardless of the mechanism involved can be induced by a variety of agents. In yeast mitotic recombination is increased among the survivors of U.V. irradiation (James, 1954; James and Lee-Whiting, 1955; Roman and Jacob, 1958; Sherman and Roman, 1963; Capaldi and Manney, 1968). U.V. irradiation also causes mitotic recombination in Aspergillus (Fratello et al., 1960; Kafer, 1963; Tector and Kafer, 1962), and in Ustilago (Holliday, 1961). In Aspergillus, U.V. irradiation induces recombination by the mechanisms of mitotic crossing-over, nondisjunction of centromeres, and haploidisation due to repeated aneuploidy (Pontecorvo et al., 1953). In Ustilago maydis, a series of experiments (Holliday, 1961, 1962, 1964) seem to indicate that mitotic crossing-over is the most probable mechanism, but the mechanism of parameiosis

was not considered. While the precise mechanism by which U.V. light acts is not understood, the mechanism by which a wide variety of other agents act is known. Those agents which stimulate mitotic recombination have been called 'recombinagens' (Holliday, 1964). Fluorouracil (Beccari et al., 1967) formalin and nitrogen mustard (Fratello et al., 1960) and bifunctional alkylating agents such as dienpoxybutane and methyl-bis (β chlorocthyl) amine (Morpurgo, 1963) are all effective inducers in Aspergillus. Mitomycin C, which specifically inhibits DNA synthesis in bacteria (Shiba et al, 1959), in yeast (Williamson and Scopes, 1962) and in animal cells (Ben-Porat et al., 1961), has been shown to stimulate mitotic segregation in Ustilago and Saccharomyces (Holliday, 1964). Another DNA inhibiter, 5 - fluoro deoxyurindine (FUDR) stimulates mitotic segregation in Ustilago (Esposito and Holliday, 1964).

Most of the investigations of mitotic recombination in yeast have been concerned with the mechanism(s) by which it takes place. There have been only a few investigations concerned with the rate at which mitotic recombination occurs spontaneously and its possible consequences in growing yeast cultures (Emeis, 1962; Johnston and Mackinnon, 1966). Approximate rates of mitotic recombination have been estimated for some genes and it has been suggested that these rates were greater than the forward mutation rates for the same genes. The consequences of mitotic recombination of genes in yeast during prolonged cultivation depend largely on the genes concerned, and on selection characteristics. For example, the rate of mitotic recombination at the ad_8 locus was estimated as approximately 6×10^{-4} per cell per generation. In this case, although there was no

difference in the nutritional requirements of the parent strain and the recombinants, there was nevertheless selection for the latter cells during cultivation (Johnston and Mackinnon, 1966). Consequently, although the various types of cells have identical nutritional requirements, the population does change quite drastically.

FIGURE 1: Proposed mechanisms of mitotic recombination

A, B, C, D, = wild type alleles
a, b, c, d, = mutant alleles

Fig. 1a Mitotic crossing-over

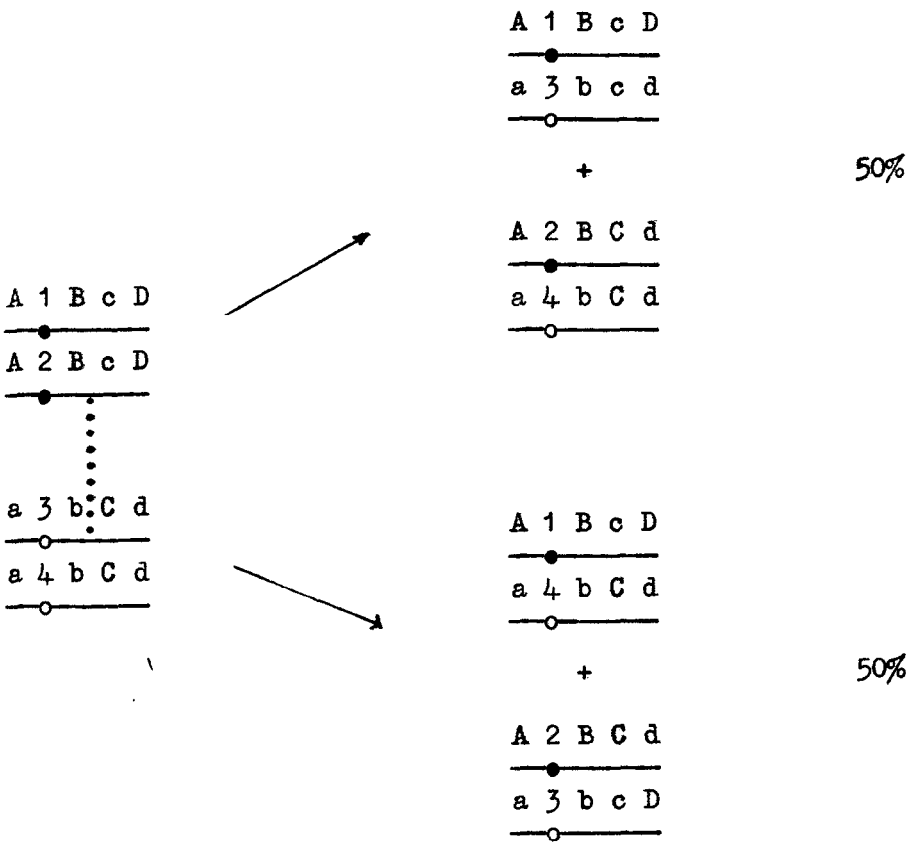


Fig. 1b Nondisjunction of centromeres at mitosis

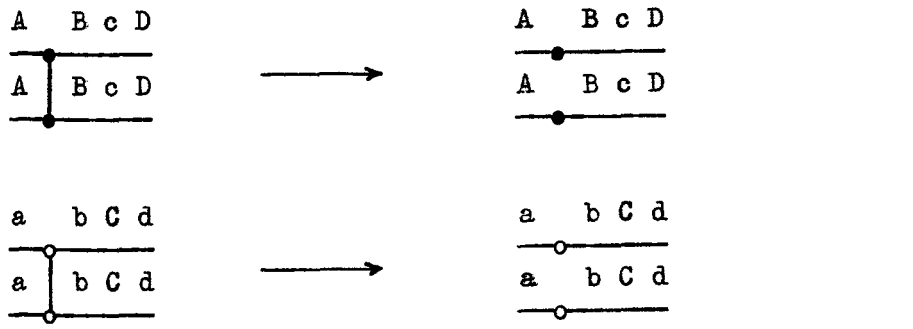


Fig. 1c Meiotic crossing-over and restitution

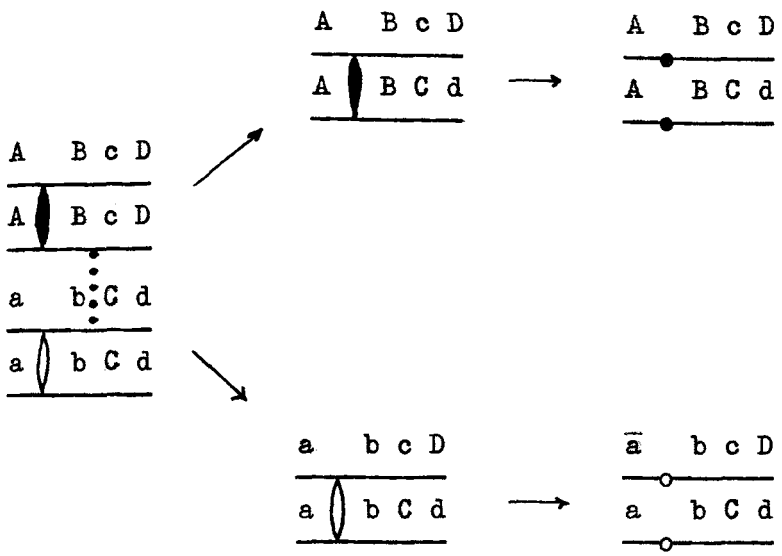


Fig. 1d Crossing-over at the two strand stage, meiotic action of centromeres and restitution.

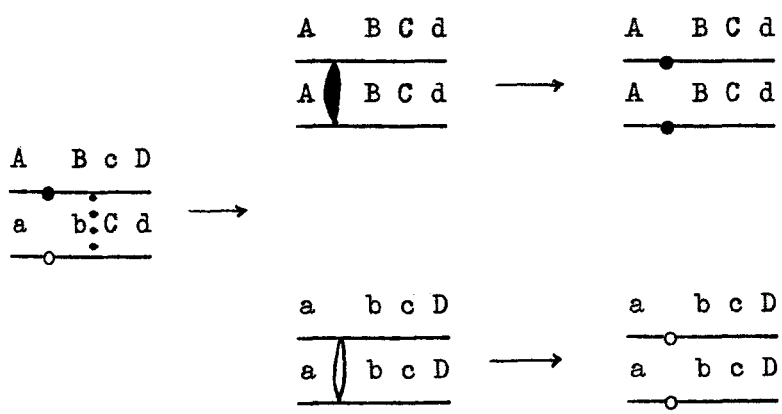
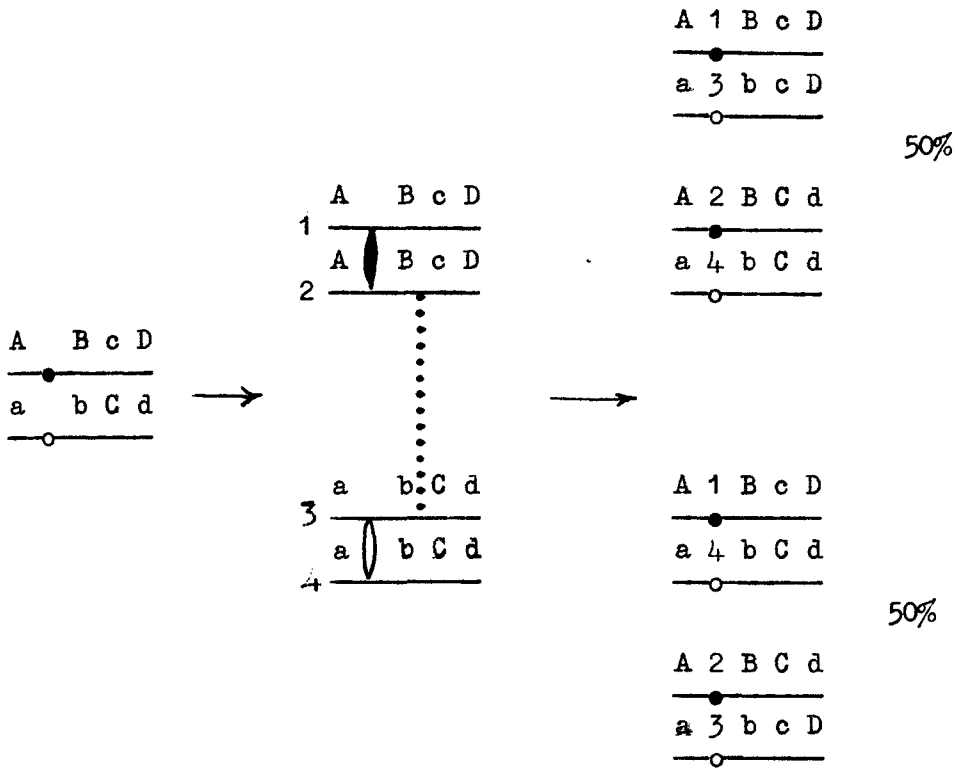


Fig. 1e Parameiosis



Continuous Cultivation

Classically, serial transfer techniques have been used to determine genetic variations such as mutation in microbial populations. However, even under good serial transfer conditions, in which both lag and stationary phases of growth are essentially absent, the cultural conditions are constantly changing. The size of the microbial population increases and the concentration of the nutrients in the medium decreases during each growth cycle. Fairly accurate determinations of mutation rates have nevertheless been made by this technique (Stocker, 1949).

The shortcomings of the serial transfer technique can be avoided by the continuous growth of microorganisms, the basic equations of which were postulated by Monod (1950). Where V=volume of culture, F=flow of media, and C=concentration of the growth factor in the culture vessel then:

(1) When the flow rate F = 0, the bacterial concentration n would rise in the culture vessel according to $\frac{1}{n}\ \frac{dn}{dt} = \alpha\ (C)$ where α = growth rate which is a function of the concentration (C) of the growth factor.

(2) In the absence of growth, the bacterial concentration in the culture vessel would decrease for a given flow rate, F, according to the formula $\frac{1}{n}\ \frac{dn}{dt} = \frac{-F}{V}$. Where $\frac{F}{V} = B$ may be called the 'washing-out rate' of the culture vessel, and $\frac{1}{B}$ the washing-out time.

(3) With growth taking place, after a while for any given flow rate, F, a stationary state will be reached at which the growth rate will be equal to the washing out rate (or dilution rate) B (and the generation time, r equal to the washing out time $\frac{1}{B}$).

i.e. $(C) = B = \frac{F}{V}$, $r = \frac{1}{B} = \frac{V}{F}$

The generation time r is also known as the culture generation time or the mean residence time.

Thus, in continuous culture, the microbial population, although growing exponentially (the culture itself exhibiting linear growth) remains of constant size in the culture vessel. Also the concentration of nutrients in the culture remains constant throughout growth. This technique is an extremely useful tool in the investigation of the physiological and biochemical factors affecting the cultivation of microorganisms. The effects of changing physical parameters such as flow rate, working volume, aeration, and agitation have been investigated by many workers and the principal results have been postulated as series of complex equations (Kono and Asai, 1969).

Continuous cultivation in an apparatus such as the 'Chemostat' or 'Turbidostat' has been used to investigate mutation rates. Novick and Szilard (1950) found that there was a relationship between mutation rate and time, independent of the generation time of the organism. This result was very controversial although partially supported by the observations of Fox (1955). An important factor in the simple determination of mutation rates, in both serial transfer and continuous culture techniques, is that the mutant has a growth rate identical to that of the parent. The possible selection of variants in a population by falling concentration of particular nutrients during batch growth is reduced in continuous culture due to the constant concentration of the nutrients. However selection for a mutant cannot be avoided if it has a greater growth rate than the parent or an inhibitory effect on its growth. It was shown (Atwood, Schneider, and Ryan, 1951) that

'periodic selection' of mutants occurs during serial transfer. A histidine requiring (H_o^-) mutant of Escherischia coli was grown by serial transfers. Spontaneous mutation to a histidine non-requiring (H_o^+) type took place. The revertant mutant, H_o^+, grew faster than the parent mutant, H_o^-, so that it tended to replace the latter in the population. However, a subsequent mutation to H_1^-, took place, this histidine requiring mutant possessing a faster growth rate than both the original mutant and the H_o^+ revertant and consequently tended to replace both in the population. This process took place several times and several different types of H^+ and H^- mutants were isolated. Each mutant was selected for by its faster growth rate and replaced its parent in the population and the process was termed 'periodic selection' of mutants. The importance of this phenomenon is the genetic stability of populations of microorganisms during continuous culture is obvious.

Commerce has shown considerable interest in continuous cultivation of microorganisms as it offers many economic advantages over the batch processes at present employed. The results of most commercial investigations have not, however, been published, although it is known, that some products of microbial fermentations such as beer, antibiotics and chemicals are being made by continuous processes. Great interest has been shown in the continuous cultivation of yeast. Beers and wines are being produced in large pilot plants and on commercial scales by continuous fermentation processes. Biomass, the production of edible protein, has been concentrated on the cultivation of yeast. Biomass production by continuous cultivation of yeast on cheap substrates such as natural gas is under active investigation.

Biomass is already being produced on a commercial scale by the continuous cultivation of yeast on the waste products of petroleum distillation. Biomass is at present being used as a cheap animal feeding stuff, but is seen as a possible long term answer to the world food shortage.

The use of continuous cultivation for genetic investigations has, with a few exceptions, been largely neglected. However more attention must be paid to the genetic stability of microbial populations during continuous cultivation as it is becoming of increasing academic and industrial importance.

MATERIALS AND METHODS

STRAINS

Several strains of Saccharomyces hybrid stock, were used in these investigations (Table 1). Except for the Brewing strains, all the strains of yeast employed have a common characteristic, namely a block in the adenine synthetic pathway resulting from the mutation of the ad_2 gene, diploid strains being homozygous for this mutation. Consequently, none of the yeast strains could synthesize adenine and this had to be present in the growth medium. Another consequence of this block is that cells form red pigmented colonies after all the adenine in the medium has been consumed. The reason for this is that an intermediate of the adenine synthetic pathway, aminoimidazole ribotide (AIR), cannot be converted into 5 - amino - 4 - imidazole carboxylic acid ribotide (CAIR) as a result of the ad_2 mutation (Silver and Eaton, 1969). The intermediate AIR accumulates in the cell and is converted into the red pigment, ribosyl - amino - imidazole (Smirnov et al., 1967). Any blocks in the adenine synthetic pathway prior to that step controlled by the Ad_2 gene i.e. due to mutation of genes Ad_3 - Ad_9, result in the accumulation of intermediates which are not converted to a pigment and hence the colonies are, as wild type, white. This sytem is extremely useful for detecting mutants of the adenine synthetic pathway and has been widely used since it was first described (Roman, 1956). All the diploid yeast strains employed included adenine loci other than ad_2 which were heterozygous. Recombination of mutant alleles at those loci results in cells which form white colonies, which are easily identified among a population which predominantly forms red colonies (plate 1).

TABLE 1

STRAINS OF SACCHAROMYCES USED IN THESE INVESTIGATIONS

STRAIN	ORIGIN	PLOIDY		GENOTYPE
X 190	J.R. JOHNSTON	DIPLOID	a	$\frac{ad_2\ ty_2\ thr_2\ ad_8\ tr_4\ ur_3\ ly_7}{}$
			α	ad_2 + + + + + +
X 190 S-91	J.R. JOHNSTON	HAPLOID	α	$ad_2\ ly_7$
X 190 S-110	J.R. JOHNSTON	HAPLOID	a	$ad_2\ ty_2\ thr_2\ ly_7$
X T5	R.J. THORNTON	DIPLOID	a	$\frac{ad_2\ ad_8\ tr_4\ ur_3 + + + +}{}$
			α	ad_2 + + $ur_3\ ad_3\ ad_6\ arg_{4-2}\ his_6$
X T14	R.J. THORNTON	DIPLOID	a	$\frac{ad_2\ tyr_4 + + + + +}{}$
			α	ad_2 + $ad_{5-7}\ ly_5\ tyr_3\ met_{13}\ ac_2^r$
A, B	ALLIED BREWERIES	NOT KNOWN		

MEDIA

The following media were used in these investigations.

Standard Growth Media

(1) MYGP. This medium was used either as a broth or solidified with the addition of 2% Oxoid No. 3 agar. In agar form this medium was used for the normal growth of the yeasts and for scoring variants in the population since it contained about 10 mg/litre adenine. This medium composition is listed below:-

Malt Extract	3 gm.
Yeast Extract	3 gm.
Glucose	10 gm.
Bacto peptone	5 gm.
Oxoid No. 3 agar	20 gm.
Deionised water	1000 ml.

(2) MAD, as above but supplemented with 20 mg/litre adenine and used for the maintenance of stock culture, and, in liquid form, for the cultivation of the yeasts by the serial transfer technique.

Synthetic Complete Medium (S.C.)

This medium was used as a control for the growth of all organisms during tetrad analysis. This medium consisted of Difco Bacto Yeast Nitrogen Base w/o amino acids and with 2% glucose as the carbon source. The medium was supplemented with various amino acids and organic bases as below:-

Yeast Nitrogen Base (Difco)	6.5	gm.
Glucose	20	gm.
Adenine	20	mg.
Argenine	20	mg.
Histidine	10	mg.
Leucine	30	mg.
Lysine	80	mg.
Methionine	20	mg.
Serine	375	mg.
Threomine	350	mg.
Tryptopham	20	mg.
Tyrosine	20	mg.
Phenylalanine	20	mg.
Uracil	20	mg.
Oxoid No. 3 agar	20	gm.
Deionised water	1000	ml.

Since this complete medium contained all the growth requirements all strains could grow on it.

Omission media

Omission media were prepared by omitting individual amino acids or bases (Hawthorne and Mortimer, 1960) e.g. the adenine requirement was tested on medium identical with SC but lacking adenine.

Pre-sporulation medium (G.N.A.)

Glucose	50 gm.
Nutrient agar	30 gm.
Yeast Extract	10 gm.
Oxoid No. 3 agar	5 gm.
Deionised water	1000 ml.

Sporulation medium (P.A.)

Potassium acetate	10 gm.
Yeast Extract	2.5gm.
Oxoid No. 3 agar	30 gm.
Deionised water	1000 ml.

Differential Media

Respiratory deficient petite colonies of Saccharomyces species can be distinguished by their small size in comparison with respiratory competent colonies on MYGP agar. This criterion is not absolutely reliable and various media have been designed to make this distinction. Glycerol and sodium lactate media depend on the fact that petites cannot utilise glycerol and sodium lactate as carbon sources (Ogur and St. John, 1956). The tetrazolium chloride overlay technique depends on respiratory enzymes present in respiratory sufficient cells acting upon the T.T.C. to produce a red colouration in one hour. Respiratory deficient petites do not produce these enzymes and the colonies remain white (Ogur, St. John

and Nagai, 1957). The exact composition and preparation of these media is listed below.

Glycerol medium

Glycerol	30 gm.
Yeast Extract	10 gm.
Bacto Peptone	10 gm.
Oxoid No. 3 agar	20 gm.
Deionised water	1000 ml.

Sodium lactate medium

Sodium lactate	10 gm.
Yeast Extract	3 gm.
Bacto Peptone	3.5gm.
KH_2PO_4	2 gm.
$MgSo_4 \cdot 7H_2O$	1 gm.
$(NH_4)_2 So_4$	1 gm.
Oxoid No. 3 agar	20 gm.
Deionised water	1000 ml.
pH adjusted to 5.5	

Tetrazolium chloride overlay

Solutions (1) 0.1% solution of Tetrazolium chloride, and

(2) 2% Oxoid No. 3 Agar in 0.07M phosphate buffer

were prepared.

Phosphate buffer:

Solution A : 0.2M KH_2PO_4 13.9 gm. in 500 ml. deionised water.

Solution B : 0.2M K_2HPO_4 17.44 gm. in 500 ml. deionised water.

To obtain a solution of pH 7.0 39 ml. Solution A were added to 61 ml. Solution B and diluted to 200 ml. to give a 0.1M solution.

10 ml. of this solution were diluted into 100 ml. deionised water to give a 0.02M solution. This solution was diluted to 280 ml. in deionised water to give a 0.07M phosphate buffer.

Sporulation

In order to achieve good sporulation, the cultures were first grown on pre-sporulation medium (GNA) slopes at 30°C for two days and transferred to slopes of sporulation medium (PA). The culture was spread thinly over the PA slopes and incubated at 25°C for 3 - 4 days (McLary, Nulty and Miller, 1959; Cox and Bevan, 1962). The purpose of growing cultures on GNA is to obtain rich growth. Sporulation medium acts as a starvation medium, with acetate as the carbon source (Fowell, 1952; Lindegren, 1949).

Tetrazolium chloride overlay

Mixtures (1) and (2) were prepared separately, melted, mixed at 55°C under sterile conditions, and held at 50°C until used. 10 ml. of this mixture were poured over an agar plate containing colonies of 2 - 3 days growth. A deep red colour develops within one hour over aerobic colonies and the respiratory deficient petite colonies remain white (plate 2).

Sterilisation

Synthetic Complete, Omission and Tetrazolium chloride media were all sterilised by autoclaving at 10 p.s.i. for 10 minutes. The other media were sterilised by autoclaving at 15 p.s.i. for 15 minutes. MAD medium for Continuous culture was sterilised by autoclaving at 15 p.s.i. for 40 minutes because of its bulk (16 litres). The glycerol medium for continuous culture was sterilised by autoclaving at 15 p.s.i. for 40 minutes on two successive days. Glass equipment such as pipettes, petri dishes, and conical flasks were sterilised in an oven at 150°C for 5 days.

Hybridisation

Selected haploid strains of opposite mating type were crossed by mixing fresh cultures fairly thickly on an MYGP agar plate. The mixture was incubated at 30°C for 6 - 8 hours and then examined microscopically for the presence of zygotes. Diploid yeast strains were obtained either by isolating individual zygotes by micromanipulation or by plating a suspension of the mixture onto selective media on which the parent strains cannot grow but diploid strains can (Pomper and Burkholder, 1949; Hawthorne and Mortimer, 1960). Where the mating type of a haploid strain was not known, it was determined by mixing freshly grown cells with cells of known mating type and of complete mentory nutritional requirements. These mixtures were either examined microscopically after 6 - 8 hours incubation at 30°C or tested on selective media for the growth of prototrophic diploids.

Tetrad Analysis

To isolate individual ascospores of four spored asci, asci were first treated with a 20% aqueous solution of a commercial preparation of snail digestive juice (Suc D'Helixi) which digests the ascus wall but leaves the spores intact and adhering together in tetrads (Johnston and Mortimer, 1959). After sufficient incubation to allow ascus wall digestion (generally 1 - 4 hours at 30°C) a loopful of the treated suspension was spread along one edge of a block of MYGP agar held on a large coverslip (40 X 20 mm). Ascospores from tetrads were then placed in a fixed position on the agar block by using a glass needle held in a De Fonbrunne Micromanipulator. The agar blocks were than removed onto an agar plate by use of a sterilised knife and was incubated at 30°C for 2 - 3 days by which time individual ascospore colonies

could be easily identified. To determine genotypes, tetrads were streaked onto MYGP 'master' plates (usually 12 tetrads per plate) and after incubation at 30°C for 24 hours, replica plated onto plates of omission media (Lederberg and Lederberg, 1952). The replicating material was sterilised velvet square held over a brass block by a brass ring. The plates of omission or other test media were incubated at 30°C and examined for growth after 24, 48, and 72 hours.

Definition of adenine loci

In order to ascertain the genotypes of adenine requiring recombinants of strain XT5, the method of 'cross-streaking' was employed. Recombinants were sporulated, tetrads dissected, and haploid segregants were streaked onto master plates. The segregants were cross-streaked with both mating types of ad_3, ad_6, and ad_8 tester strains and incubated for 24 hours at 30°C. The cross streaks were then replica plated onto Ad omission media and incubated at 30°C. Since tester strains contained only one particular adenine mutation, segregants streaked with a particular tester strain which did not grow on adenineless media were assumed to carry the same mutation as the tester strain.

Serial transfer technique

For investigations at 30°C, 10 ml. of media in 50 ml. conical flasks were incubated in an orbital shaker at 175 revolutions/minute. A growth cycle of 24 hours was normal and generally a 0.1 ml. sample of a 1:100 dilution of a culture was transferred to fresh medium. Variations from this method are noted in the results.

For investigation at, or below, 21°C, 10 ml. media in 150 ml. conical flasks were incubated on an orbital shaker at 60 revolutions/

minute in a water bath. The length of the growth cycle and size of inoculum varied, details of which are noted in the results.

Chemostat

The complete continuous culture apparatus of 'Chemostat' type is shown in plate 3 and a close-up of the culture vessel is shown in plate 4. All the glass apparatus used in the Chemostat was of Quickfit manufacture, with the following specifications:

Wide Neck Reaction Vessel FR500 : F	Volume 500 ml.				
Spring clip					
Air/steam inlet tube, bent with cone	MF/2B				
Multi socket/flat flange lid MAF1/75	Centre socket 19/26				
	Side sockets				
	Parallel	5°	10°	15°	
	14/23	19/26	19/26	19/26	

Air/steam inlet tubes were adapted for various purposes:

(a) a sintered glass bulb was welded onto an inlet tube to make an air inlet tube. The sintered glass breaks the air into small bubbles and gives good oxygen diffusion in the culture.

(b) an inlet tube was shortened to make a media inlet tube well above the level of the culture.

(c) an inlet tube was lengthened to make an overflow and a variety of working volumes can be given by altering the length.

(d) an inlet tube was lengthened to reach to the bottom of the reservoir. Air was supplied to the culture by a HyFlo air pump at a rate of 2 litres per minute. The air was sterilised by passing through a glass fibre and cardboard filter made by Microflow Ltd. This filter was sterilised by autoclaving at 15 p.s.i. for 15 minutes.

Media was supplied to the culture vessel from the reservoir and

controlled by a peristaltic metering pump made by Edmund Buhler of Tubingen Ltd.

The temperature of the culture was maintained to within half a degree Centigrade by placing the culture vessel in a water bath (Grant Instruments, Cambridge) or by use of a water bath whose temperature was controlled by a Circotherm (Shandon, London).

All the non-glass connections were of silicone rubber tubing which is resistant to repeated autoclaving.

The culture vessel and its connections were sterilised by autoclaving at 15 p.s.i. for 15 minutes. After sterilisation the culture vessel and the reservoir were connected aseptically, generally in a beaker of alcohol. Media was pumped into the culture vessel up to the appropriate working volume and the culture vessel was then inoculated. The culture was incubated for 24 hours to build up a suitable size of population at 30°C. The waterbath was then adjusted to the working temperature and the metering and air pumps turned on. The culture was maintained in a homogeneous state by the vigourous aeration and a sample was taken immediately. The connections of the culture vessel were made airtight by use of autoclave tape and as the air pressure builds up effluent is forced out. The effluent was collected in a large flask and samples were taken directly from the end of the effluent tube. Samples were taken at 24 hour intervals, and the reservoir was aseptically replaced by another when depleted. It should be noted that in this method one does not have complete control over the quality of the initial population. In all cases a high quality inoculum from a good stock culture was used. However, there was a 24 hour incubation period before the continuous culture

was set in motion and during that period there is a possibility that the quality of the starting population can change.

This method of continuous cultivation was adequate for all the investigations that were made but some difficulties exist and should be considered.

Contamination can be a major problem during all experiments using continuous culture. The sterilisation techniques employed in the preparation of the apparatus were quite satisfactory. Once the apparatus was running the main danger lay in contamination from the environment. The area from the reservoir to the metering pump is under negative pressure and contaminants could be sucked into the apparatus in this area unless all the connections are airtight. Negative pressure in the reservoir is prevented by the use of a cotton wool filter, which, providing it is properly packed and sterilised, allows only sterile air to enter the reservoir and occupy the space which is left by the consumption of media. The reservoir must be connected to the culture vessel and the joining of the tubes under the surface of alcohol gives a sterile environment, but nevertheless is a possible source of contamination. The air filters were very efficient unless they became damp.

The MAD medium was very susceptible to foaming by the vigourous aeration employed. This had the effect of reducing the working volume as a large head of foam was formed. Foaming was prevented by the addition of 1 ml./litre of Silicone Emulsion Antifoam to the medium. Unfortunately the antifoam and the MAD medium could not be sterilised together since some reaction takes place and the antifoam fails to re-emulsify properly. The antifoam was sterilised seperately by autoclaving at 15 p.s.i. for 15 minutes and was then added by normal aseptic techniques

to the reservoir. Although this method was generally efficient more risk of contamination at this stage exists. There was always some slight risk when the culture vessel was inoculated but this could not be avoided.

The vigourous aeration provided good mixing of the culture and obviated the need for mechanical agitation which, through its vibration, is a frequent source of contamination. The air also provided a positive pressure in the effluent tube and reduced the possibility of contamination by that avenue.

Although all these precautions were observed contamination did occur from time to time and the most likely source was considered to be the silicone rubber/glass connections. Although care was taken with these connections it was not always possible to ensure that they were airtight. The results of every continuous culture experiment has not been reported since some had to be terminated prematurely because of contamination, and the results only confirmed the observations of an early part of an experiment.

Elaborate apparatus with sophisticated equipment for measuring pH, internal temperature, dissolved oxygen etc. was not considered necessary for these preliminary investigations. Good control of temperature, working volume, and flow rate, were the prime considerations. Only the culture generation time or mean residence time $(\frac{V}{F})$ was of significance to these investigations.

Estimation of Recombination Rates

The mutation rate in a population during exponential growth can be estimated as the slope of the line given when proportion of mutants in a population is plotted against the number of generations (Stocker,

1949). This method was adopted to find the rate of mitotic recombination during exponential growth in serial transfer (Johnston, and Mackinnon, 1966). Their method was refined by calculation of the best straight line by the 'Line of Regression'. The slope of the line can be calculated from the formula:

$$b = \frac{\Sigma xy - n \bar{x} \bar{y}}{\Sigma x^2 - n \bar{x}^2}$$

Where n = number of observations and $\bar{x}$ and $\bar{y}$ are the mean values of the variables x and y. The accuracy of this estimate can be determined by calculation of Confidence Limits. The 95% confidence limit for b is given by:

$$t_{n-2} \frac{S}{\sqrt{\Sigma x^2 - n \bar{x}^2}}$$

where t is a constant and can be found from statistical tables and:

$$S = \sqrt{\frac{y^2 - \hat{a} \Sigma y - \hat{b} \Sigma xy}{n - 2}}$$

where a and b are the estimated values of a (intercept on the x axis) and b from the experimental observations.

Thus the recombination rate is given in the form:

$$\hat{b} \pm t_{n-2} \frac{S}{\sqrt{\Sigma x^2 - n \bar{x}^2}}$$

R E S U L T S

Population changes during extended growth by serial transfer

(1) Observation of a high level of petites in strain X 190 populations cultivated at 15°C

During experiments to determine the generation time of strain X 190 of Saccharomyces cerevisiae at 15°C, a large proportion of respiratory deficient petite colonies were observed to develop on the plates. These petite colonies are small in comparison with normal colonies of X 190 and are white (plate 5). Another variant of strain X 190 which is orange in colour (plate 5) was also observed at a relatively high frequency. The colony size of this orange variant is intermediate between petite and normal and a more complete analysis of its respiratory ability is given later.

The increased frequency of petites was an unexpected result but a literature search revealed the report of a similar result (Ogur, Ogur and St. John, 1960). They studied the growth of five strains of Saccharomyces over a temperature range of 15°C to 38°C and found that temperature induction of petites at both high and low temperatures appeared to be strain dependent.

(2) Investigation of the increased frequency of petites in strain X 190 populations at 15°C

The reason for the increased frequency of petites was not apparent from the initial experiments, so strain X 190 was cultivated under differing conditions at 15°C.

Static cultures of strain X 190 in MAD medium were cultivated by the serial transfer technique at 15°C, with control cultures at 30°C, and samples were plated out once every 24 hours. After 4 days of serial transfers the culture at 15°C consisted entirely of petite

cells (fig. 2A). The control culture at 30°C remained fairly stable. Although the level of parent type colonies (plate 6) fell to a minimum of 84%, this was due to a rise in the level of orange variants up to 16% and no petites were detected (fig. 2B). It could be argued that these results are a consequence of inadequate diffusion of oxygen into the medium. Insufficient diffusion might result in deoxygenation of the medium and the induction of anaerobic respiration in the yeast cells. These conditions would favour a respiratory deficient variant and explain the rise in the frequency of petites at 15°C. As will be shown later the orange variant is also respiratory deficient and these conditions would explain the rise in the level of orange variants in the culture at 30°C. These conditions, combined with the low temperature, could also explain the difference between the cultures at different temperatures.

To test the possible role of anaerobiosis in these population changes, the cultural conditions were changed to include vigourous aeration. Strain X 190 was cultivated at 15°C in MAD medium by serial transfers in a growth tube which incorporated an air bubbler which diffused sterile air into the growth medium. The results (fig 3A) were practically identical with those observed in standing cultures of strain X 190 at 15°C. Hence it appears that lack of oxygen in standing cultures is not responsible for the increased frequency of petites. In a control experiment at 30°C in a growth tube, petites were observed at levels between 1% and 5% which is in the range of the average spontaneous frequency (Sherman, 1959). Orange variants were observed at a level between 4% and 12% and the parent type fell below a level of 90% on only one occasion (fig 3B). These results suggest

Figure 2. Population changes during serial transfer of a stationary culture of strain X 190 in MAD medium.

A : At 15°C

B : At 30°C

O = parent type

Δ = petite

▲ = orange

FIG. 2

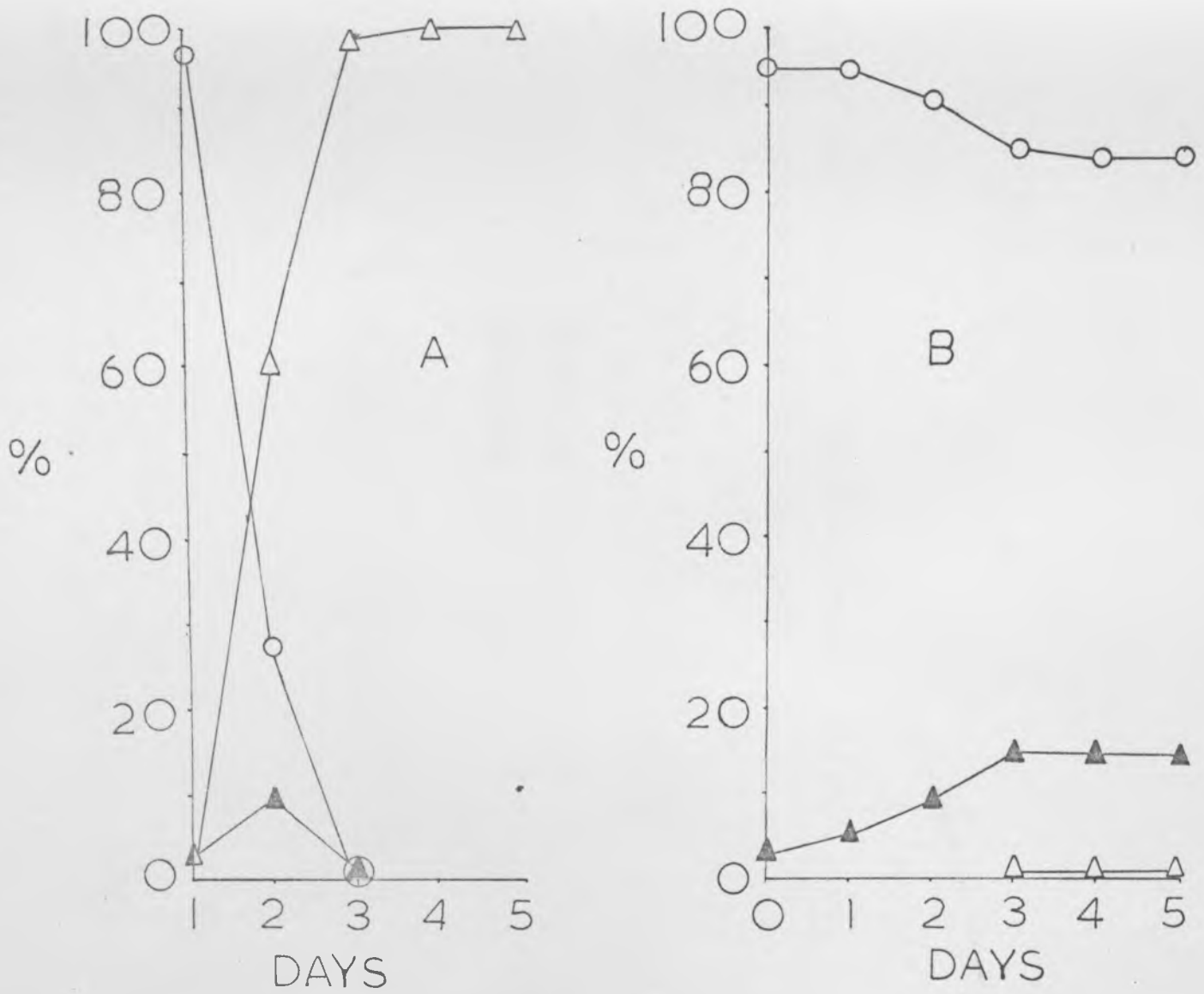


Figure 3. Population changes during serial transfer of an aerated culture of strain X 190 in MAD medium.

A : At 15°C

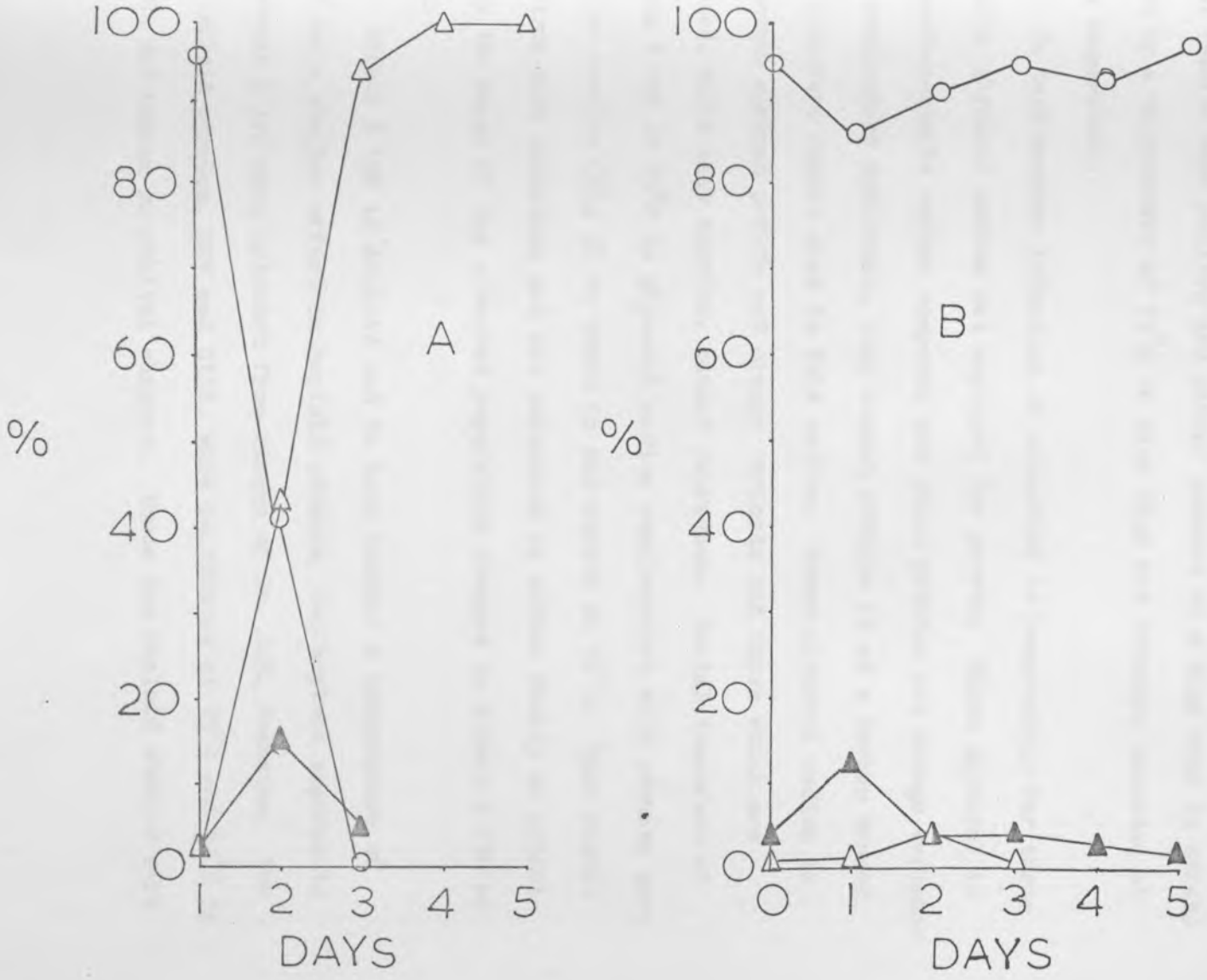
B : At 30°C

O = parent type

Δ = petite

▲ = orange

FIG. 3



that temperature itself, it most likely responsible for the appearance of petites in strain X 190 at 15°C. This view was supported by the results obtained after another variation of the cultural conditions. Strain X 190 was cultivated by the serial transfer technique in 10 ml. MAD medium in conical flasks at 15°C and 30°C on orbital shakers. The results (fig 4) were practically identical with those already obtained under other cultural conditions. It can be concluded from these results that petites are either induced at a high rate in strain X 190 by a temperature of 15°C or else they are strongly selected at this temperature.

To test whether induction or selection is responsible for these results, glycerol medium was employed for growth. Since glycerol is a non-fermentable carbon compound and since petites and orange variants are respiratory deficient, they cannot utilise it as a carbon source and therefore cannot grow in this medium. Hence glycerol medium is selective against petite and orange variants and those which are induced, while they survive, cannot reproduce. Serial transfers of strain X 190 at 15°C in glycerol medium supplemented with adenine gave similar results (fig 5) to these in MAD medium at 15°C. This result confirms that induction and not selection is either wholly or principally the cause of the observed population changes in strain X 190 at 15°C.

Strain X 190 is diploid and to test whether a temperature of 15°C has a similar effect on haploid strains, two haploid segregants of strain X 190 were selected from stocks of Dr. J.R. Johnston. The two haploid strains, S91 and S110, were cultivated at 15°C and 30°C in 10 ml. MAD medium on orbital shakers. These two haploid strains were

Figure 4. Population changes during serial transfer of a shaking culture of strain X 190 in MAD medium at 15°C.

○ = parent type

Δ = petite

▲ = orange

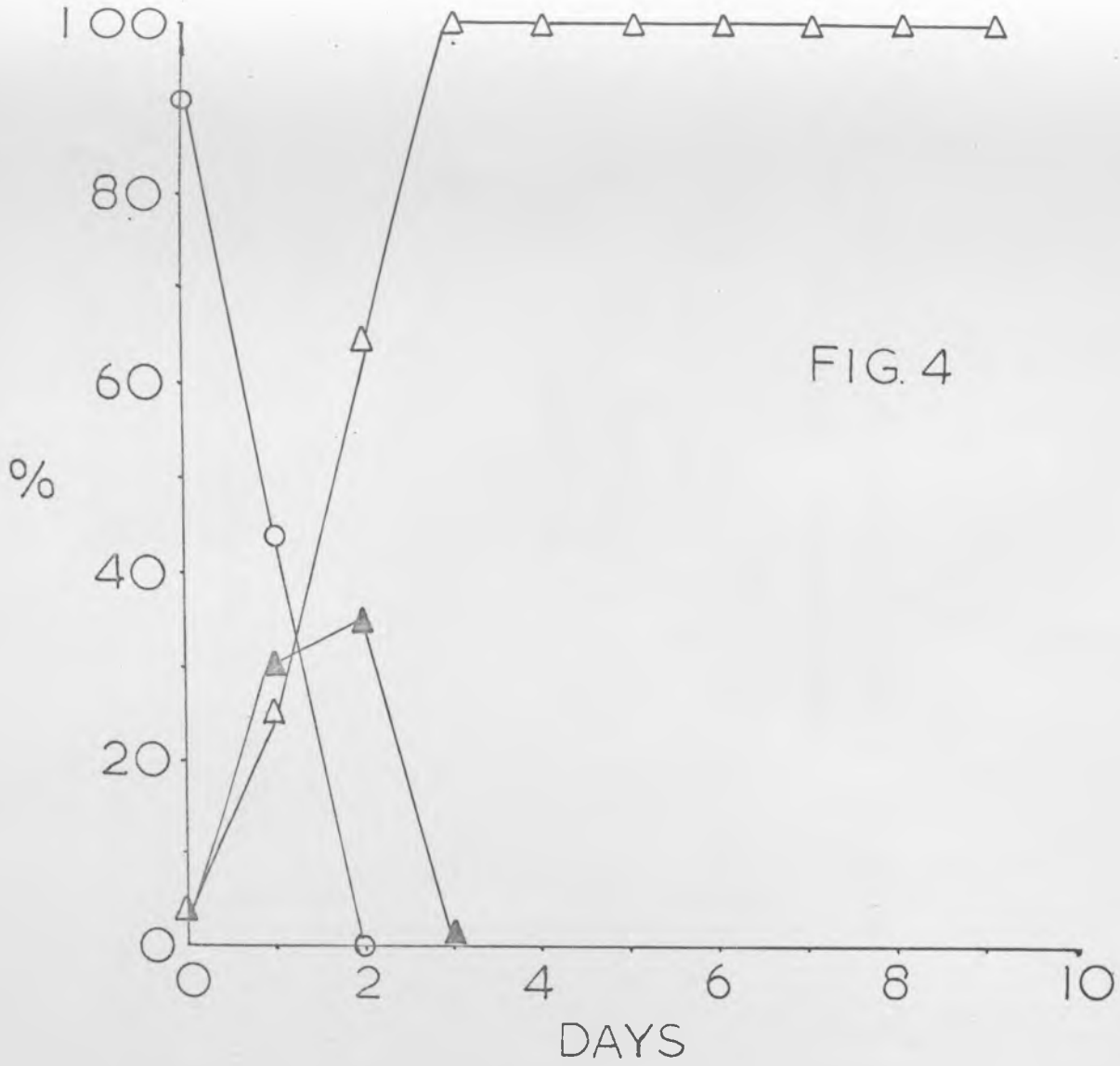


FIG. 4

Figure 5. Population changes during serial transfer of a shaking culture of strain X 190 at 15°C in glycerol medium.

O = parent type

Δ = petite

▲ = orange

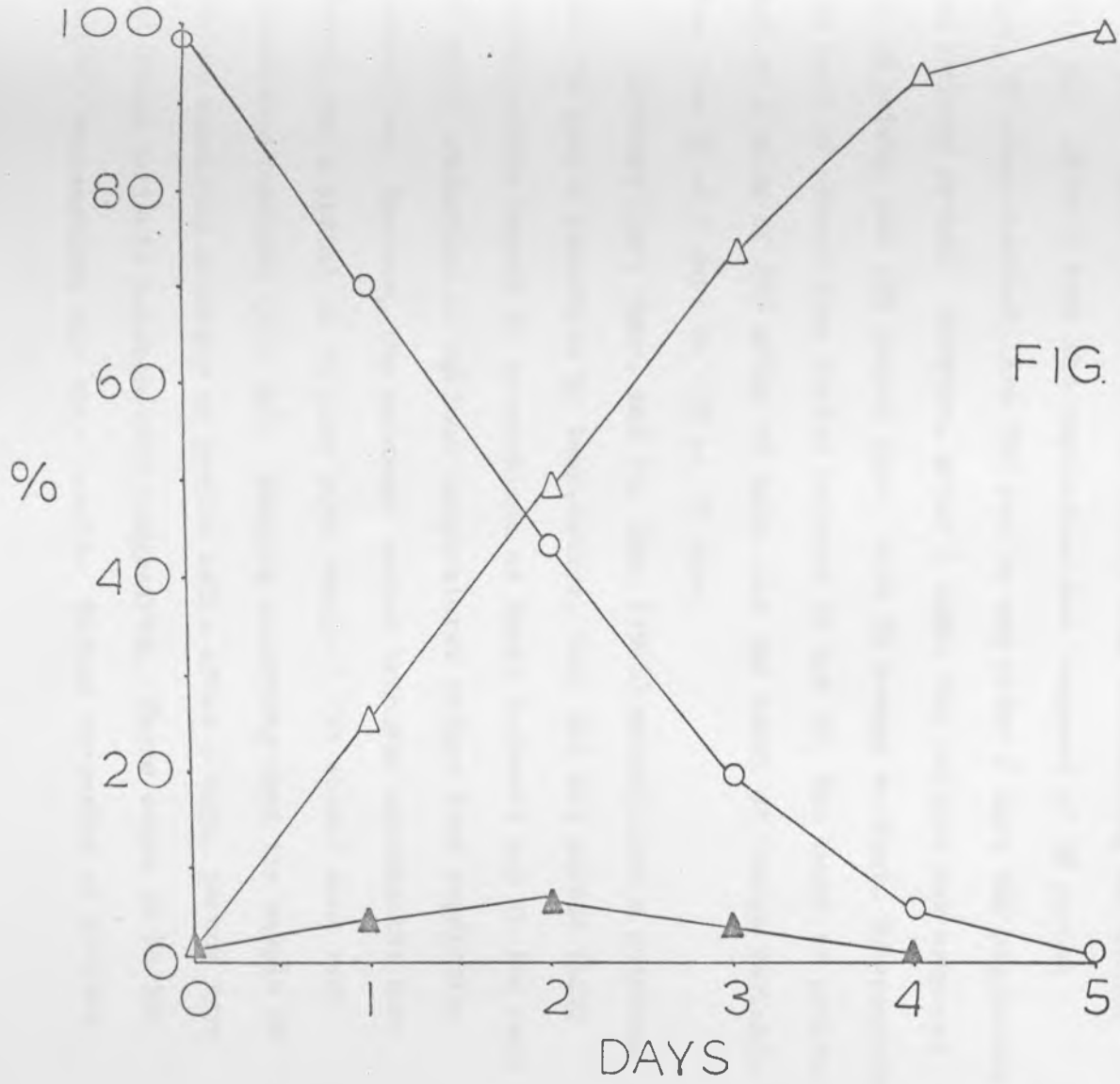


FIG. 5

fairly stable at 30°C, but at 15°C exhibited the same pattern of induction of petites as observed in the diploid strain (fig 6A, 6B).

To examine whether petites were continually induced by a temperature of 15°C over a period of time, serial transfer experiments were extended. Strains X 190, S91, and S110, were cultivated in 10 ml. of MAD medium on an orbital shaker at 15°C. Transfers were made every 48 hours and samples were plated out every 72 hours. After three days cultivation the two haploid strain cultures were composed entirely of petite cells (fig 7B, 7C) and remained essentially stable over periods of 12 and 18 days. Strain X 190 showed some interesting variations (fig 7A). After 3 days the population was composed of 3% parent type, 9% orange variant, and 88% petite and after 6 days the population was entirely petite. However, after 9 days, the culture was composed of 60% petite, and 32% parent type, with 8% orange variant. Subsequently the level of parent type varied between 6% and 9%, the level of petites fell to a value of 25% after 18 days, and the level of orange variants rose from 7% at 9 days to 66% at 18 days.

Although Ogur, Ogur, and St. John (1960) established a mutation rate for petite induction by temperature, they did not pursue their investigations beyond 10 generations as their interest lay in the rate of petite production at various temperatures rather than population composition. However, the extended serial transfer experiments conducted over a period of 18 days with strain X 190 showed some very interesting features (fig 7A). Despite observing that the sample of culture consisted entirely of petite cells after 6 days, parent type and orange variants subsequently reappeared. There seems to be two possible explanations for this result. Either reversion of petites

Figure 6. Population changes during serial transfer of stationary cultures of strain X 190 segregants in MAD medium at 15^{0}C.

A : S 91

B : S 110

O = parent type

Δ = petite

▲ = orange

FIG. 6

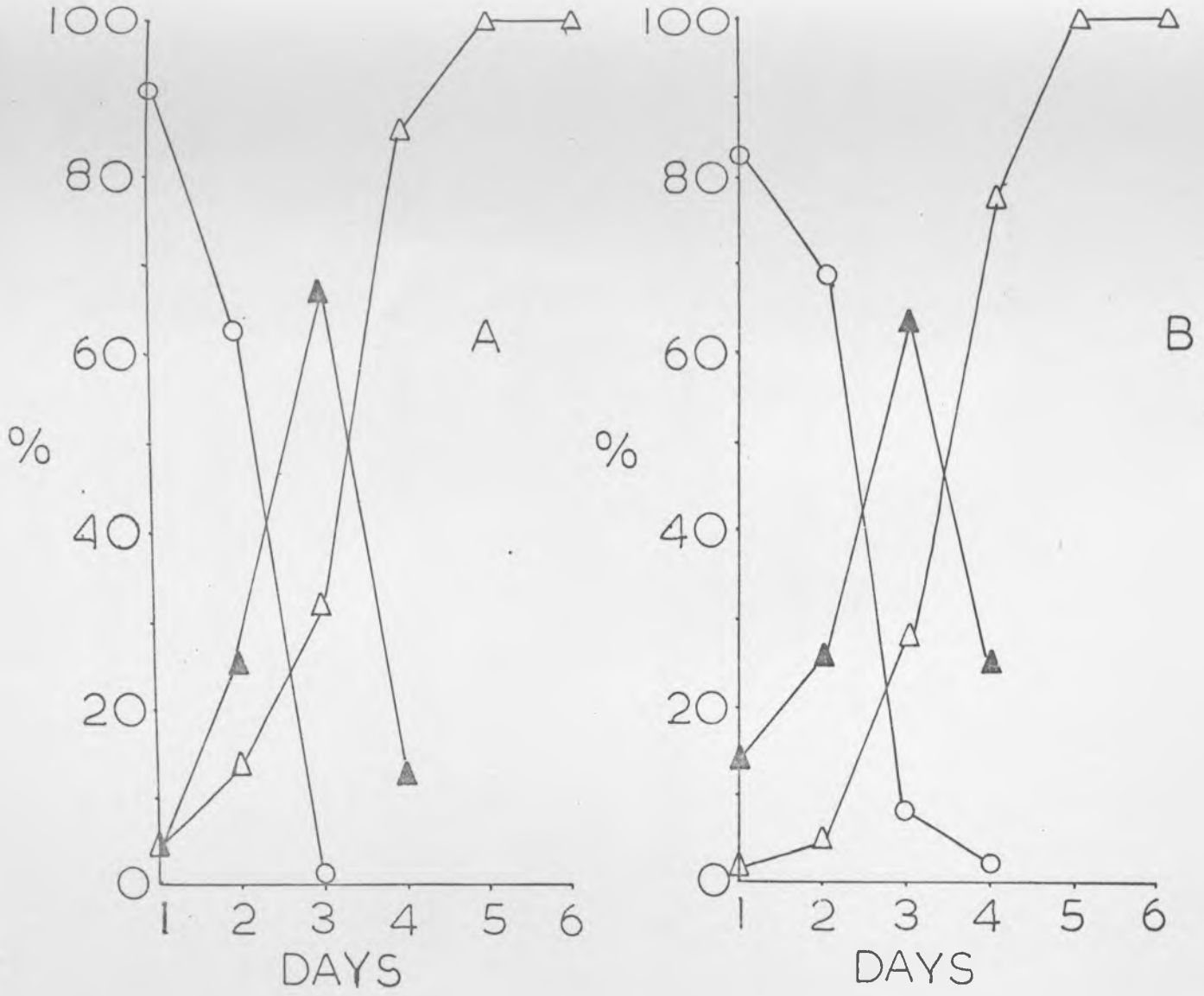


Figure 7. Population changes during extended serial transfer of shaking cultures of strain X 190 and segregants S 91 and S 110 in MAD medium at 15°C.

A : Strain X 190

B : S 91

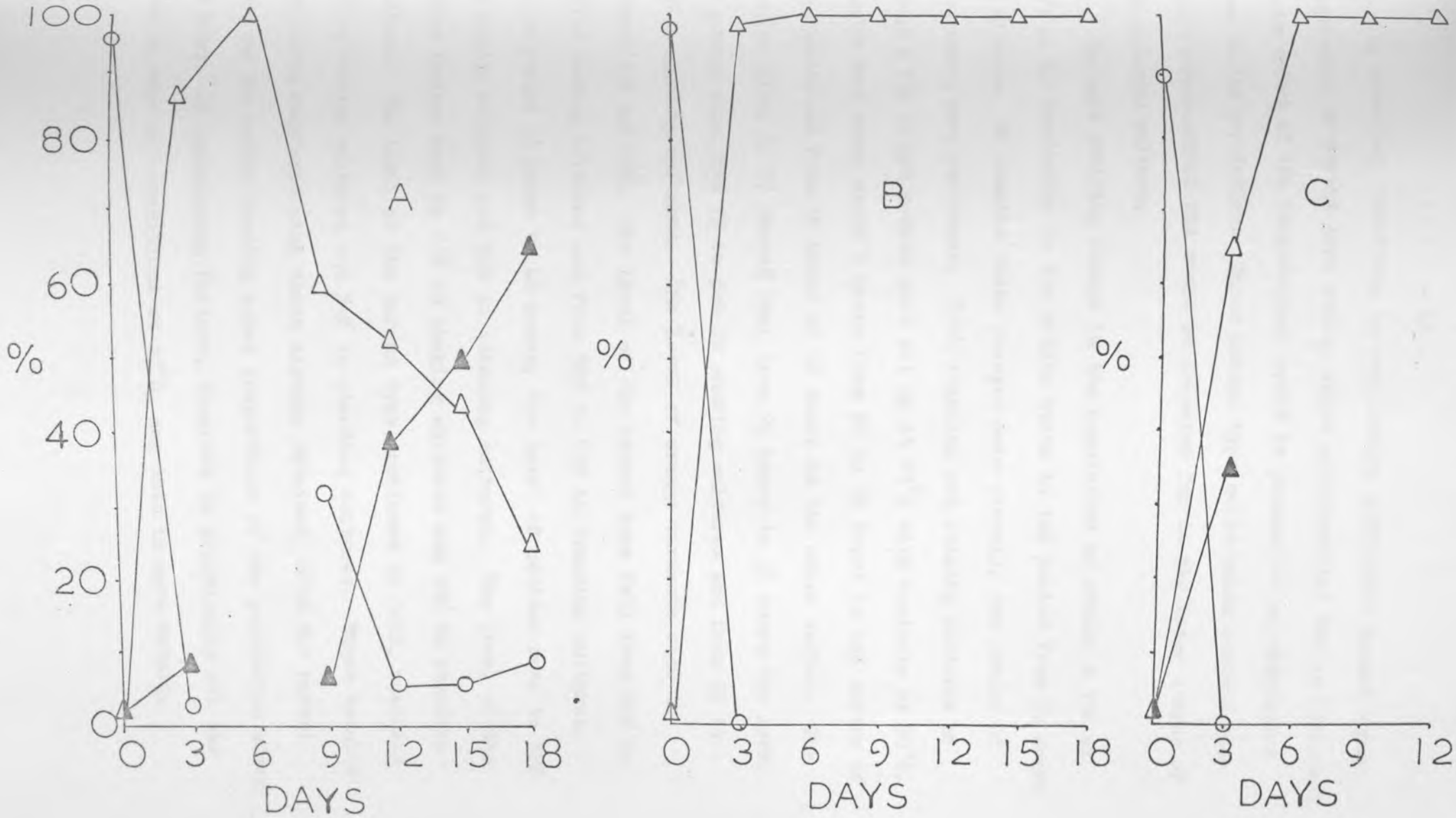
C : S 110

O = parent type

Δ = petite

▲ = orange

FIG. 7


100
80
60
40
20
%
A
0 3 6 9 12 15 18
DAYS
%
B
0 3 6 9 12 15 18
DAYS
%
C
0 3 6 9 12
DAYS

could be occurring, resulting in respiratory sufficient parent types reappearing, or parent type cells, whose mitochondrial DNA is resistant to the effect of low temperature, could be present at an undetected level in the population. These parent type cells which contain resistant mitochondrial DNA would be selected for in the later stages of the prolonged culture.

The most striking change in the population of strain X 190 at 15°C is the conversion to the petite state in the period from 24 hours to 48 hours. To examine these changes more closely, two series of experiments were performed. Both standing and shaking cultures of strain X 190 in MAD medium were set up at 15°C with controls at 30°C. Samples were taken every 2 hours from 24 to 36 hours in one series of experiments and from 36 hours to 48 hours in the other series. The results (figs. 8, 9) showed that from 24 hours to 36 hours the level of petites rose from 1% to 34% in shaking cultures and from 7% to 17% in standing cultures. The level of orange variants rose to between 15% and 20%. The level of the parent type fell from 94% to 50% in shaking cultures and from 90% to 65% in standing cultures. In the period 36 hours to 48 hours, the level of petites rose to 38% in shaking cultures and 36% in standing cultures. The level of the orange variant rose to 25% in shaking cultures and 30% in standing cultures. The level of the parent type continued to fall, reaching 36% in shaking cultures and 34% in standing cultures. These results correlated very well with those already obtained, with the parent type and the petite forming equal proportions of the population after 48 hours. An interesting feature, observed in practically all the results under all conditions at 15°C, was seen in more detail.

Figure 8. Population changes during the periods 24 - 36 hours, and 36 - 48 hours cultivation in aerated cultures of strain X 190 in MAD medium at 15°C.

O = parent type

Δ = petite

▲ = orange

FIG. 8

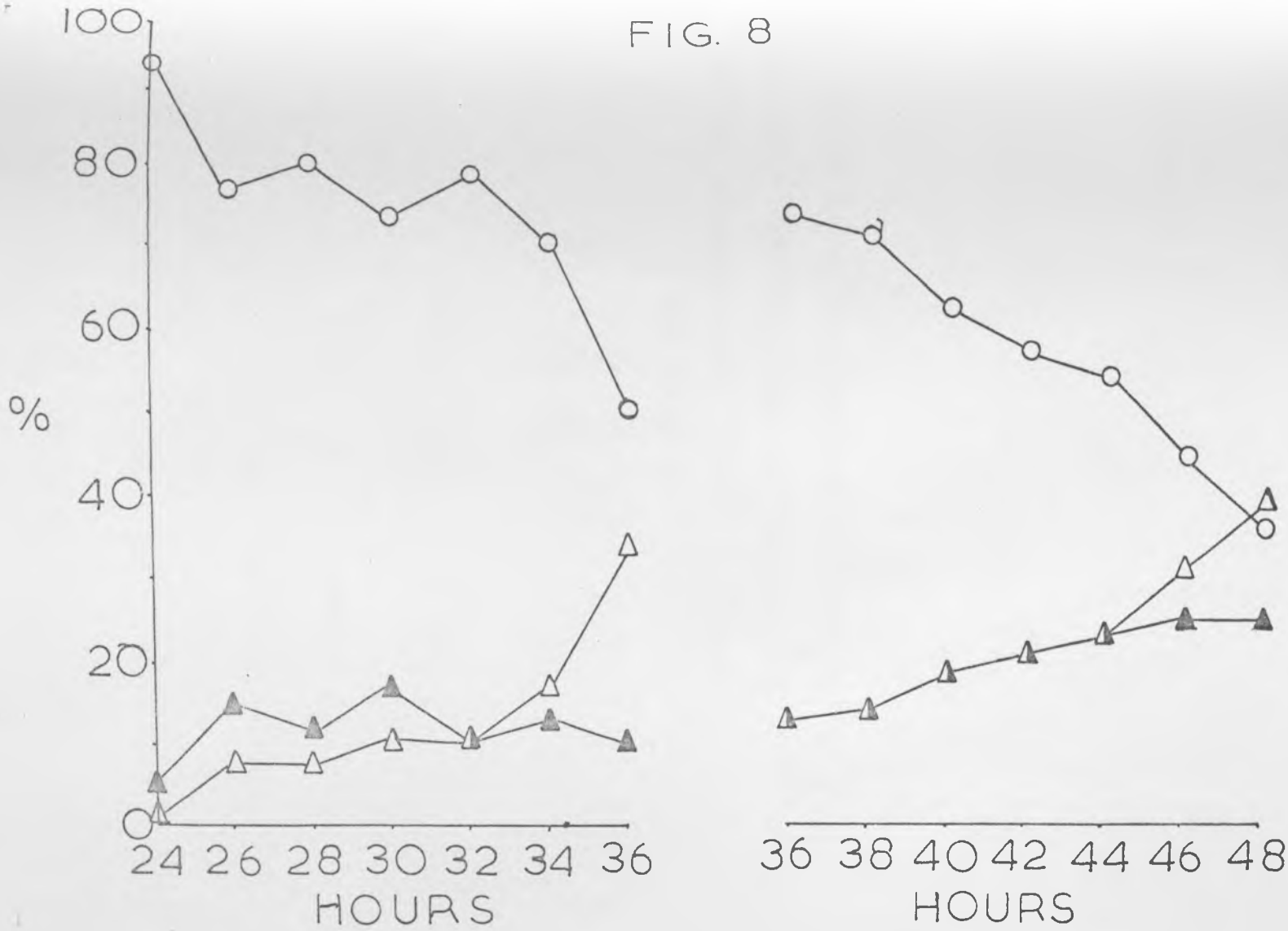


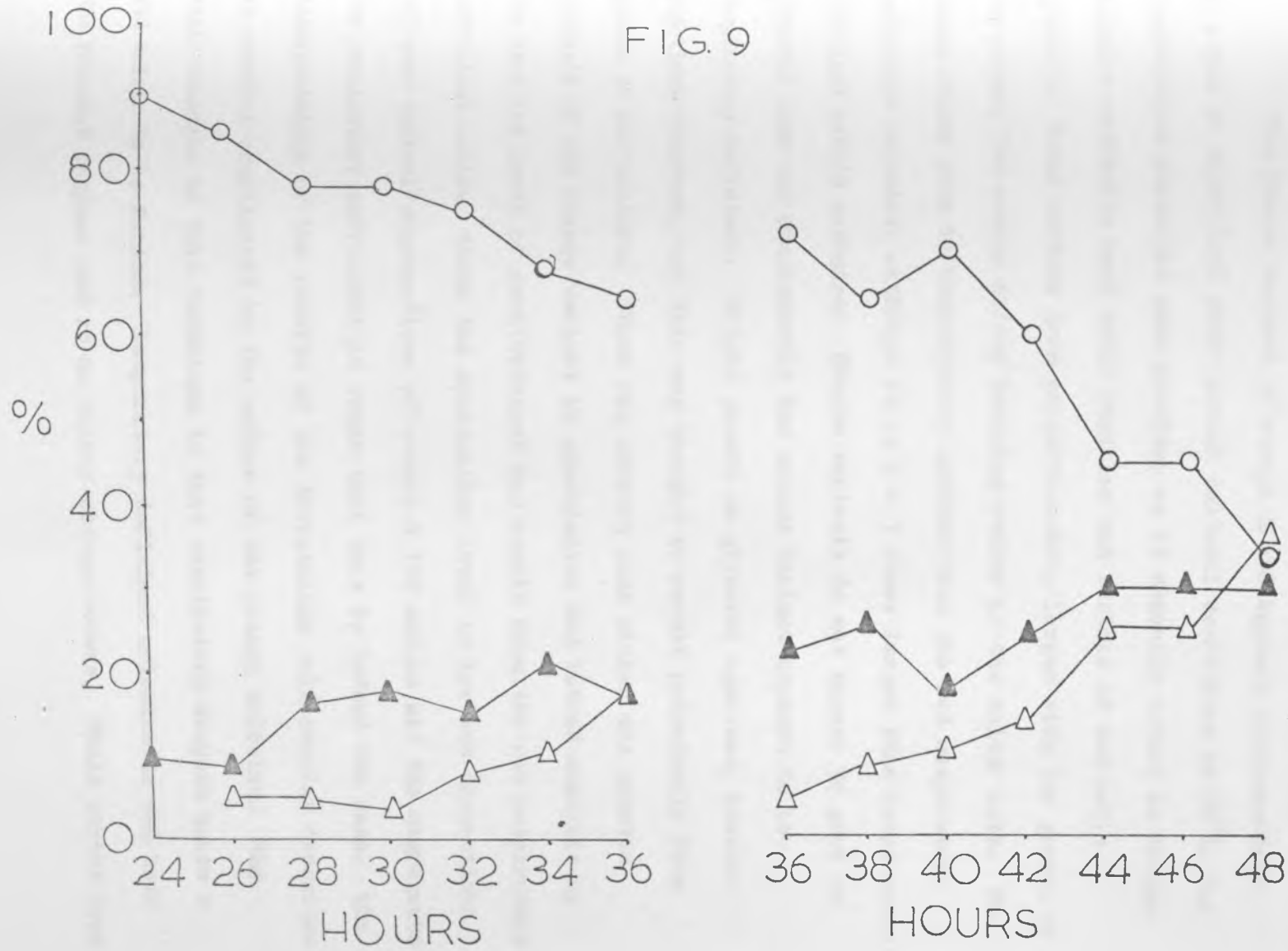
Figure 9. Population changes during the periods 24 - 36 hours and 36 - 48 hours cultivation in stationary cultures of strain X 190 in MAD medium at 15°C.

0 = parent type

Δ = petite

▲ = orange

FIG. 9


100
80
60
%
40
20
0
24 26 28 30 32 34 36
HOURS
36 38 40 42 44 46 48
HOURS

This was the initial rise in the level of orange variants in the 0 - 48 hours period, which in many cases preceded the rise shown by petites. The nature of this orange variant was further investigated.

(3) Investigation of the orange variant of Strain X 190

The orange variant of strain X 190 appears spontaneously at a rate of about 0.5% under normal cultural conditions at 30°C. Its investigation presented some problems as it commonly occurs as orange colonies containing many small papillae and sectors of red cells (plate 5). These sectors grow proportionately larger with the growth of the colony, the orange colony becoming redder as the colony ages. The orange colony size is consistently smaller than the red respiratory sufficient colonies, although it is 2 - 3 times larger than respiratory deficient petite colonies. Orange variants do not appear to grow on glycerol agar and consequently the orange variant appears to be respiratory deficient. Slight growth on glycerol agar was, however, sometimes observed, but this was thought to result principally from growth of red sectors. These red sectors also hinder the genetic analysis of the orange variant by sporulation and tetrad analysis as the very low level of asci obtained may result from the red respiratory sufficient cells. Since the sporulation level is low and dissections all show typical segregations of strain X 190 and as all the segregants are respiratory sufficient it seems that this is indeed the case. The interpretation of the results of the tetrazolium salt overlay technique is somewhat complicated by the colour of the orange colonies. The basic reaction in this technique is that respiratory enzymes cause a red colour to be formed. Respiratory deficient colonies do not have the relevant enzymes and so no colour change occurs. While parent type

colonies of strain X 190 and petites were clearly distinguished by this technique (plate 7), the reaction of the orange colonies was more difficult to interpret. The orange colonies did not turn bright red but they did change to a light shade of pink and the red sectors were easily distinguished from the large orange areas. Estimation of oxygen uptake by the Warburg technique showed that the orange variant is respiratory deficient as oxygen was not absorbed by a culture of orange variants. However the Warburg results do not indicate whether this respiratory deficiency is due to the absence of cytochromes a + a_3 and b, as in petites, or the absence of some other respiratory enzyme. Since there is a considerable difference in colony size and growth rate between orange variant and petite, their respiratory deficiency seems certain to be due to different causes.

To investigate the stability of the orange variant in more detail, six orange colonies, identified after only 48 hours incubation on MYGP agar at 30°C, were inoculated into MAD medium, cultivated at 30°C by daily serial transfers and samples were plated out every 24 hours. Appropriate dilutions were made to give approximately 150 colonies per plate. A sample of 0.1 ml. of a 10^{-2} dilution of the culture was transferred to a fresh culture vessel containing 9.9 ml. MAD medium. In practice this meant about 10 generations of growth every 24 hours. The results (table 2) showed that in two of the six cultures only red and orange colonies were found after 24 hours cultivation. In the remaining four cultures, red colonies were present at a level of 1 - 3% but petites were also present at a level of 6 - 7%. It is evident from the results of the following days that these populations were very unstable and the failure of repeated attempts to obtain pure cultures of orange variants

TABLE 2

COMPOSITION OF POPULATIONS OF SIX ORANGE COLONY CULTURES DURING SERIAL TRANSFER AT 30°C

CULTURE	DAY	PERCENT OF POPULATION				TOTAL NUMBER
		RED	ORANGE	WHITE	PETITE	OF COLONIES
1						
	1	1	98	0	0	1173
	2	23	74	1	2	1052
	3	41	54	1	3	775
	4	59	32	8	1	737
	5	57	14	26	2	1197
	6	30	4	65	1	753
2						
	1	12	87	0	0	1068
	2	32	64	0	3	963
	3	40	55	0	5	433
	4	57	40	1	2	775
	5	67	30	1	1	756
	6	81	15	3	0	354
3						
	1	1	92	0	6	433
	2	3	92	1	4	821
	3	6	89	2	3	829
	4	14	79	4	2	699
	5	21	58	18	2	799
	6	20	25	55	0	612
4						
	1	1	92	0	6	573
	2	6	90	0	4	677
	3	14	79	1	3	939
	4	22	52	24	2	670
	5	32	58	6	4	685
	6	23	43	13	0	536
5						
	1	2	90	0	7	663
	2	7	86	1	6	546
	3	10	85	1	4	901
	4	21	72	4	3	754
	5	35	52	10	3	727
	6	41	29	30	0	440
6						
	1	2	90	1	6	913
	2	7	88	1	3	631
	3	15	80	2	2	875
	4	24	70	3	2	762
	5	40	51	7	2	768
	6	55	30	15	0	578

by various techniques confirms this inherent instability. The tendency in these cultures was for petites to stay at a level between 3 - 5% irrespective of the initial value, several times higher than the normal rate observed for strain X 190. The principal trend was for the level of red and large white colonies to increase, with a corresponding decrease in the level of the orange variant. The actual proportions in which red and white colonies appeared, varied from culture to culture and probably depended on selective factors. The red and large white colonies are both respiratory sufficient and in consequence, as the orange variant is respiratory deficient, have a selective advantage over both orange and petite variants.

The large white colonies were analysed phenotypically for their adenine and tryptophan requirements by replica-plating onto adenine and tryptophan omission media (table 3).

TABLE 3

Phenotypic analysis of white colonies observed during culture of 6 orange colonies

	NUMBER OF COLONIES		
DAY	AD^-TRY^-	AD^-TRY^+	AD^+
1	3	7	0
2	26	13	0
3	31	7	17
4	54	27	84
5	99	69	173
6	90	54	146

The rate of mitotic recombination for the ad_8 mutation (ad^- and ad^-try^- colonies) was determined graphically (fig 10) and was estimated as 8.8 X 10^{-4} per cell per generation. This rate is similar to, although somewhat higher than, the rate of 6.7 X 10^{-4} per cell per generation estimated in strain X 190 at 30°C by Johnston and Mackinnon (1966). The principal trend in these cultures, however, was from orange to parent type, the average rate from orange to parent type being estimated as 8 X 10^{-3} per cell per generation, and individual rates varying in the six cultures from 5 X 10^{-3} to 1.6 X 10^{-2} per cell per generation (fig 11). This very high rate of reversion to parent type, coupled with reversion to adenine independence, a relatively high frequency of petites, and a fairly high rate of recombination for the ad_8 mutation explain the high level of instability of the orange variant, and this instability could be an important factor in the variations observed in strain X 190 at 15°C.

The serial transfer technique must be subject to scrutiny when considering variations in the population of strain X 190 at 15°C. In serial transfer the main intention is to maintain conditions of exponential growth and a stationary phase should not occur at the end of the growth cycle. The dilution factor of 10^{-4} between each culture is entirely consistent with this principle for the growth of strain X 190 at 15°C. This gave approximately 14 generation in 48 hours with a generation time of just under 3½ hours. The generation time of parent type cells of strain X 190 was extimated as 3 hours 10 minutes and that of X 190 petites as 4 hours 40 minutes at 15°C (fig 12). The overall generation time of strain X 190 based on results in figures 8 and 9 was estimated as 3½ hours.

The actual transfer inoculum contained approximately 10^5 cells and was considered a representative sample of the population unless the level

Figure 10. Estimation of the rate of recombination of the ad_8 mutation in the orange variant of strain X 190. R = Line of regression

FIG. 10

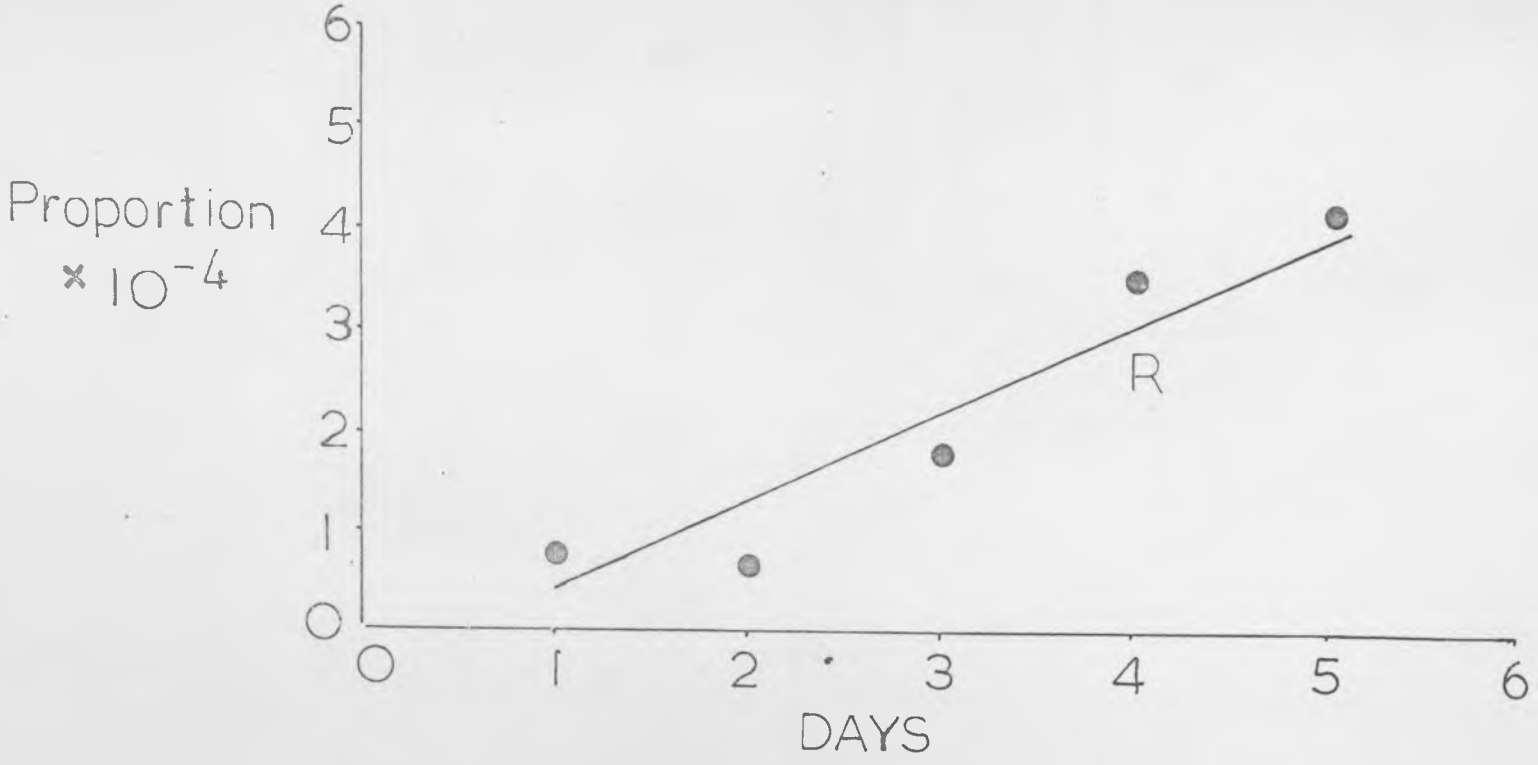


Figure 11. Estimation of the rate of change of orange cultures to parent type at 30°C.

A : maximum observed rate

B : minimum observed rate

M : mean rate based on observations in six orange cultures

1 cycle = 10 generations

FIG. 11

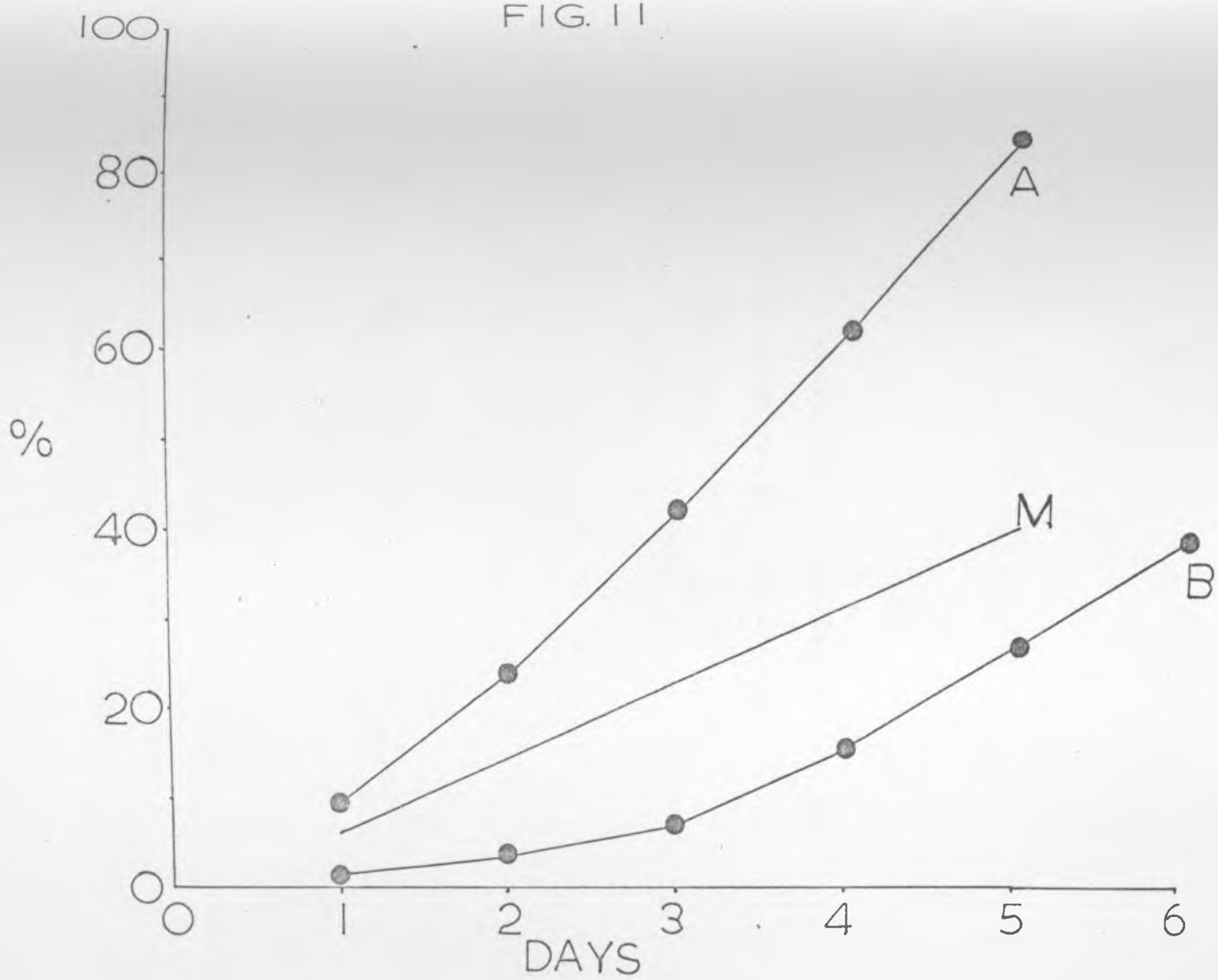
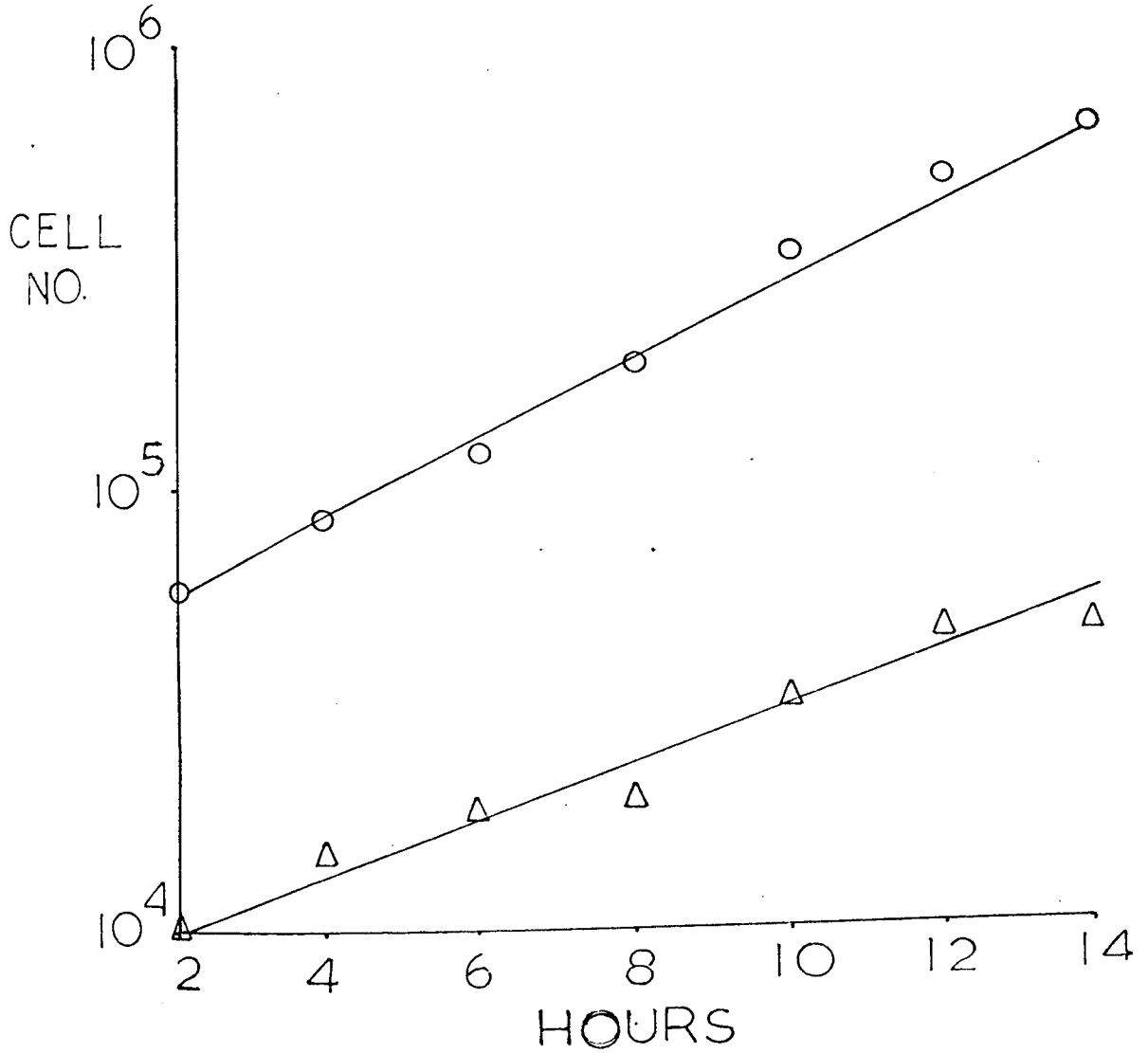


Figure 12. Estimation of the generation time of strain X 190 parent type and petite at 15°C.

O = parent type

Δ = petite

FIG 12



of a variant was very low. However, the level of parent type cells in strain X 190 was very low after 2 - 3 days of serial transfer at 15°C, when the sample population was entirely petite. As only 10^5 cells were transferred to a fresh flask of media then the serial transfer technique could be selective against the parent type if these composed $< 0.01\%$ of the population.

The growth conditions were highly favourable to aerobic respiration with glucose as a carbon source and good aeration. The results of extended serial transfers of strain X 190 could be explained by supposing that nuclear genetic material can code for the direct formation of new mitochondrial DNA. In thes circumstances the results could be explained as a lag phase in the production of mitochondrial DNA until adaptation to the new temperature took place. However, it could be argued that since induction of petites was taking place then the gene controlling mitochondrial DNA replication was directly affected and could not code correctly for the formation of new mitochondrial DNA. Such direct chromosomal control has not yet been reported and the evidence at the moment suggests that mitochondrial DNA has a large degree of autonomy. Mitochondrial DNA has been shown to control the formation of cytochromes $a + a_3$ and b and the inner membrane of the mitochondrion (Prezbindowski et al., 1968). Moreover, if all petite induction is due to mutation at the chromosomal level then this site is unusually susceptible to a large variety of mutagens. Assuming the evidence to be correct and that mutation of mitochondrial DNA is the main source of petite production, then another explanation of these results must be sought.

It is thought that petite induction is the result of a mutagen converting mitochondrial DNA to 'nonsense' DNA (Woodward and Munkres, 1966;

Mounolou et al., 1966). There is also evidence to suggest that mitochondrial DNA is heterogeneous (Avers et al., 1965; Avers, Pfeffer and Rancourt, 1965; Avers, 1967; Avers et al., 1968). If this is the case then the possibility exists that not all the mitochondrial DNA in strain X 190 is susceptible to the mutagenic effect of low temperature. A fraction of the population could remain respiratory sufficient and, under cultural conditions which convey a selective advantage, be re-established in the population. Alternatively, reversion of petites to respiratory sufficient parent types may be taking place as has been reported by Kraepelin (1964).

Should similar variations in the population of strain X 190 at 15°C occur during continuous cultivation then the effects could be far-reaching. The stability of strain X 190 at 15°C during continuous cultivation was therefore examined.

Continuous cultivation of Strain X 190 at 15°C

Strain X 190 was cultivated continuously in a simple Chemostat apparatus with a working volume of 200 ml. and flow rate of 25 ml. per hour at 15°C. These conditions gave a generation time of 8 hours, this being the time required for the complete replenishment of the growth medium in the culture vessel. Samples were taken directly from the effluent tube once every 24 hours.

The results of the first experiment (fig 13) showed that in the initial period of 24 hours the level of petites rose from 6% to 30% and remained at a level between 14% and 32% for the duration of the experiment. The orange variants followed a similar pattern to petites and, after an initial rise from 3% to 26%, remained at a level of 22 - 34% for the duration of the experiment. The parent type level fell to 44% in the first 24 hours and remained at a level fluctuating between 43% and 57%. It seems that a crude equilibrium quickly became established in the population with the proportions of parent type, petite, and orange variant, remaining in a fairly constant relationship after the initial changes.

The results of the second experiment (fig 14) were similar to those already obtained. The initial phase of 24 hours gave results very similar to those obtained in the first experiment, and, from the results of the next two days, it seemed that an equilibrium had been established. However, after 3 days the level of petites rose steadily to a level of 90% after 8 days. The levels of both parent type and orange variant fell to a level of 5% after 8 days. Unfortunately only one more sample was taken and it was not possible to tell whether an equilibrium, or a pseudo-equilibrium, had been established.

The results from both experiments were based on plate counts of

Figure 13. Population changes during continuous cultivation of strain X 190 in MAD medium at 15°C.

V = 200 ml. F = 25 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 13

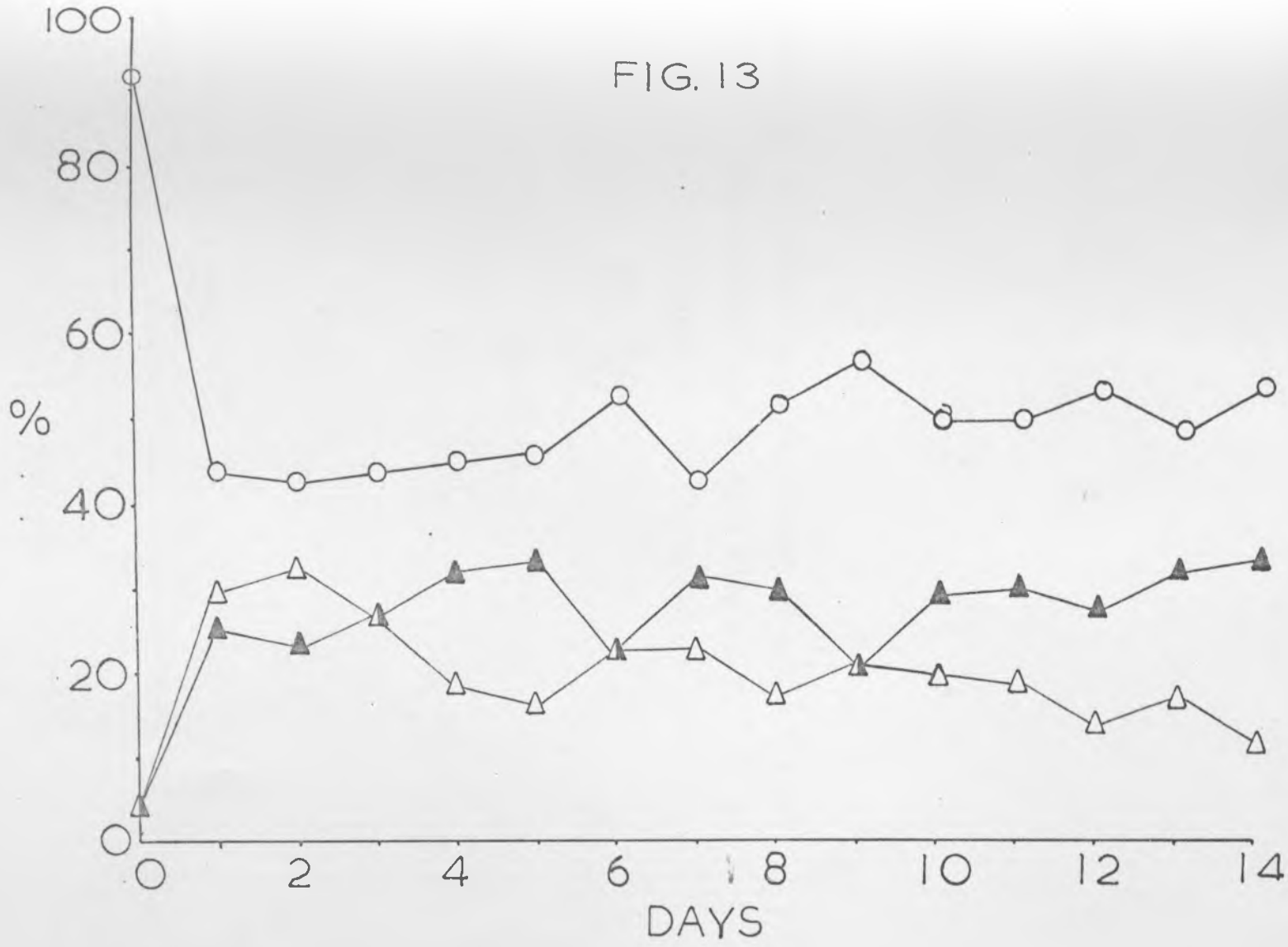


Figure 14. Population changes during continuous cultivation of strain X 190 in MAD medium at 15°C.

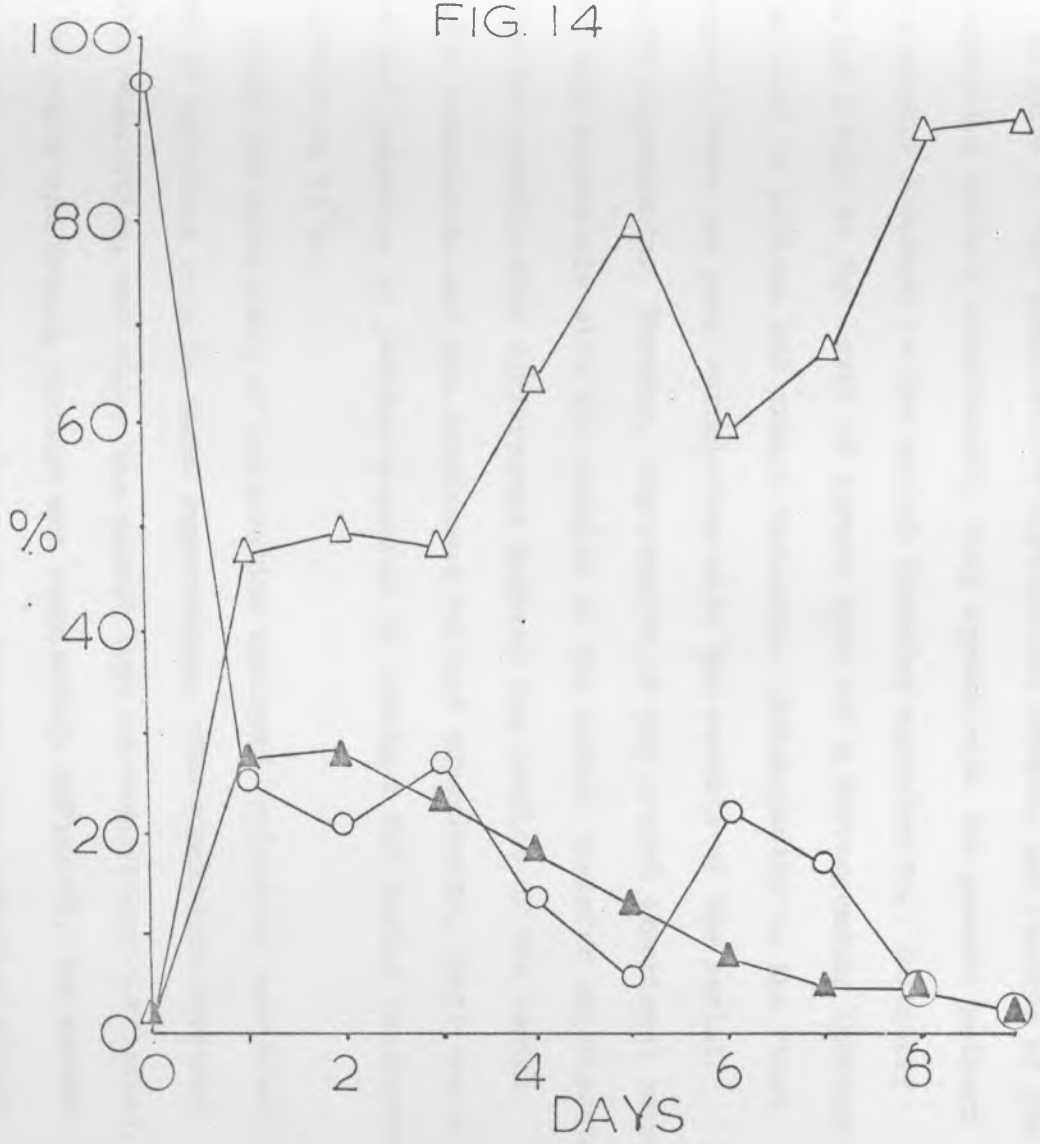
V = 200 ml. F = 25 ml./hour

0 = parent type

Δ = petite

▲ = orange

FIG. 14



approximately 200 colonies per plate, with the total number of colonies per sample counted in excess of 400 with one or two exceptions. The cultures contained approximately 3×10^7 cells per ml. throughout, and spot checks made on the effluent showed that no glucose was present. These checks, coupled with the constant cell density, indicated that glucose was the growth limiting nutrient. This was not unexpected since the flow rate of 25 ml. per hour gave a culture generation time of 8 hours. Since the generation time of strain X 190 at 15°C in the 'unlimited' conditions of batch growth was estimated as $3\frac{1}{2}$ hours then it was reasonable to expect that all the glucose would be consumed.

In spite of the quantitative differences between the results of the two continuous culture experiments, they agreed with the general pattern of the results obtained in the serial transfer experiments. Initially there was a fall in the level of parent type and a corresponding increase in the level of petites and orange variants. Subsequently in the first experiment there was poor correlation with the results of the serial transfer experiments. However, the results of the second experiment correlated more accurately with the results of the serial transfer experiments. Despite the quantitative differences between the results of the serial transfer experiments and the continuous culture experiments, there was no doubt that induction of petites occurred in strain X 190 during continuous cultivation at 15°C.

Under the conditions of cultivation employed, selection undoubtedly played an important role in these experiments. The conditions favoured aerobic respiration, and since the parent type is respiratory sufficient, and the petite and orange variant are respiratory deficient, the parent type should enjoy a selective advantage. However, the inductive effect

of temperature in producing petite and orange variants seemed to balance this selective advantage in the first experiment. In the second experiment the inductive effect apparently exceeded the selective advantage and petites became the major component of the population.

It was decided to alter the cultural conditions by increasing the working volume to 300 ml. and the flow rate to 75 ml. per hour. These alterations were made for several reasons. The working volume was increased from 200 ml. to 300 ml. because of two possible effects arising from an aeration rate of 2 litres per minute. Firstly, volatile intermediates of the glucose oxidation pathway might be removed from the cells and this could lead to depression of the growth rate of respiratory sufficient types of cells. Secondly, with low working volume, low flow rate, and high aeration, the working volume might be reduced by evaporation. As MAD medium is not selective against petites, the cultural conditions were altered to be more selective against respiratory deficient variants. The relationship between the flow rate and the working volume controls the culture generation time. The generation time of petite cells of strain X 190 at 15°C has been estimated as $4\frac{3}{4}$ hours, and that of strain X 190 parent type as $3\frac{1}{2}$ hours. The flow rate was adjusted to 75 ml./hr., to give a culture generation time of 4 hours ($\frac{V}{F} = \frac{300}{75}$), which should be selective against petites but not against the parent type.

Two cultures were set up under these modified conditions and two features of the results (figs 15A,B) were immediately apparent. Firstly, induction of petites proceeded at a very high rate and, under conditions which were strongly selective against petites, reached a constant value in excess of 90%. Secondly, a feature already noted in other experiments was highlighted, the orange variants showed a rapid increase in the first

Figure 15A and B. Population changes during continuous cultivation of strain X 190 in MAD medium at 15°C.

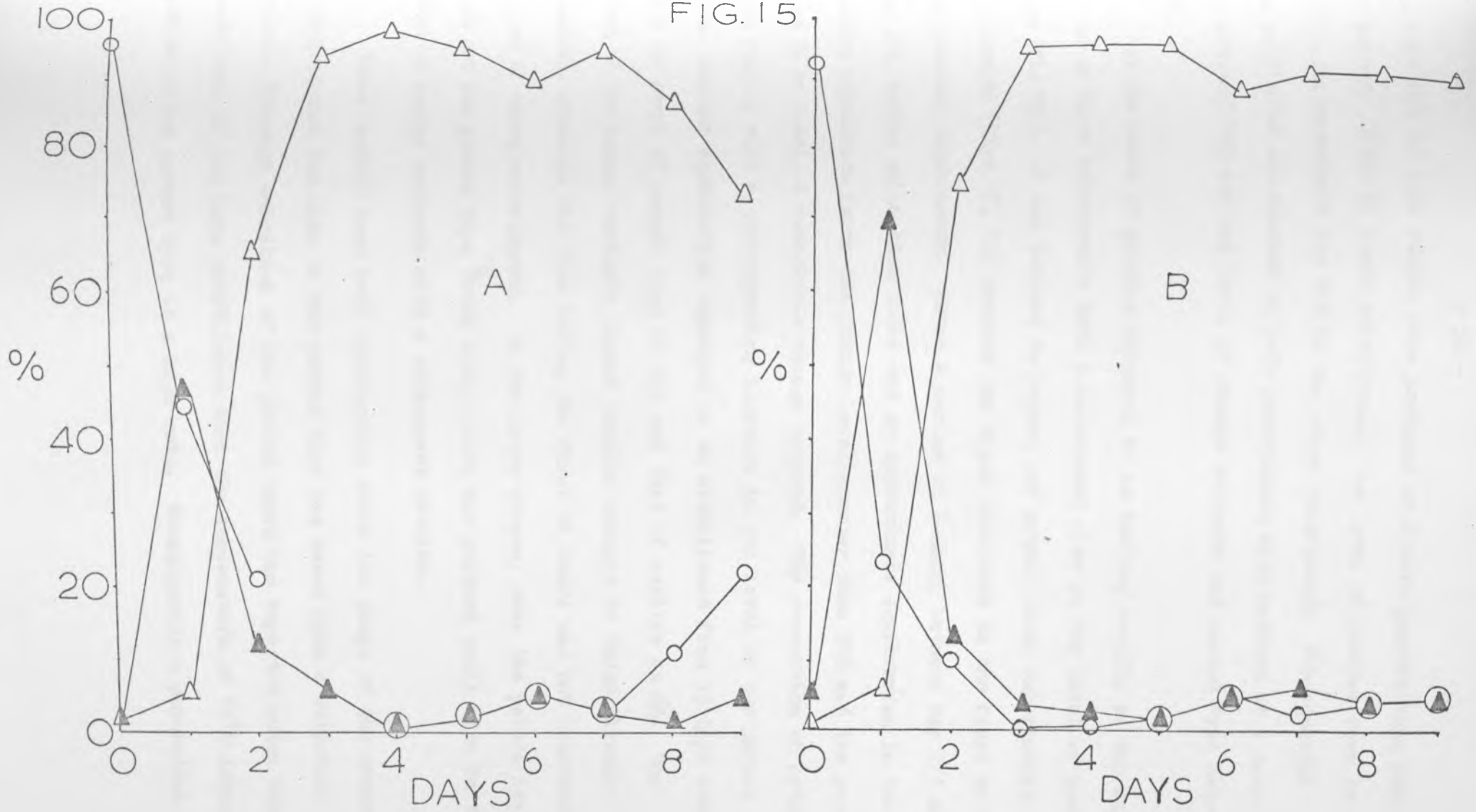
V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 15



24 hours and, at this stage, were produced at a rate greater than that of petites. After 48 hours cultivation, the level of petites rose to 66% in one experiment and 76% in the other experiment. Subsequently an equilibrium was reached in both experiments with petites at a level in excess of 90% and the level of orange variants and parent type below 5%.

As the level of petites appeared to be falling rapidly at the end of one of these experiments with a consequent rise in the level of parent type (fig 15A), it was decided to repeat and extend these experiments. The results (figs 16, 17) confirm the trend indicated in the first of the two previous experiments. After a period of 5 days, between day 2/3 and day 7/8, during which time there was an approximate equilibrium in the culture population (with the petite level greater than 90% and the parent type 5% or less), a remarkable change occured. The proportion of petites fell rapidly with a corresponding increase in the level of the parent type. Another equilibrium appeared to be established from 15 days onwards, with the level of parent type at 90% and that of petites at 6%. The level of the orange variants showed similar changes to those already observed, although the rise during the first 24 hours was not observed in one of these experiments. In the later stages, when the petite level fell and the parent type level rose, there was another small rise in the level of orange variants with a subsequent decline.

These results were very interesting since the shape of the graphs indicated that the rise of the parent type was based upon a selection process. Although selection of the parent types was expected under these conditions, it had been established that the temperature of 15°C induced petites in the parent type at a high rate. Consequently a population

Figure 16. Population changes during continuous cultivation of strain X 190 in MAD medium at 15°.

V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 16

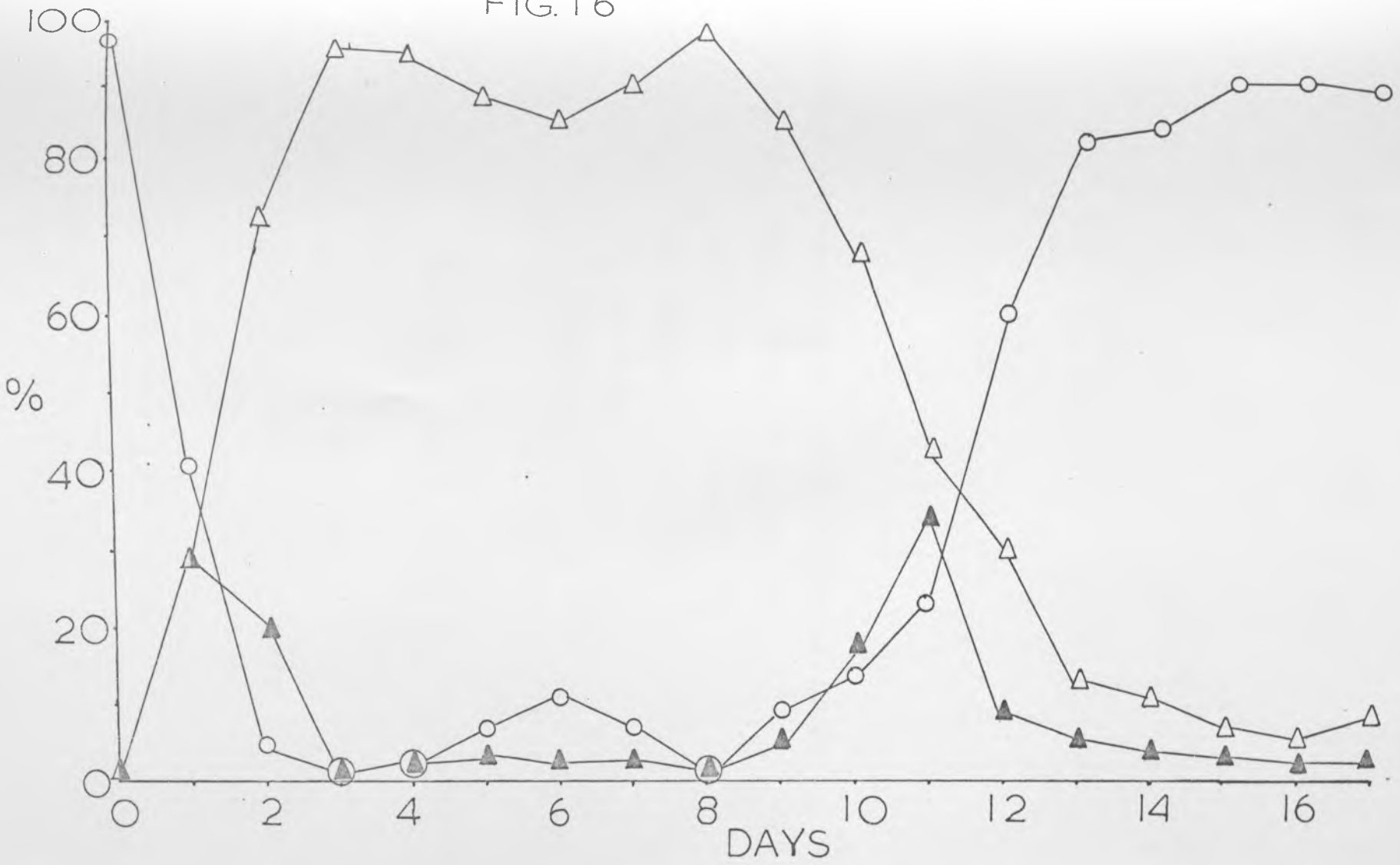


Figure 17. Population changes during continuous cultivation of strain X 190 in MAD medium at 15°C.

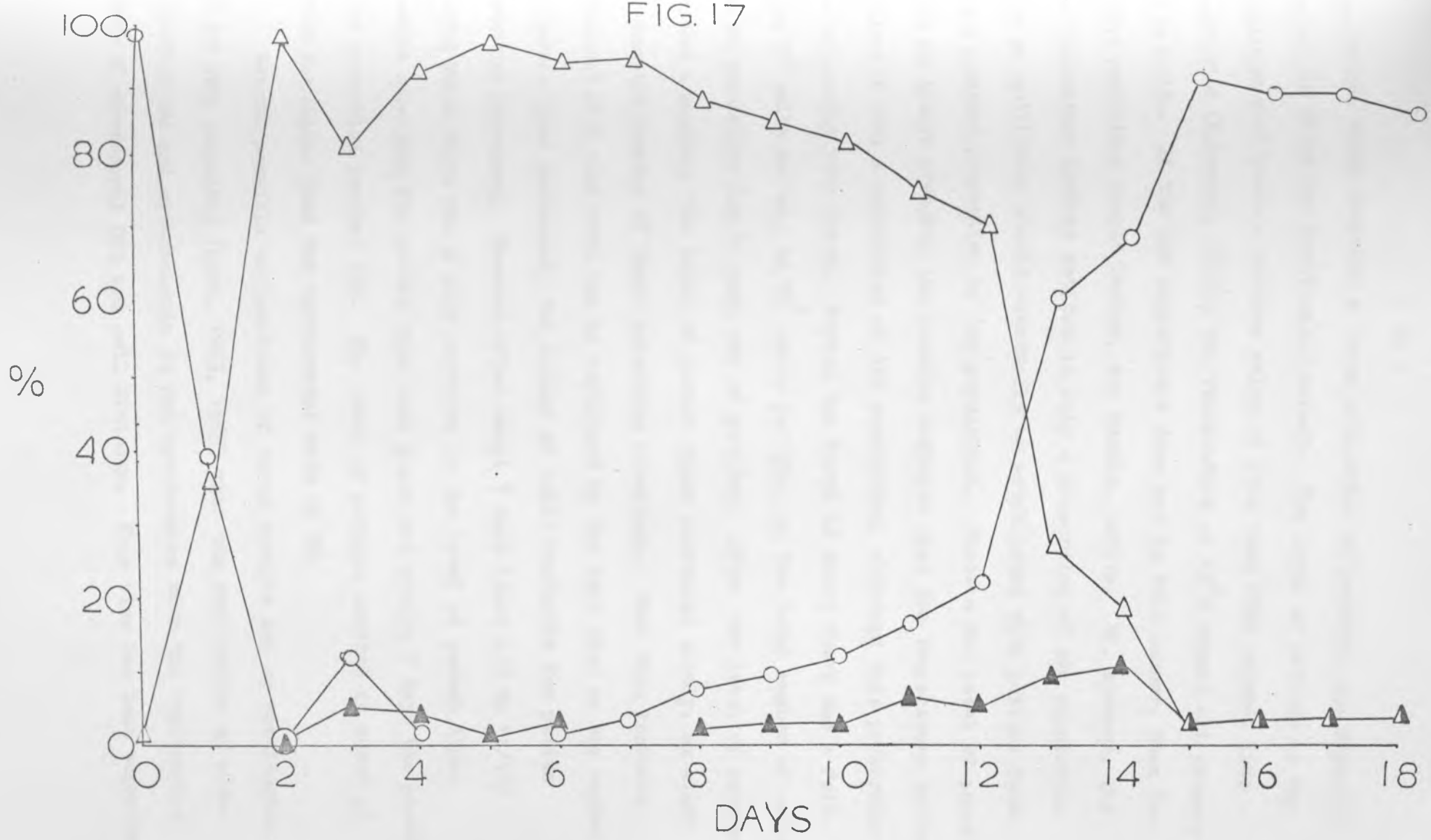
V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 17



equilibrium, which included a large proportion of petites, was expected, and not one which was practically normal. The level of petites in the population must reach a maximum value of less than 100% unless, like acriflavine (Ephrussi, 1953), the temperature of 15°C caused all progeny to be petite. If the low temperature does act in this manner, then the entire population should become, and remain, petite. If, however, the low temperature induces petites in only a proportion of the population, then an equilibrium should eventually be established with petites forming a constant proportion of the population. Since a low level of parent type was always present, the results indicate that the temperature induced petites in only a proportion of the population, although this proportion was apparently very large. During the first 48 hours there was a fall, from 10^8 cells per ml. to 10^6 cells per ml., in the total number of cells in the population due to wash out of petites. After the level of petites reached a maximum, the level of parent types increased slowly, as might be expected because of their selective advantage. That this increase proceeded at a slow rate can be explained by the fact that as the number of parent types increased, the number of cells available for petite induction increased. However after about 7 days (days 4/5 to 11/12) during which there was a slow increase in the level of parent types, massive selection for parent type took place and within 3 days the parent type proportion reached 90%. The level of petites settled at about 4% which was higher than the spontaneous rate of 1%.

Several possible explanations of these results can be considered. It has been suggested (Luck, 1963, 1965) that the replication of mitochondrial DNA and mitochondria is not synchronous with the replication cycle of chromosomal DNA and cell division. This view has been supported

by Smith et al., (1968) who found that in synchronous cultures of yeast, replication of mitochondrial DNA was periodic but occurred at different times to replication of chromosomal DNA. It was also found that the size and number of mitochondria varied during the lifetime of a cell in response to the environmental conditions. If the rate of replication of mitochondrial DNA and mitochondria was reduced by the low temperature of 15°C by a greater factor than that of chromosomal DNA and cell division, then an increasing proportion of the daughter cells would be petite due to lack of mitochondria. This would explain the rise in the proportion of petites. Subsequently, if the rate of replication of mitochondrial DNA were to adapt to the temperature and increase, then an increasing number of daughter cells would be respiratory sufficient. This explanation assumes that the decrease in temperature affects differentially the rate of replication of mitochondrial and chromosomal DNA. As yet there has been no evidence to support this assumption and there appears no obvious reason why the rate of replication of mitochondrial DNA should be slowed more than that of chromosomal DNA or, for that matter, vice versa.

Another possible explanation of these results is that not all mitochondrial DNA need have the same sensitivity to temperature. Although all mitochondrial DNA has the same basic functions, there is evidence to suggest that it is heterogeneous (Avers, 1967). This being so, the possibility exists that not all the mitochondrial DNA is susceptible to mutation by temperature. If a fraction of the mitochondrial DNA present in the culture was not susceptible, then variation in the culture could follow the pattern shown in the results. A small fraction of the population would always be respiratory sufficient and there would

be selection for the fraction which contains mitochondrial DNA resistant to the low temperature. Eventually the population would consist of respiratory sufficient cells whose mitochondrial DNA was not temperature-sensitive, along with a fraction of cells whose mitochondrial DNA was sensitive. Cells of the latter fraction would show a high rate of petite induction and petites would still appear in the population at a rate greater than the spontaneous rate.

A further explanation which cannot be discounted is reversion of petites to respiratory sufficiency. Reversion of petites at 23°C under conditions of restricted growth has been reported for a strain of Saccharomyces cerevisiae (Kraepelin, 1964). Revertants took 18 - 30 days to appear in Raut-Hebbs' medium (Raut-Hebb and Slebodnik, 1958) and only a fraction of the population became respiratory sufficient. The 'reversion time' was shortened to 11 - 14 days when a medium of yeast extract, glucose, and peptone, was used. This latter experiment shares similar features of medium and low temperature with the continuous culture experiments. During the first 24 hours of continuous cultivation of strain X 190 at 15°C, and again when the level of petites was falling rapidly after 11 - 13 days cultivation, a rise was observed in the level of orange variants. The orange variant, while being respiratory deficient, appears to be an intermediate type, as its growth rate is much greater than that of petites and only slightly inferior to that of parent type. Possibly the orange variant is an intermediate for two processes: from respiratory sufficient to respiratory deficient and, reversion from respiratory deficient to respiratory sufficient parent type. These possibilities will be considered further following the description of additional experiments.

Continuous cultivation of Strain X 190 at 18°C

Since many brewing processes are performed at, or about, a temperature of 18°C, it was decided to investigate the continuous cultivation of strain X 190 at this temperature. In order to compare the results with those obtained under similar conditions at 15°C, a working volume of 200ml. and a flow rate of 25ml. per hour were first used.

The results (figs 18, 19) resembled those obtained at 15°C under conditions of larger working volume and faster flow rate (figs 16, 17). However it should be noted that the time scale at 18°C is twice that employed at 15°C. In essence, the results indicated that the induction of petites was apparently much slower than at 15°C. A rise in the level of orange variants was observed during the first 2 - 4 days of cultivation at 18°C. This rise was, however, more marked and it reached a higher level (56 - 82%) and laster longer (2 - 3 days) than at 15°C. The level of petites reached maxima of 75% and 84% in the two experiments but fell from these values much more quickly than at 15°C. The high level of petites was maintained for only 2 - 3 days at 18°C compared with 5 - 6 days at 15°C. Basically, however, the pattern of changes in the population was the same at both temperatures with the variations in the duration of each change probably being due to a combination of temperature and culture generation time. The ultimate fall in the level of the parent type was due to the appearance of adenine recombinant and revertant types in the population. The recombinants, while still requiring adenine, have been found to have a selective advantage over the parent type (Roman, 1956). The revertants are adenine independent and have a selective advantage over all other types in the population. The results showed that 18°C also induced petites in strain X 190.

Figure 18. Population changes during continuous cultivation of strain X 190 in MAD medium at 18°C.

V = 200 ml. F = 25 ml./hour

○ = parent type

● = adenine recombinants and revertants

△ = petite

▲ = orange

FIG. 18

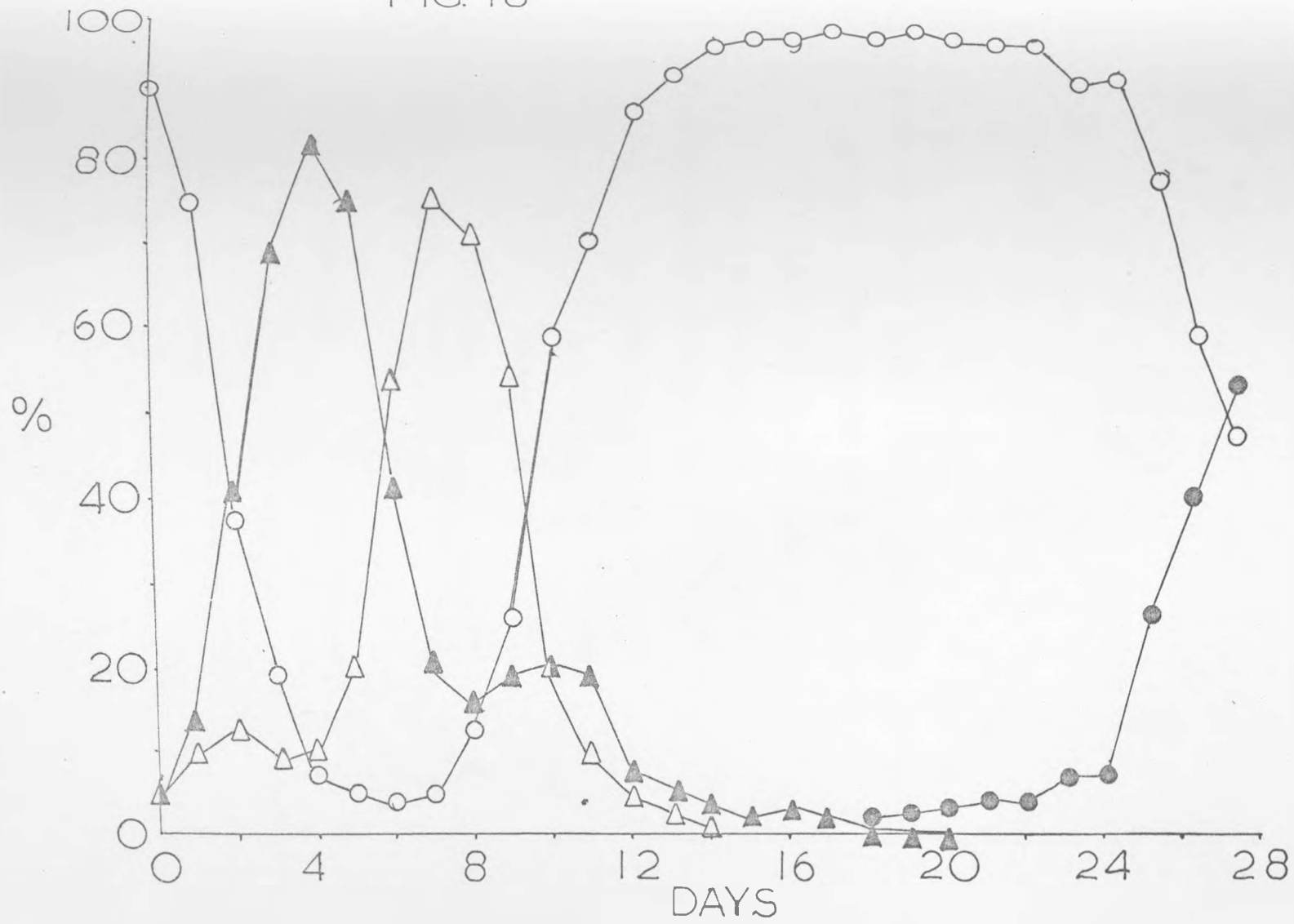


Figure 19. Population changes during continuous cultivation of strain X 190 in MAD medium at 18°C.

V = 200 ml. F = 25 ml./hour

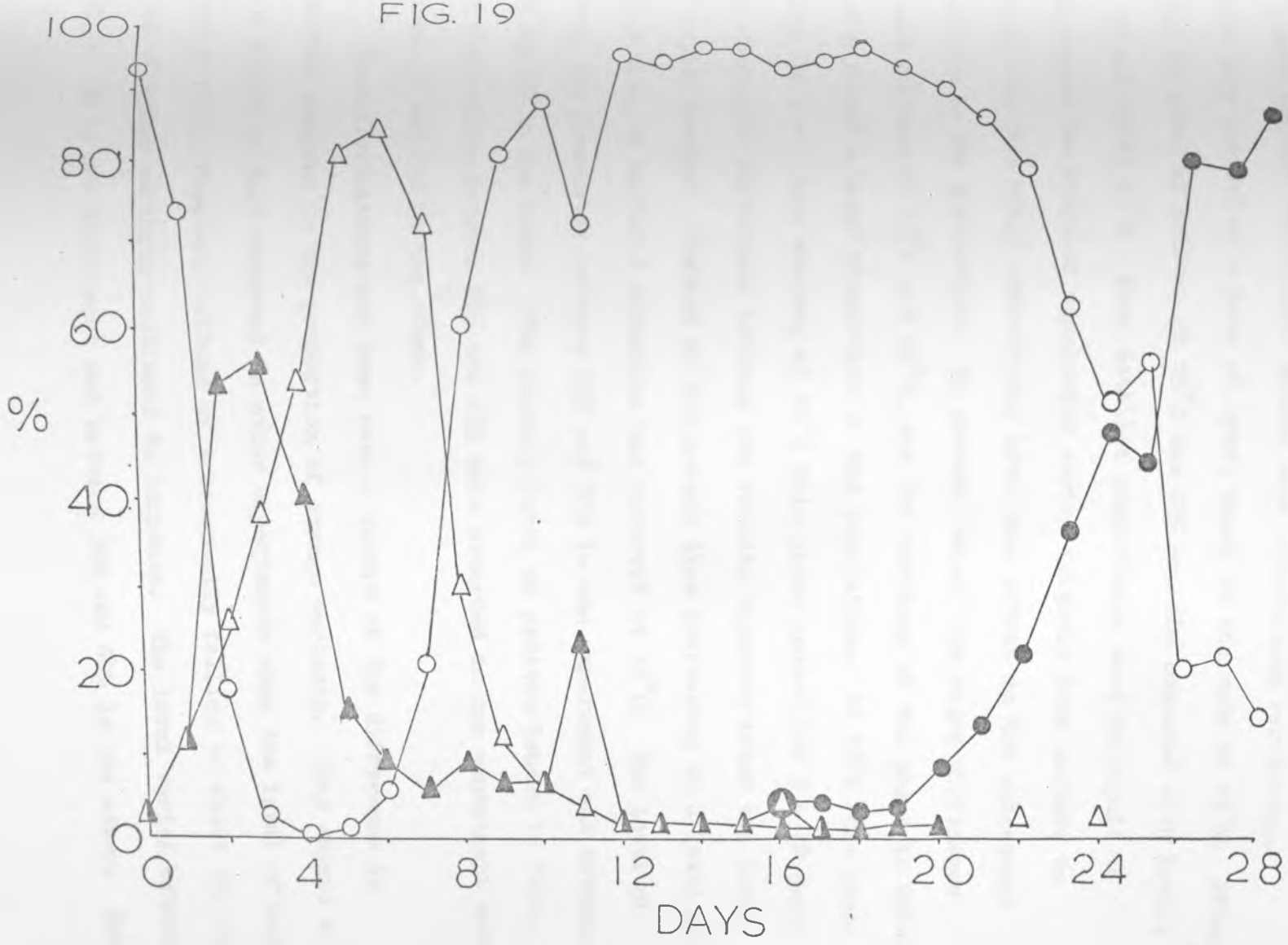
O = parent type

● = adenine recombinants and revertants.

Δ = petite

▲ = orange

FIG. 19



For a more accurate comparison of the effect of temperature on strain X 190 at 15°C and 18°C, the experiments at 18°C were repeated using a working volume of 300 ml. and a flow rate Of 75 ml. per hour. The results (figs 20, 21) while basically similar to those obtained at 15°C under similar conditions, showed some interesting variations. Petites were induced at a rate at least equal to the rate at 15°C. After 2 days the level of petites at 18°C was 85% and 88% compared with levels of 73% and 98% at 15°C. More detailed comparisons were difficult to make because the origianl population varied slightly from culture to culture, and this would undoubtedly have some effect on the subsequent variations in the population. In general terms, the major difference between cultures at 15°C and 18°C, was the duration of the phase in which petites formed a major proportion of the population. At 15°C this phase lasted for 5 - 6 days whereas at 18°C this phase lasted for 9 - 10 days. The other major difference between the results occurred after the level of petites dropped. Instead of the parent type increasing to a level of 90% or more, an unstable situation was observed at 18°C. The level of parent type fluctuated between 25% and 30% in one experiment and between 20% and 60% in the other. The overall level of petites tended to fall, but fluctuations between 10% and 40% were observed in one experiment and between 5% and 15% in the other.

These fluctuations may have been a result of the difference in behaviour observed in the proportion of orange variants. They showed a rise similar to that observed in other experiments when the level of petites began to fall. However, instead of subsequently falling to about 5%, the level of orange variants continued to increase. The level varied between 35% and 75% in one experiment and between 30% and 60% in the other. The

Figure 20. Population changes during continuous cultivation of strain X 190 in MAD medium at 18°C.

V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 20

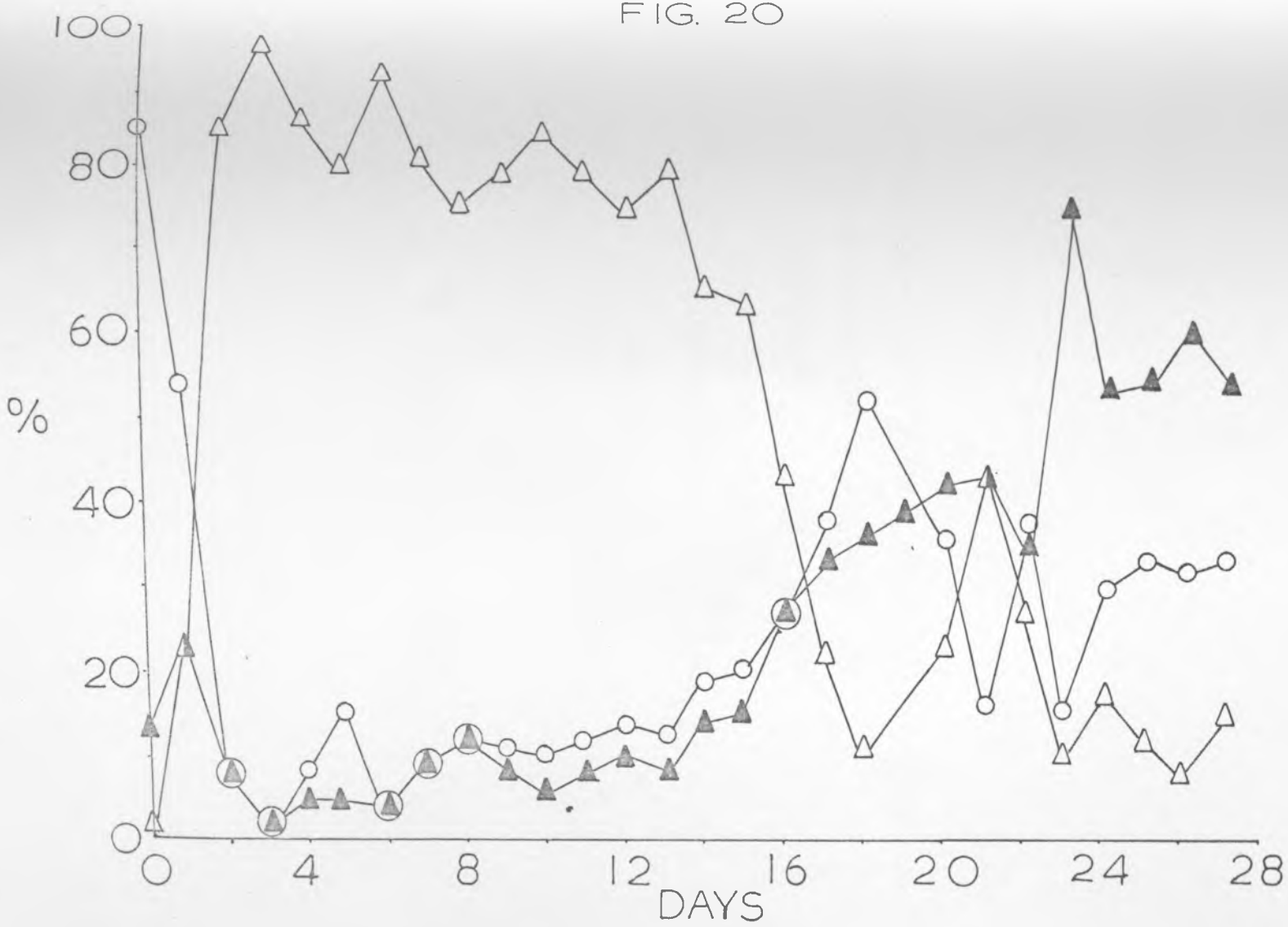


Figure 21. Population changes during continuous cultivation of strain X 190 in MAD medium at 18°C.

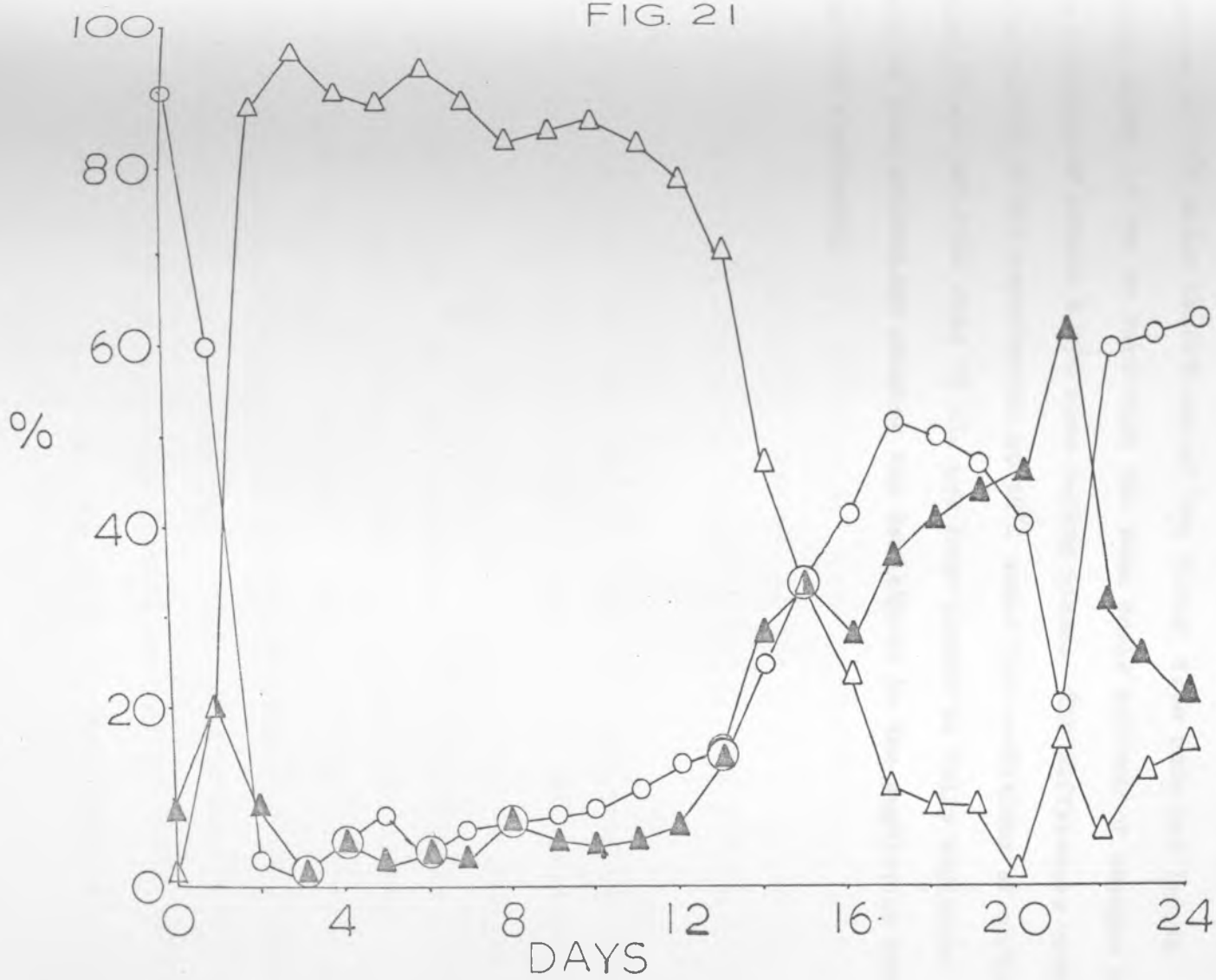
V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 21



results in the later stages of one of the experiments presented a very unstable pattern (fig 20) but the pattern of results of the other experiment was considerably more stable (fig 21). In the latter experiment it seemed that the changes in the population were following the same pattern as that observed in the other experiments at 15°C and 18°C, where ultimately the parent type formed the major proportion of the populations.

By comparing the results of all the experiments at 18°C and those performed at 15°C under conditions of the faster flow rate and larger working volume, it can be seen that the same basic pattern of changes in the population of strain X 190 were taking place. The differences observed in the results of the experiments at 18°C under the conditions of working volume 300 ml. and flow rate 75 ml. per hour cannot be fully explained until the basic mechanisms causing the variations in the population have been fully elucidated.

Continuous cultivation of strain X 190 in glycerol medium at 18°C

The serial transfer of strain X 190 at 15°C in glycerol medium indicated that low temperature induced the production of petites in this strain of Saccharomyces cerevisiae. To determine whether a temperature of 18°C also induced petites, strain X 190 was cultivated continuously in glycerol medium supplemented with adenine, with a flow rate of 75 ml. per hour and a working volume of 300 ml.

The results (figs 22, 23) confirmed that the appearance of a high level of petites in a population of strain X 190 at 18°C was due to induction and not selection. Petites reached a maximum between 66% and 71% after 4 days cultivation. During the same period the level of the parent type fell to 25 - 27%. The orange variants showed a small rise up to 10% in the first two days and thereafter fell to a steady value of 5% for the duration of the experiments. After 4 days the level of petites fell and there was a proportional increase in the level of parent type. The parent type eventually reached a level of 90% and the petites a stable level of 5%.

The rate at which the proportion of petites in the population increased was much slower in glycerol medium than in MAD medium, and the maximum level of petites was lower in glycerol medium than MAD medium under similar cultural conditions. This was not surprising since theoretically, petites cannot utilise glycerol as a carbon source and, therefore reproduce in glycerol medium. In practice, however, it has been observed that newly formed petites undergo a few divisions in sodium lactate medium before division ceases (Ogur, St. John and Ogur, 1959). Sodium lactate medium is similar to glycerol medium, having lactate as the sole carbon source which petites cannot utilise. Ogur, St. John, and Ogur suggested that

Figure 22. Population changes during continuous cultivation of strain X 190 in glycerol medium at 18°C.

V = 300 ml. F= 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 22

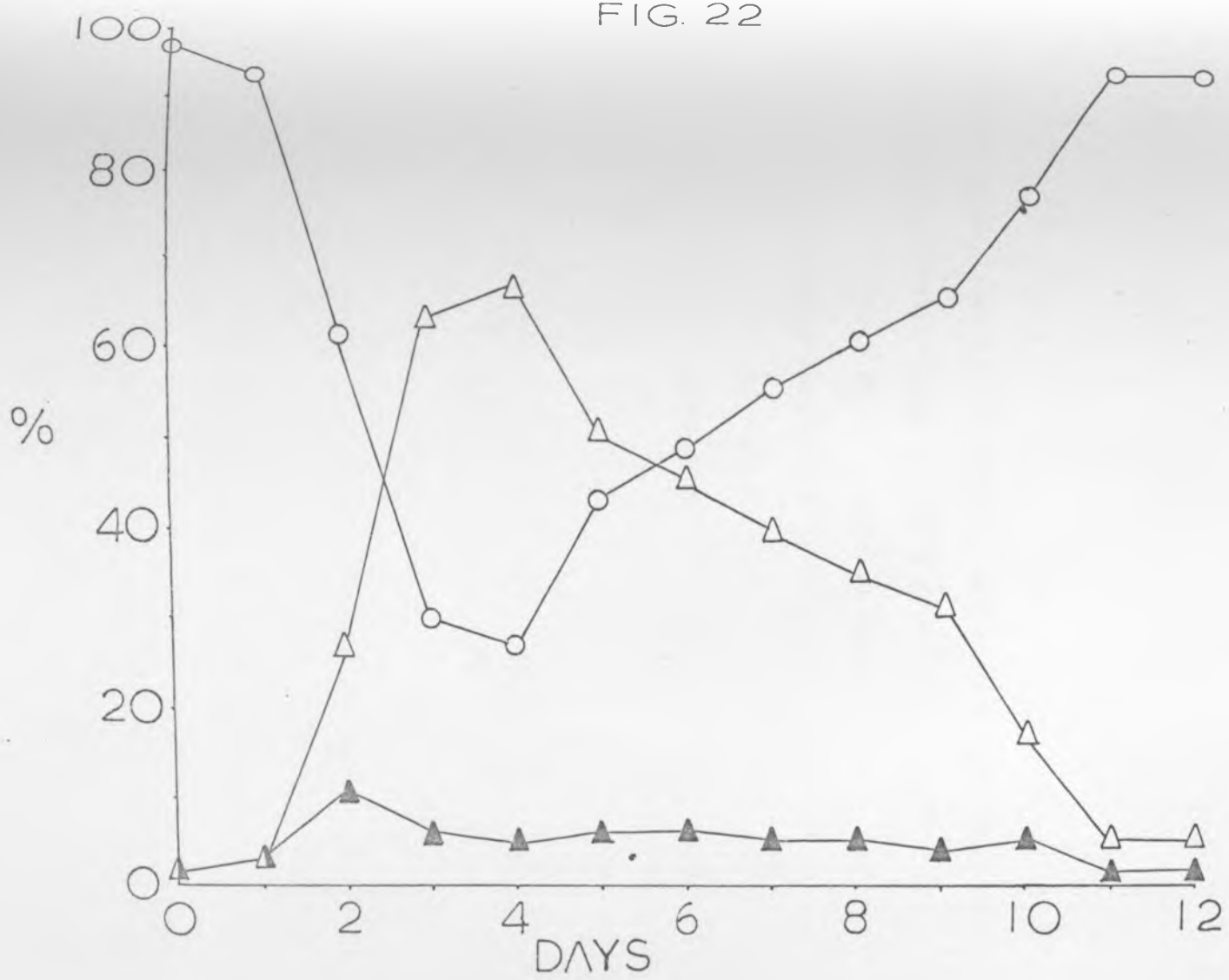


Figure 23. Population changes during continuous cultivation of strain X 190 in glycerol medium at 18°C.

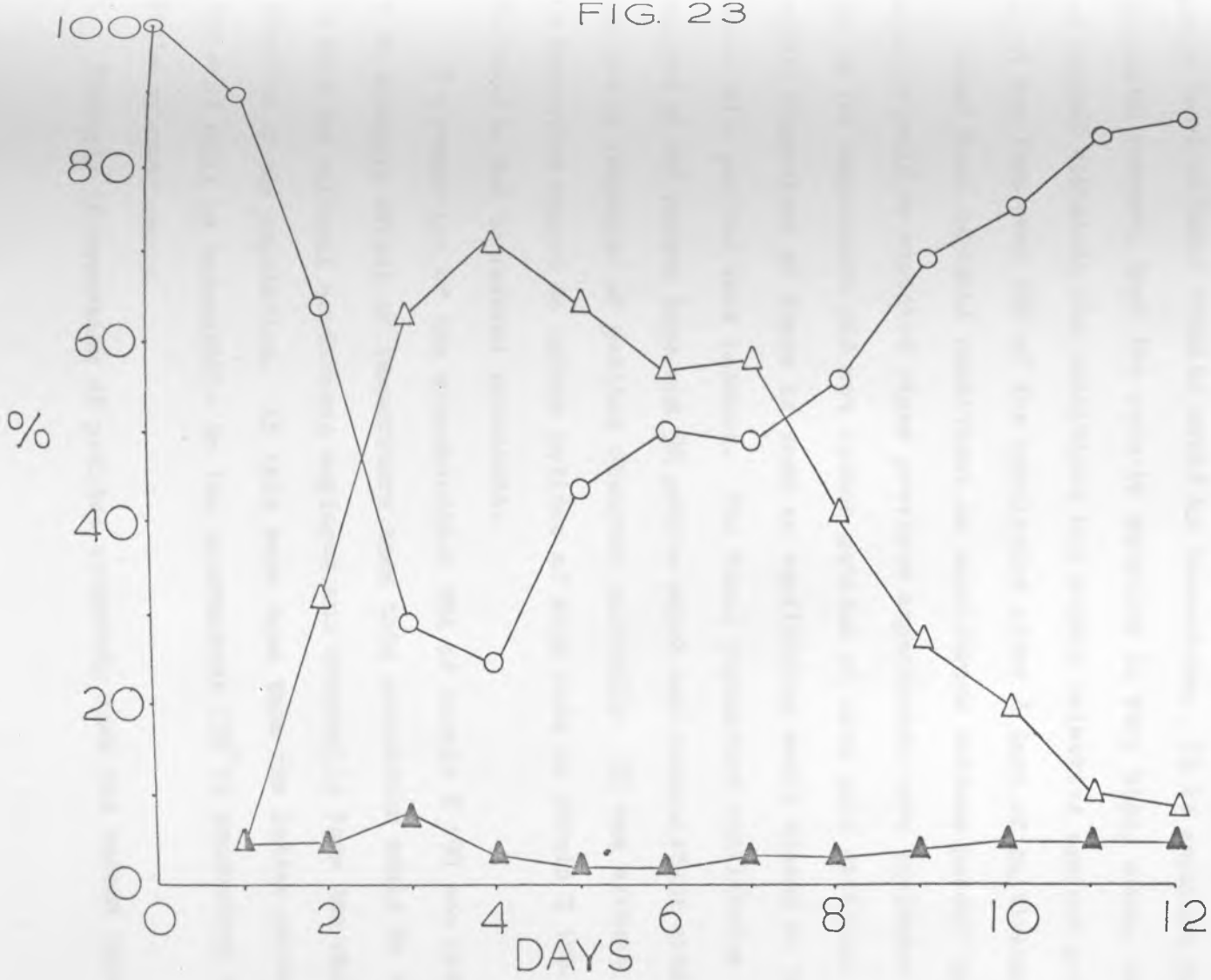
V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 23



newly formed petites underwent as many as five divisions in lactate medium before reproduction ceased, and suggested the reason that petites possess residual cytochrome activity derived from the respiratory sufficient parent cells. This phenomenon could also take place in glycerol medium and as a result affect the determination of the rate of mutation of parent type to petite, since the number of petites observed could be higher than the actual number of petites induced. Consequently an estimated rate of mutation based on these results would be inaccurate. It is apparent from the results, however, that the rate of mutation is very high, since, despite some residual division, the conditions are highly selective against petites and yet they form over 50% of the population after 3 days of cultivation.

Under these cultural conditions an equilibrium between parent type and petite would be expected since previous experiments have indicated that the low temperature did not induce petites at each cell division. The relative proportions of types in such an equilibrium would depend on the rate at which petites were induced. The final population equilibrium consisted of 90% parent type and 5% petite which was inconsistent with the high rate of induction of petites observed initially. It was evident that the temperature ceased to induce petites at high rate in strain X 190. This could be due to several processes.

If a proportion of the mitochondrial DNA of strain X 190 was resistant to the mutagenic effect of temperature then this proportion would be selected for under the cultural conditions employed and eventually form the major proportion of the population. If this were true then the latter parent type would still be susceptible to the spontaneous ($30^{\circ}C$) production of petites by other means.

Similarly if reversion of petites occurred, then the parent type

would increase in the population. Moreover, if the mitochondrial DNA in these revertants was resistant to the induction of petites by low temperature then this revertant would be selected for and form the major proportion of the population at 18°C. However this population would still be susceptible to petite induction by any of the other mechanisms by which petites arise spontaneously .

Adaption of the replication rate of mitochondrial DNA to the temperature of 18°C would also result in parent type becoming the major proportion of the population.

Each of these mechanisms could explain these results but the technique employed does not allow one to differentiate between them.

Continuous cultivation of strain X 190 at 30°C

Control experiments in serial transfer at 30°C confirmed that significant induction of petites in strain X 190 did not occur at this temperature. A similar control experiment at 30°C was performed in continuous culture.

In order to compare the results at 30°C as closely as possible with those obtained at 15°C and 18°C, the same working volume of 300 ml. and flow rate of 75 ml. per hour were employed. These cultural conditions were not selective against peties at 30°C , as they were at 15°C and 18°C, as the generation time of petites at 30°C is less than 4 hours. The results (fig 24, 25) confirmed that induction of petites did not occur at 30°C during continuous cultivation of strain X 190. Petites were observed in almost every sample but only at a level of 1% or less which confirmed the observations of Sherman (1959). The orange variants were observed only sporadically and the level varied between 0 - 4%. In general, orange variants were not observed in at least half of the samples. The level of parent type remained in the order of 97 - 100% for at least 5 days (see also Thornton, Law, and Johnston, 1969). Subsequently the level of parent type fell rapidly as the proportion of large white colonies rose rapidly. Phenotype analysis (table 4) revealed these large white colonies were a mixture of ad_8/ad_8 recombinants and ad_2 revertants. These types of variants have a selective advantage over the parent type (Johnston and Mackinnon, 1966) and consequently they quickly outgrew the parent type in the population. Because selection for the mitotic recombinants and against the parent type took place in strain X 190 in MAD medium (Johnston and Mackinnon, 1966), it was not possible to estimate the rate of ad_8 recombination because of the indeterminate degree of selection which operated.

Figure 24. Population changes during continuous cultivation of strain X 190 in MAD medium at 30°C.

V = 300 ml. F = 75 ml./hour

O = parent type

● = adenine recombinants and revertants

Δ = petite

FIG. 24

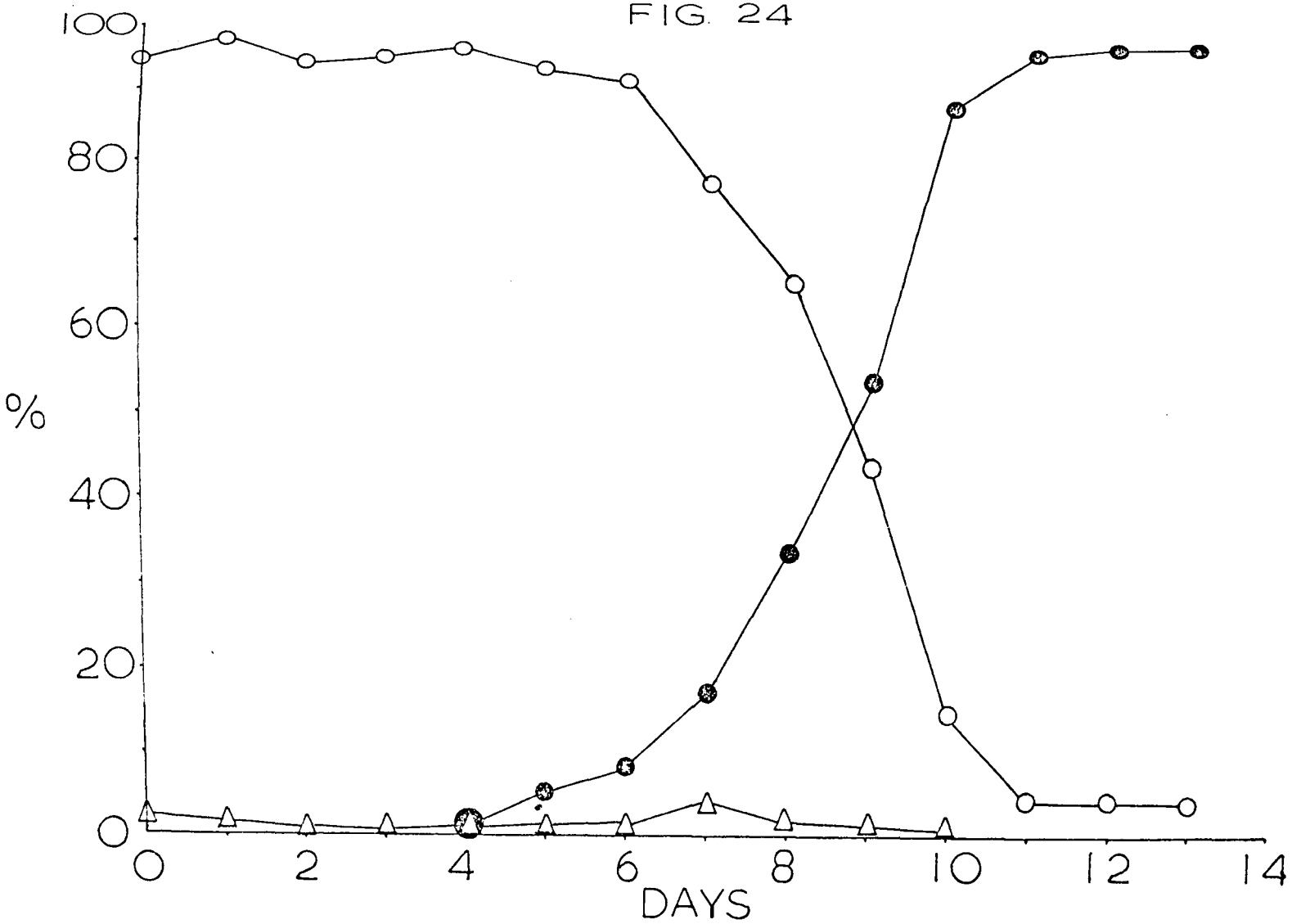


Figure 25. Population changes during continuous cultivation of strain X 190 in MAD medium at 30°C.

V = 300 ml. F = 75 ml./hour

O = parent type

● = adenine recombinants and revertants

Δ = petite

FIG. 25

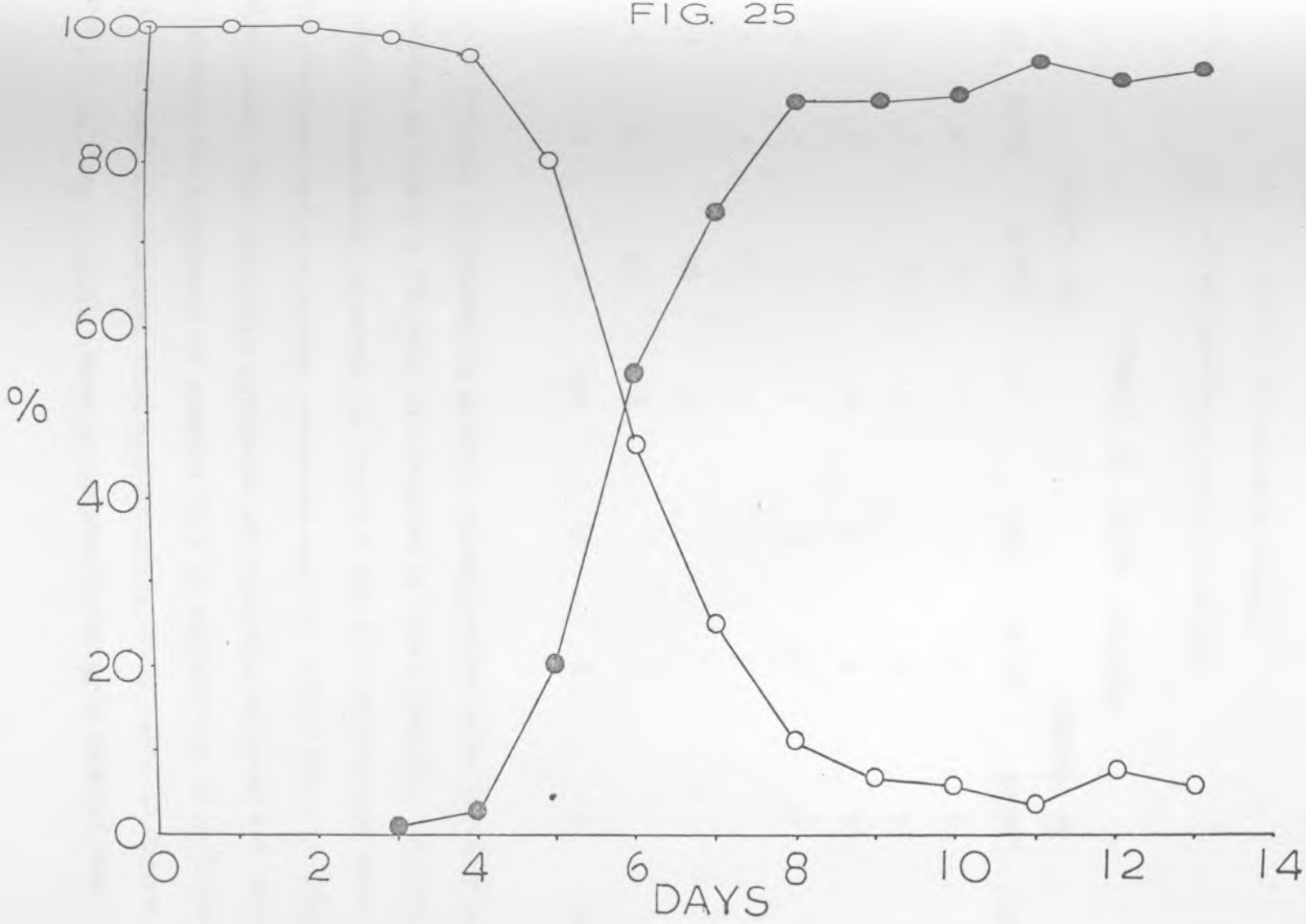


TABLE 4

Phenotypic analysis of large white colonies from continuous cultures of strain X 190 at 30°C.

(+) = growth on omission media.

(-) = no growth on omission media.

NUMBER OF WHITE COLONIES

	FIGURE 24				FIGURE 25		
DAY	AD^-TR^-	AD^-TR^+	AD^+	DAY	AD^-TR^-	AD^-TR^+	AD^+
2	2	0	0	2	0	1	0
3	1	0	0	3	2	1	0
4	0	3	0	4	4	7	0
5	1	2	0	5	37	37	4
6	5	11	0				
7	7	18	3				
8	22	23	2				
13	0	0	100	13	0	0	100

Two problems in estimating mitotic recombination rates by continuous cultivation in strain X 190 were illustrated by these results. Reversion to adenine independence occurred in strain X 190 at a significant rate and these revertants had a selective advantage over all other types in MYGP and MAD media. This selective advantage was reduced, although not negated, by increasing the supplement of adenine from 20 mg/litre to 40 mg/litre i.e. total adenine was raised from approximately 40 mg/litre to approximately 60 mg/litre in MYGP. Even so adenine revertants enjoyed some

selective advantage which made it highly improbable that either the parent type or any other variant of strain X 190 survived at any significant level in the final population.

Similarly increasing the adenine supplement did not reduce the selective advantage of the white ad_8 recombinants to a level at which the growth rates of the parent type and the recombinant were equal, so the rate of recombination could not be accurately measured. Synthetic complete medium (Johnston and Mackinnon, 1966) was found to be suitable for the measurement of mitotic recombination rates in strain X 190 in serial transfer. To measure mitotic recombination rates by use of the continuous culture technique synthetic complete medium should therefore be used in preference to MYGP or MAD media.

Continuous cultivation of strain X 190 at 21°C

The results of experiments performed at 15°C and 18°C showed that petites were induced by these temperature in strain X 190. Moreover, experiments at 30°C confirmed that induction of petites did not occur at this near-optimum temperature for growth, under the same cultural conditions. Evidently induction of petites ceases at some temperature between 18°C and 30°C. In an attempt to determine the temperature at which petite induction ceases, strain X 190 was continuously cultivated at 21°C. Generally, in yeasts, the rate of reproduction increases with the temperature, up to an optimum temperature for growth at about 30°C. In order to make the cultural conditions, using MAD medium, as selective as possible against petites, and taking into account the increase in temperature from 18°C to 21°C and the consequent increase in the growth rate of strain X 190, the flow rate was increased to 100 ml. per hour. This flow rate, with a working volume of 300 ml., gave a culture generation time of 3 hours, and a total of 8 generations in every 24 hour cycle. These conditions were selective against petites (see later section) and the population density of 10^8 cells per ml. was very similar to those at the lower temperatures. Checks on the sugar content of the effluent indicated that glucose was again the growth limiting nutrient.

The results at 21°C (figs 26, 27) bore a close resemblance to those obtained at 15°C. The only differences were the rate of production of petites and orange variants in the first 24 hours and quantitative differences in the proportion of each variant present in the population at any specific time. Absence of a rise in the level of orange variants during the first 24 hours of cultivation was observed in only one experiment (fig 27) and several other experiments which were prematurely terminated

Figure 26. Population changes during continuous cultivation of strain X 190 in MAD medium at 21°C.

V = 300 ml. F = 100 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 26

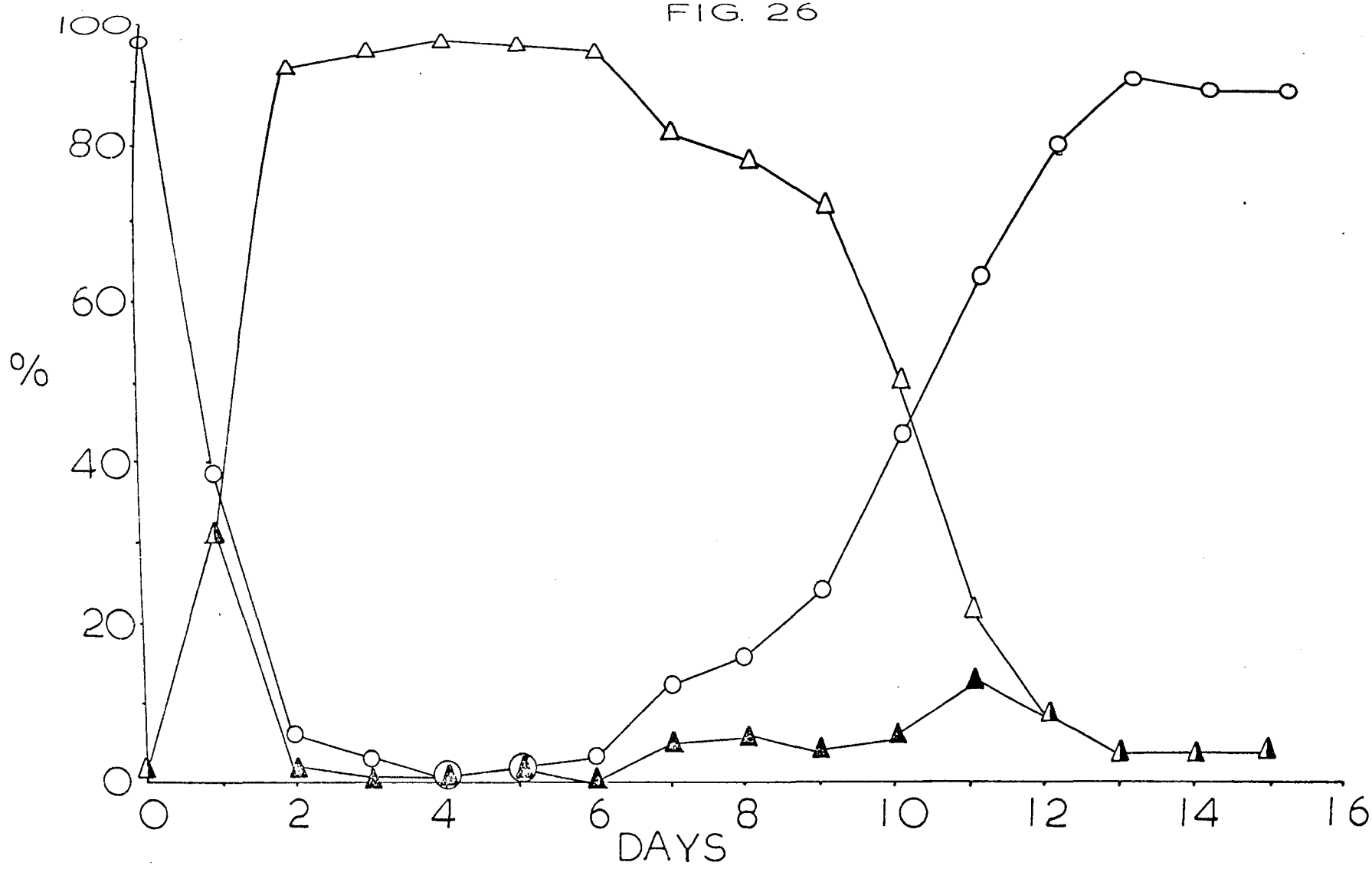


Figure 27. Population changes during continuous cultivation of strain X 190 in MAD medium at 21°C.

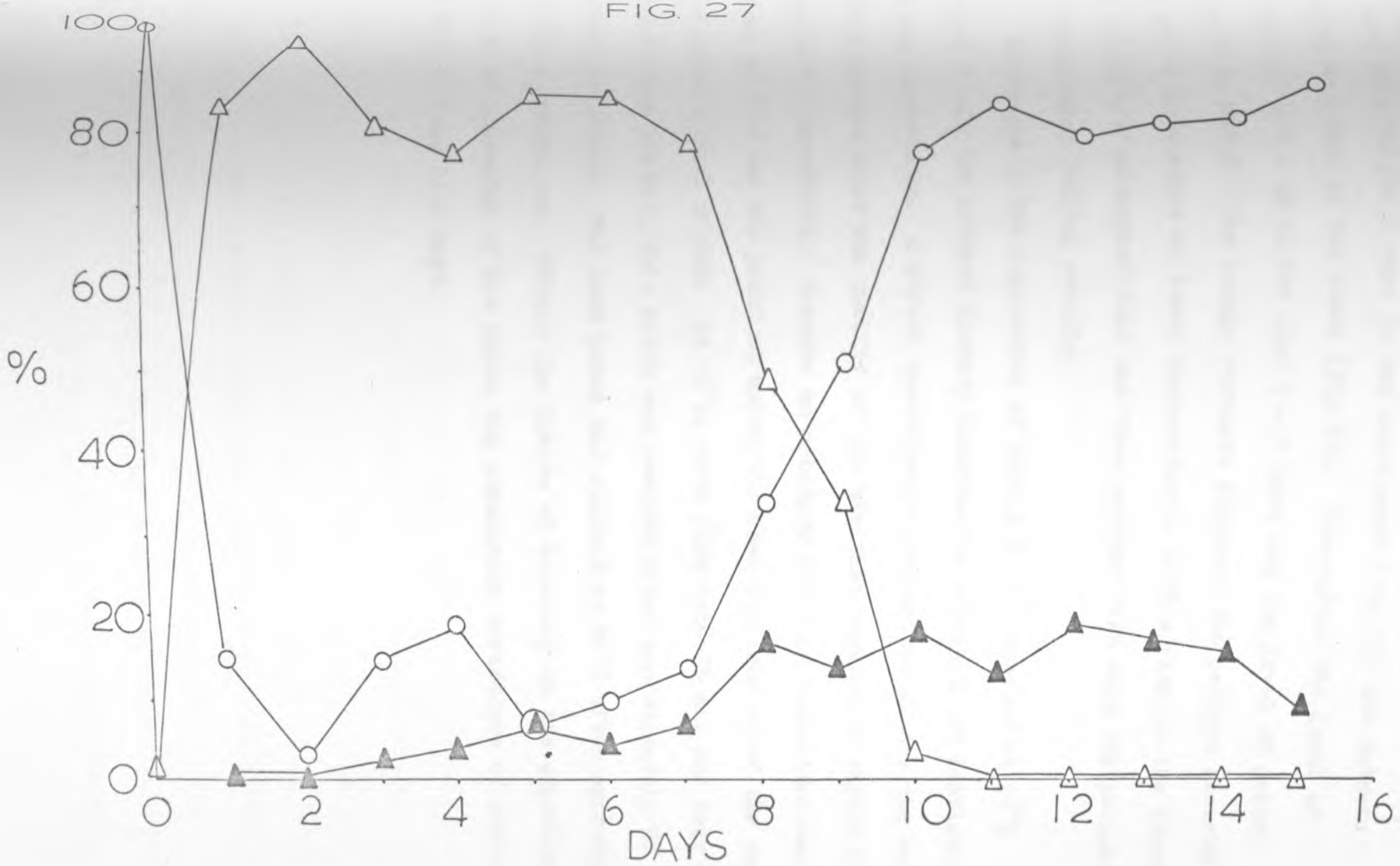
V = 300 ml. F = 100 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 27



all showed that this increase normally occurred. The level of petites remained above 90% for 4 days in one experiment (fig 26) and between 78 - 95% for 5 days in the other (fig 27). Thereafter the level of petites fell to 1 - 4% in the next 5 - 6 days and the level of parent type rose to 80-90%. The orange variants followed the pattern observed in previous experiments at lower temperatures, with a rise in the first 24 - 48 hours, a subsequent fall and then another rise when the proportion of petites begins falling rapidly.

The changes in the population of strain X 190 observed at 21°C followed closely the pattern already observed in strain X 190 populations at lower temperatures. A strict quantitative comparison of all the results was not possible since the quality of the starting population varied from experiment to experiment. However an arbitary point of comparison was chosen, and this was the point at which the re-establishment of the parent type reached a level of 50%. At 18°C, with flow rate 75 ml. per hour and working volume 300 ml., this point was reached after approximately 90 culture generations. The same point was reached at 21°C after approximately 80 culture generations. Within the limits of accuracy of the experiments this was an indication of how close the population variations of strain X 190 at 18°C and 21°C were.

Continuous cultivation of strain X 190 at 25°C

Since it was found that growth at 21°C induced petites in strain X 190, it was decided to determine whether the temperature of 25°C also produced population changes involving respiratory deficient variants. Identical culture conditions to those at 21°C were used, although these were not as selective against petites.

The results of four experiments (fig 28, 29) indicated that petites were not induced in strain X 190 to any great extent by growth at 25°C. In only one case (fig 28B) did the level of petites rise above a few per-cent. In this exceptional case petites rose to 25% of the populations, although the result at 5 days indicated that there might have been a rapid return to a low level of petites in this population. This increase, however, did not compare either in rate or maximum level with the increase observed at lower temperatures during the same period. In fact, the level of petites showed no increase at all in the first 24 hours of cultivation, in contrast to observations at lower temperatures.

In the other three experiments (fig 28A, 29A, B) the level of petites remained effectively constant at 1 - 2% with the exception of one obser-vation of 6% (fig 28A). There were, however, marked variations in the proportions of parent type and orange variant in all of these experiments. In general, the level of the parent type fell in the first two days to levels ranging between 33% and 65%. In two experiments (figs 28A, 29B), the level of parent type continued to fall and reached approximately 20% after 5 - 6 days. In the other two experiments (figs 28B. 29A) the parent type level fluctuated about a level of 50 - 60%. Simultaneously with the fall in parent type, the orange variant rose to levels between 35 - 65% after 2 days, and 20 - 65% after 5 - 6 days. The final populations observed

Figure 28A and B. Population changes during continuous cultivation of strain X 190 in MAD medium at 25°C.

V = 300 ml. F = 100 ml./hour

O = parent type

● = adenine recombinants and revertants

Δ = petite

▲ = orange

FIG. 28

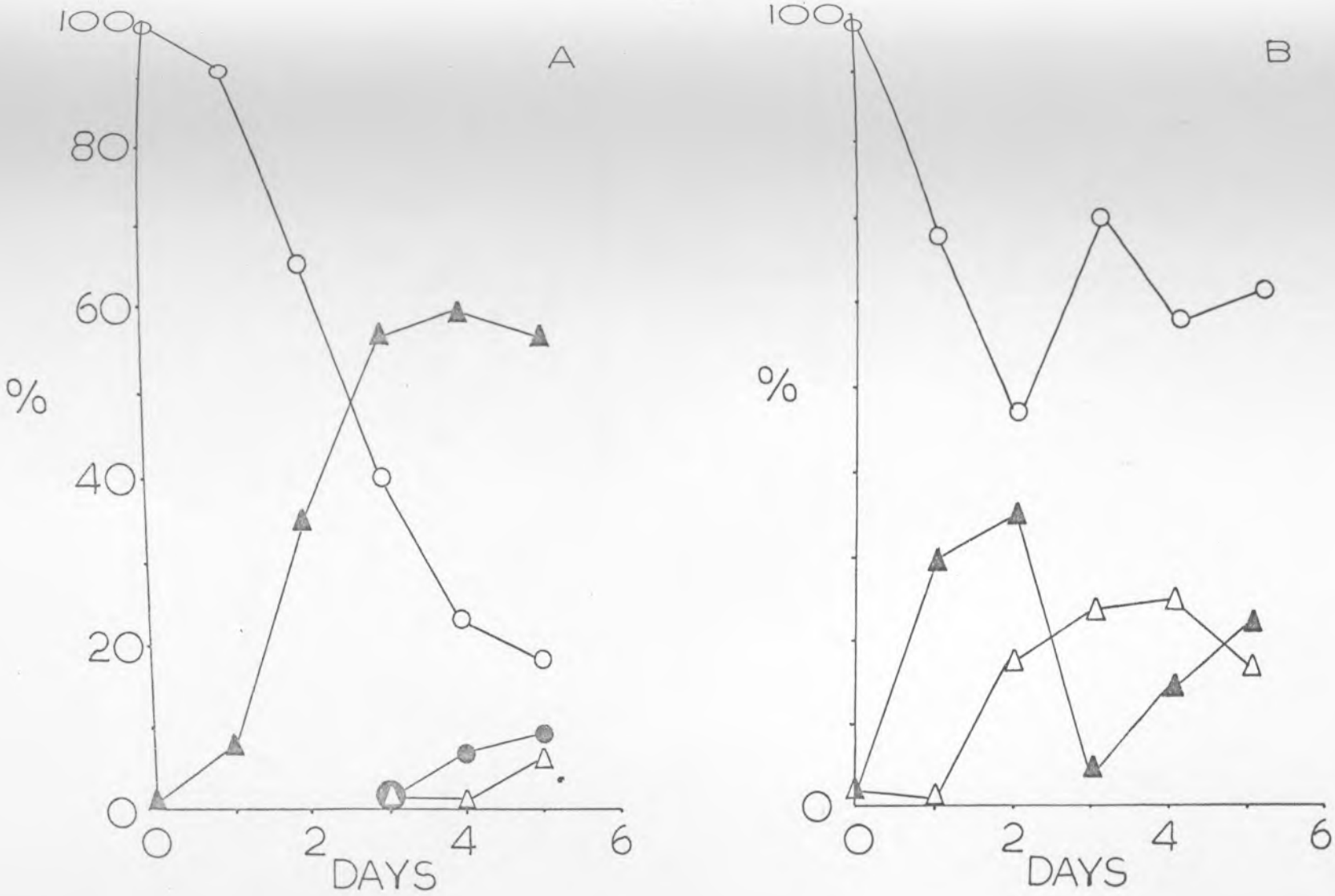


Figure 29A and B. Population changes during continuous cultivation of strain X 190 in MAD medium at 25°C.

V = 300 ml. F = 100 ml./hour

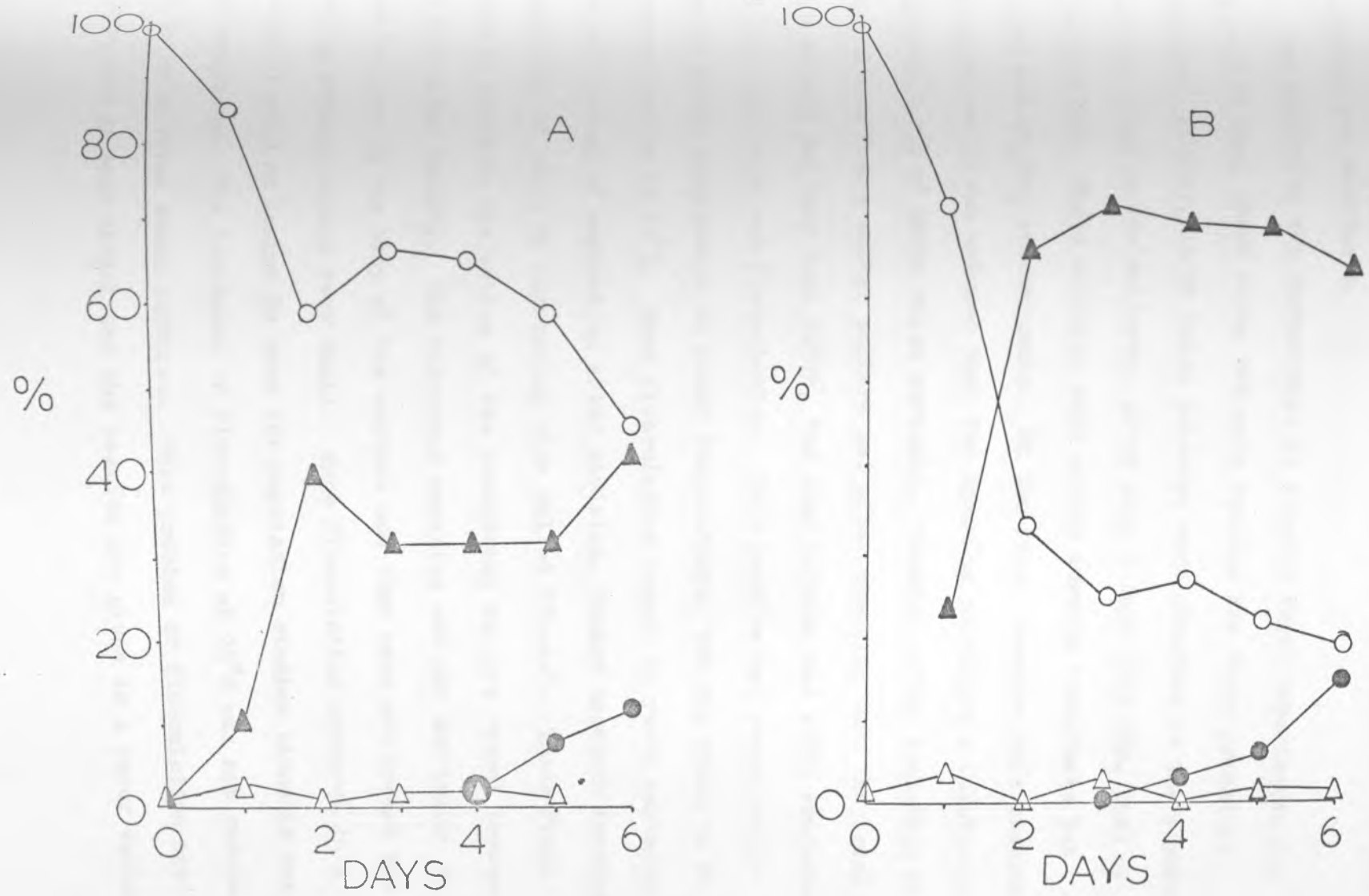
O = parent type

● = adenine recombinants and revertants

Δ = petite

▲ = orange

FIG. 29



were possibly in equilibrium, for parent type, petite and orange variant, the value of which was different from one experiment to the next, although the populations were still dynamic because of the appearance of adenine recombinants and revertants.

Some difficulty was encountered in running these experiments for any length of time, there being two main reasons for their premature curtailment. Firstly, large white colonies were observed in the population at an early stage in the cultures, after only 3 days (fig 28A, 29B) or 4 days (fig 29A). These colonies were mainly adenine revertants but included some ad_8/ad_8 recombinants. On the first occasion this problem was encountered it was assumed that the inoculum contained a relatively high level (0.1%) of these white variants. However, after isolating fresh stock cultures from a master culture and after checking that the level of white variants was less than 0.01%, the same problem was still encountered. The other difficulty was flocculation. This problem had occasionally been met during experiments at lower temperatures, but was found to be a recurrent problem at 25°C. When flocculation occurs in yeast cultures, the cells instead of separating after division, remain together forming aggregations of cells of increasing size called 'flocs'. These flocs tended to settle to the bottom of the continuous culture vessel because of their higher density. The vigourous aeration was not sufficient to keep the flocs in the body of the culture and they were not pushed out with the effluent unless very small. Once flocculation occurred in a culture it could no longer be used for population studies since it was not homogeneous. The incidence of flocculation at 25°C was not reduced by the use of fresh stock cultures. This problem of flocculation was investigated in some detail and the results are given in a later section.

It was evident from these results that the temperature of 25°C did not induce petites to any great extent during the growth of strain X 190. However, there was an increase in the level of orange variants, and this increase persisted throughout the experiments, without falling as observed in the experiments at lower temperatures. So although the temperature of 25°C did not induce respiratory deficient petites, it did induce a respiratory deficient type in the orange variant.

The temperatures of 15°C, 18°C and 21°C, induced petites at a high rate during the growth of strain X 190 and orange variants at a much lower rate. In contrast, the temperature of 25°C induced respiratory deficient orange variants at a high rate but did not induce respiratory deficient petites. Neither petites nor orange variants were induced by the temperature of 30°C. Evidently between the temperatures of 21°C and 25°C there was a marked change in the rate at which petites and orange variants were induced. Although both petites and orange variants have been shown to be respiratory deficient, the nature of this deficiency is different since the orange variant has a much greater growth rate than that of petites. Considering the range of temperatures studied it is evident that, as the temperature falls below the near optimum growth temperature of 30°C, an increasing degree of respiratory deficiency was induced in cultures of strain X 190.

Recycling of cultures of strain X 190 at 18°C

The population of cultures of strain X 190 after 16 - 18 days continuous cultivation at 18°C consisted mainly of respiratory sufficient parent type cells. These parent type cells were thought to be resistant to the inductive effect of low temperature and therefore should not produce respiratory deficient variants, at a high rate. On this assumption, if these cells were recycled into a fresh continuous culture at low temperature, induction of petites should not be observed. The population of strain X 190 after 18 days of continuous cultivation at 18°C did not, however, consist entirely of parent type cells but also contained 3% petite and 2% orange variant cells. To avoid recycling a mixed culture which could complicate the results, a sample of this population was subcultured into glycerol medium at 30°C and serial transferred for 3 days, by which time the population consisted entirely of parent type cells. This population was inoculated into continuous culture at 18°C which was started immediately.

The result of sampling at intervals of 24 hours (fig 30) showed that, although the level of petites remained at approximately 1% for 6 days, there was a gradual increase to 12% over the period from 7 - 10 days. The level of orange variants, however, rose to 30% after 5 days, and to 45% after 11 days. The level of the parent type gradually fell to 50% after 11 days. The results indicated that the population which formed the inoculum was susceptible to temperature-induced mutation to intermediate respiratory deficient orange variants and, to a lesser degree, petites. It was thought that this induction effect may have been due to the inoculum having been grown for a time at 30°C.

To test this idea another sample was taken from a continuous culture

Figure 30. Population changes during continuous cultivation of a recycled population of strain X 190 at 18°C.

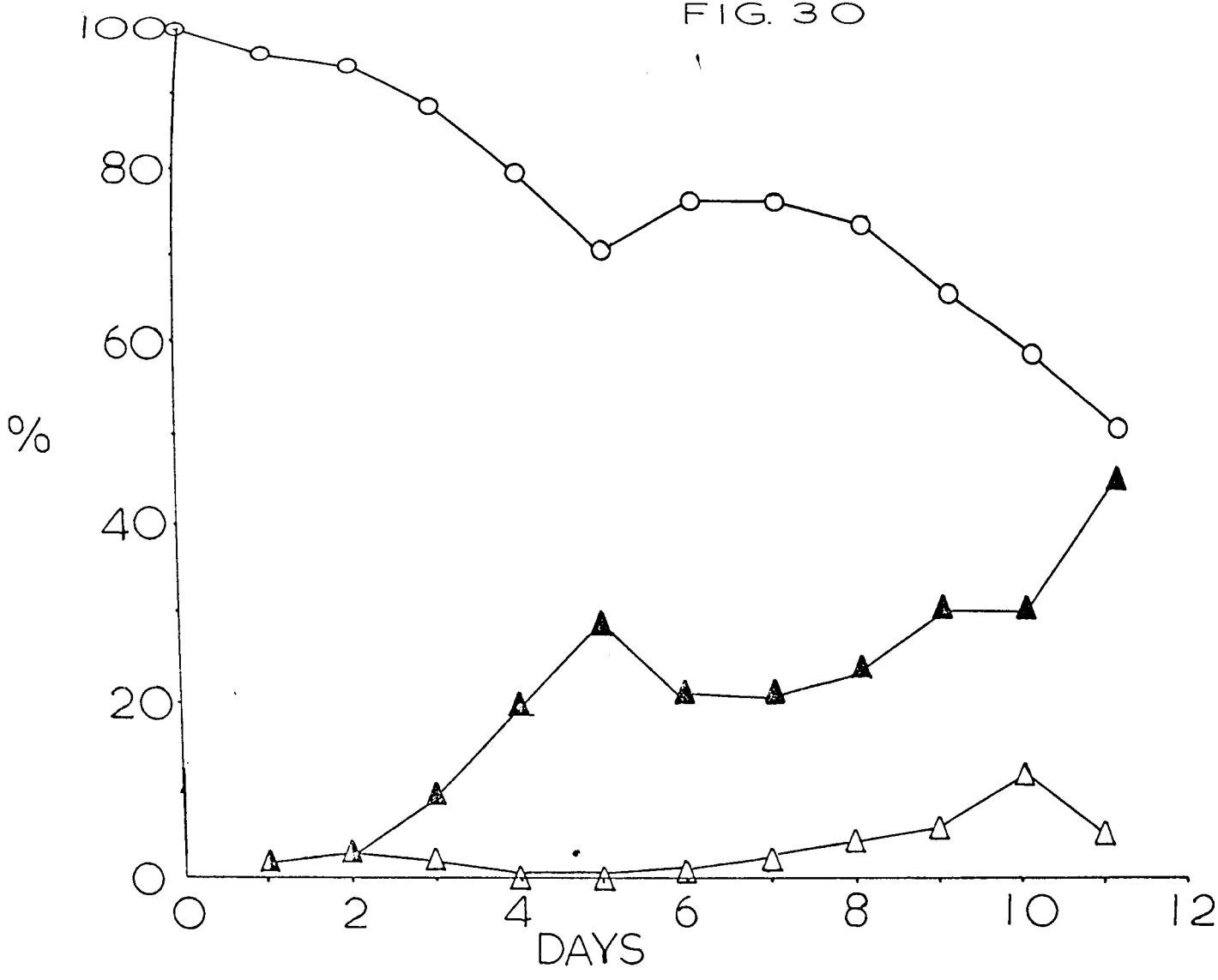
V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 30



of strain X 190 at 18°C after 17 days, whose populations was 96% parent type, 2% petite, and 2% orange variant. This sample was then subcultured in glycerol medium at 18°C for 3 days and subsequently inoculated into continuous culture at 18°C. The results of samples taken at 24 hour intervals of two continuous cultures (figs 31, 32) showed that no large scale induction of respiratory deficient variants was occurring. In one case (fig 31) the level of the parent type reached an approximate equilibrium of 85 - 90% after 3 days and stayed at this level until the end of the experiment at 14 days. The level of the orange variant rose slowly over 5 days to a similar type of equilibrium around 10 - 12%. The level of petites remained low at a typical background level of 1 - 2% throughout the experiment. Only slight quantitative differences were observed in the second experiment (fig 32). The ultimate fall in the level of the parent type was due to the appearance of adenine recombinants and revertants which have a faster growth rate than that of the parent type.

The results of these recycling experiments were very interesting since they gave some indication of the causes of the changes observed in the continuous cultivation of strain X 190 at 18°C. In one recycling experiment respiratory deficient variants were induced in the population and in the other two experiments the population remained stable. The temperature at which the sample was subcultured in glycerol, at 30°C in the first experiment and at 18°C for the latter two experiments, was the only difference between the experiments. If all the parent type cells in the samples contained the same type of mitochondrial DNA, which was not susceptible to temperature, then one would not expect to observe the induction of respiratory deficient variants on recycling in continuous

Figure 31. Population changes during continuous cultivation of a recycled population of strain X 190 at 18°C.

V = 300 ml. F = 75 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 31

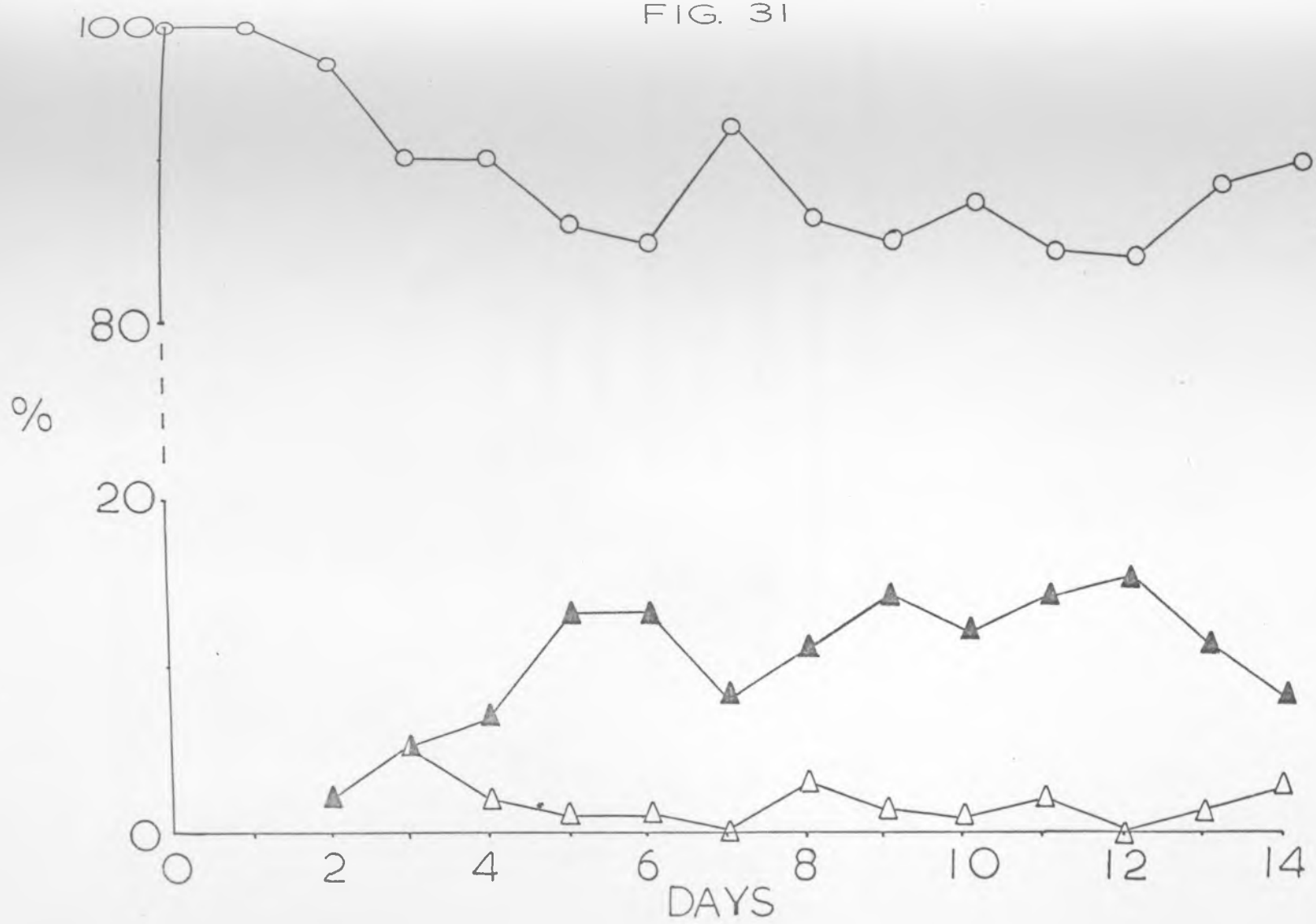


Figure 32. Population changes during continuous cultivation of a recycled population of strain X 190 at 18^{0}C.

V = 300ml. F = 75 ml./hour

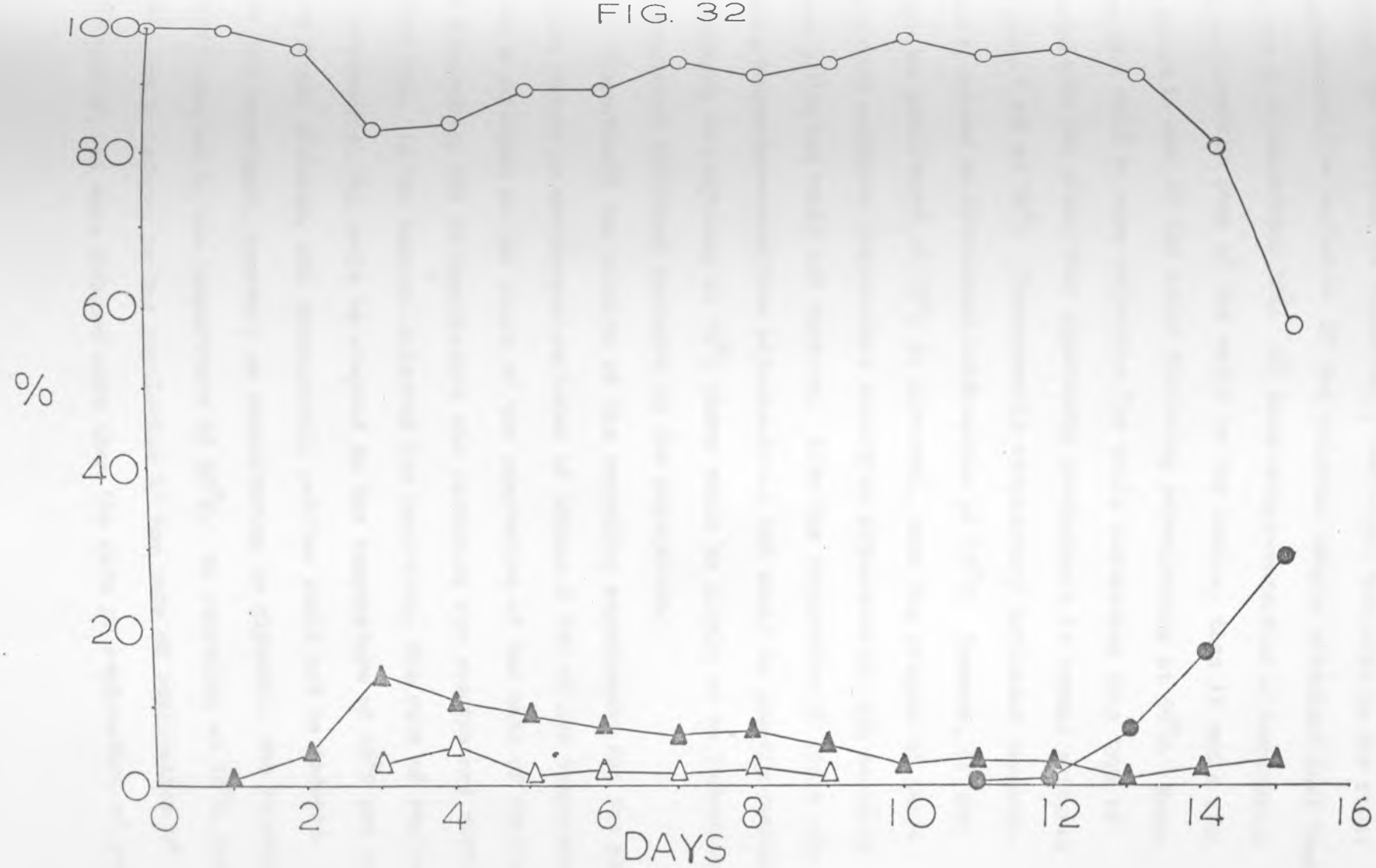
O = parent type

● = adenine recombinants and revertants

Δ = petite

▲ = orange

FIG. 32



culture since 'de novo' synthesis of mitochondrial DNA has not been observed. The increase in respiratory deficient variants in the first experiment could be explained if the original sample contained more than one type of mitochondrial DNA. If temperature-sensitive mitochondrial DNA was present in some of the cells in the sample, then it would also be present in some of the cells following subculturing at 30°C. Moreover there could be some selection for cells containing this type of mitochondrial DNA since they apparently predominate in normal cultures of strain X 190 at 30°C. Consequently respiratory deficient variants could be induced on continuous cultivation at 18°C. However, if the sample was subcultured at 18°C in glycerol, then the progeny of these cells which contained temperature sensitive mitochondrial DNA would be mainly petite and would not survive. Also the proportion of cells containing temperature-sensitive mitochondrial DNA would be greatly reduced. Consequently on recycling at 18°C there would be little or no induction of respiratory deficient variants in the population.

Alternatively the results of the recycling experiments and the population changes in continuous cultures of strain X 190 at low temperatures could be explained on the basis of the adaptation of the rate of replication of mitochondrial DNA to temperature and selection for respiratory sufficient parent type. In the samples selected for recycling, the rate of replication of mitochondrial DNA would be adapted to the temperature of 18°C and to the rate of cell division, and consequently petites would not be induced. In the first experiment, however, on subculturing in glycerol, the respective rates re-adapted to the temperature of 30°C. On recycling at 18°C, petites would then be induced in the population if the rate of replication of mitochondrial DNA were reduced more than the rate of replication of chromo-

somal DNA. In the other two experiments, subculturing in glycerol was performed at 18°C and adaptation to different temperatures would not occur. Consequently petites would not be induced in these experiments as they were in the first.

The effect of dilution rate on petites of strain X 190 at various temperatures.

An important factor in all the experiments involving continuous culture of strain X 190 at various temperatures was that the conditions were selective against the petite variant.

Theoretically, during continuous culture of an organism the cell density is a constant over a range of dilution rates, and the culture is in a 'steady state'. It has recently been found that in fact the cell density falls slightly as the flow rate is increased over the 'steady state' range (R.S. Holdom, personal communication). There is a critical dilution rate above which the organism is washed out of the culture vessel. The critical dilution rate is directly related to the working volume and the minimum generation time of the organism.. However, if the culture generation time ($\frac{1}{D}$) is less than the minimum generation time then the cells will be washed out of the culture vessel. In a mixed culture, each type is likely to have a different minimum generation time and there should be a different critical dilution rate for each type. Consequently a normal dilution rate for one type may be greater than the critical dilution rate for another type in the culture.

Selection against petites in cultures of strain X 190 at various temperatures was achieved by adjusting the dilution rate so that 'steady state' conditons for the parent type were 'wash out' conditions for petite.

Pure cultures of petite cells of strain X 190 were grown continuously in MAD medium in order to ascertain that the flow rates employed in experiments at various temperatures were 'wash out' rates for petites. The culture generation time was reduced as the temperature was increased in order to compensate for the increase in growth rates of the various types

in the population.

At 15°C, with V = 200 ml., F = 25 ml. per hour, wash out of petites did not occur (fig 33). This was not unexpected since the culture generation time was 8 hours, and the generation time of petites during batch growth at 15°C is $4\frac{3}{4}$ hours. At both 15°C and 18°C under these cultural conditions, selection therefore did not operate against petites. However with V = 300 ml., F = 75 ml. per hour, rapid wash out of petites occurred at 15°C (fig 33). So the experiments at 15°C under these cultural conditions were highly selective against petites.

At 18°C, with V = 300 ml., F = 75 ml. per hour, 'steady state' conditions were observed (fig 34), but when the flow rate was increased to 80 ml. per hour, wash out of petites occurred (fig 34). So although the conditions employed at 18°C did not cause wash out the flow rate was very close to critical.

At 21°C, with V = 300 ml., F = 75 ml. per hour, wash out of petites was not observed (fig 35), but when the flow rate was increased to 100 ml. per hour, wash out of petites took place (fig 35). Thus the experimental conditions at 21°C were selective against petites. The same conditions did not, however, lead to wash out of petites in pureculture at 25°C (fig 35).

These experiments at various temperatures showed that, when the flow rate was sufficiently high to cause wash out of petites in pure culture, then in experiments on strain X 190 under similar cultural conditions there was strong selection against petites. The comparitive effects of selection against petites and their temperature-induction were emphasized by the continuous cultivation of artificial mixed populations of strain X 190 at 30°C and 18°C (Tables 5, 6).

Figure 33. The effect of dilution rate (F) on the density of petites of strain X 190 in continuous cultivation at 15°C.

A. V = 200 ml. F = 25 ml./hour

B. V = 200 ml. F = 75 ml./hour

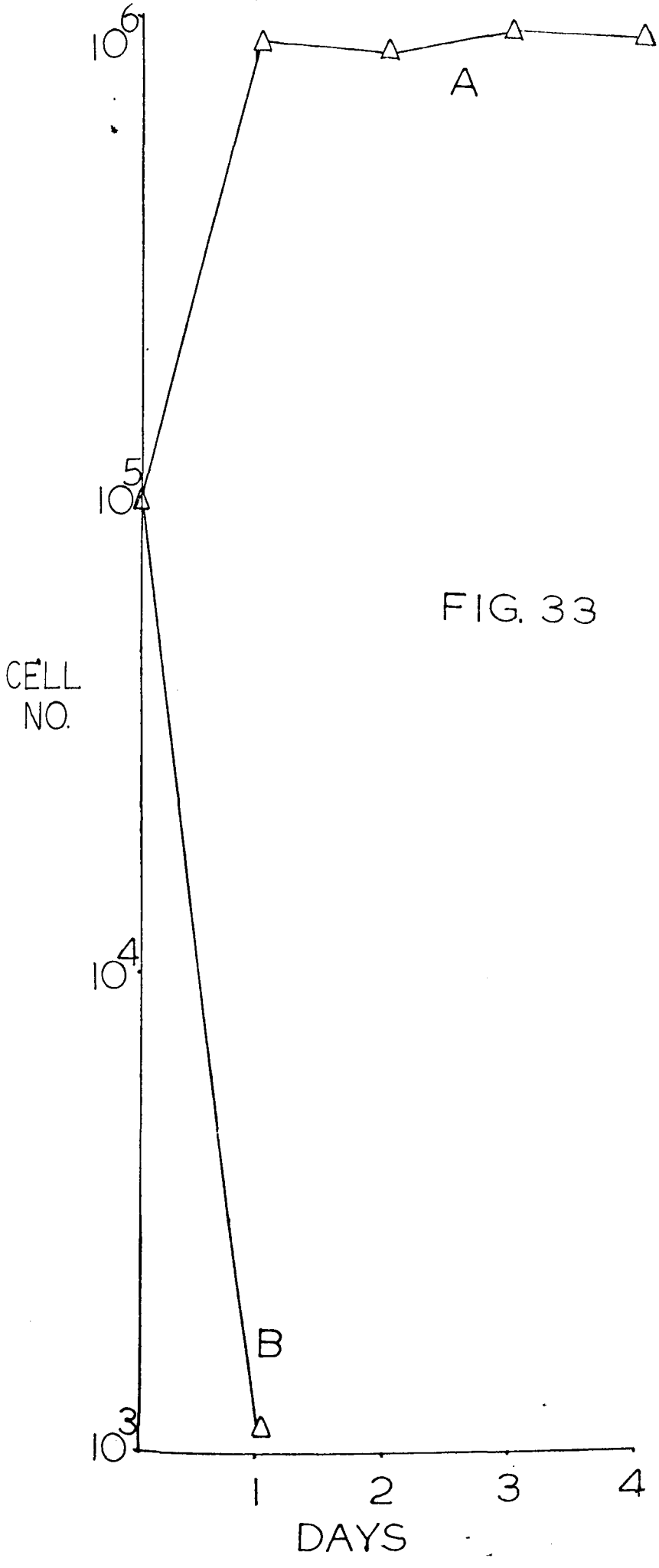


FIG. 33

Figure 34. The effect of dilution rate (F) on the density of petites of strain X 190 in continuous culture at 18°C.

A. V = 300 ml. F = 75 ml./hour

B. V = 300 ml. F = 80 ml./hour

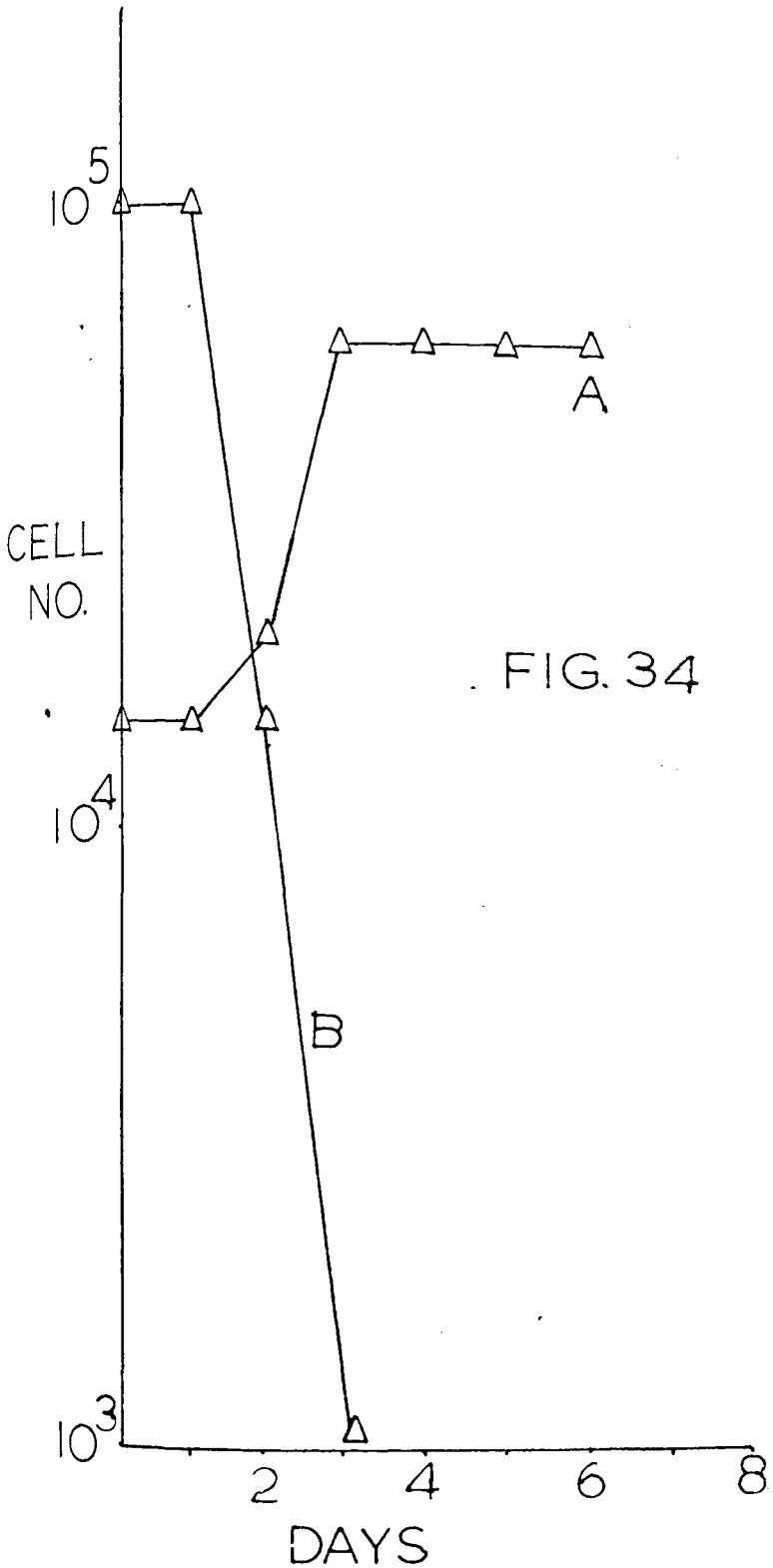


FIG. 34

Figure 35. The effect of dilution rate (F) on the density of petites of strain X 190 in continuous culture at 21°C and 25°C.

A. 21°C V = 300 ml. F = 100 ml./hour

B. 21°C V = 300 ml. F = 75 ml./hour

C. 25°C V = 300 ml. F = 100 ml./hour

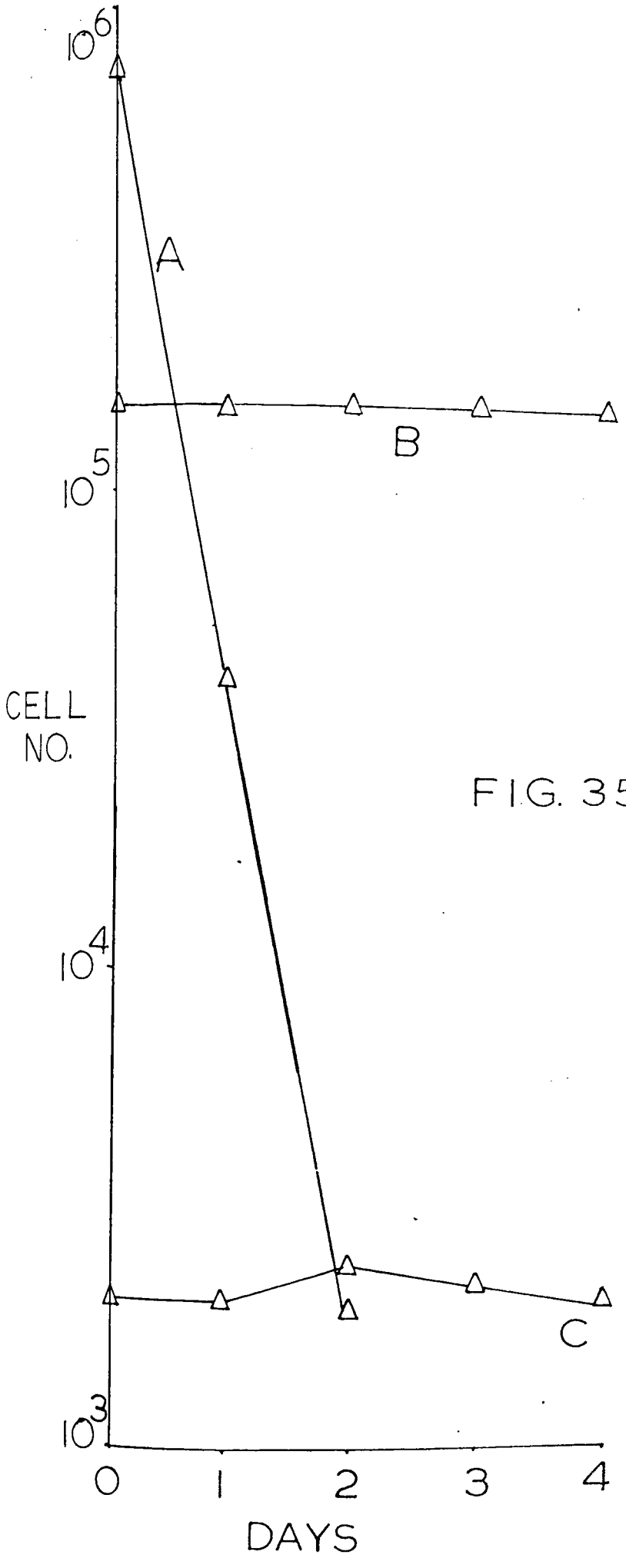


FIG. 35

TABLE 5

Population changes during continuous culture of an artificial population of strain X 190 at 30°C

V = 300 ml., F = 100 ml./hour

	PERCENTAGE OF POPULATION							
	CULTURE A				CULTURE B			
	RED	ORANGE	WHITE	PETITE	RED	ORANGE	WHITE	PETITE
INOCULUM	10.5	0.5	0	89	10.5	0.5	0	89
DAY: 1	48.5	3.4	0.2	47.9	47.7	3.3	0.7	48.3
2	87.2	5.9	0.7	6.2	86.3	4.7	1.8	7.2
3	85.5	9.5	3.3	1.7	-	-	-	-

TABLE 6

Population changes during continuous culture of an artificial population of strain X 190 at 18°C

V = 300 ml., F = 50 ml./hour

	PERCENTAGE OF POPULATION							
	CULTURE A				CULTURE B			
	RED	ORANGE	WHITE	PETITE	RED	ORANGE	WHITE	PETITE
INOCULUM	8	0.2	0	91.8	8	0.2	0	91.8
DAY 1	5.3	0	0	94.7	6.1	1.6	0	92.3
2	25.8	3.6	0.6	70.0	7.7	4.9	0.4	87.0
3	3.6	2.8	0	93.6	1.6	0.7	0	97.7
4	4.2	3.4	0.7	91.7	1.8	0.2	0.4	97.6

At both temperatures the inoculum was an artificial population of strain X 190 composed of 10% parent type and 90% petite. At 30°C, selection took place for the parent type and within 2 days the population was reversed with 87% parent type and 7% petite. At 18°C selection for the parent type was outweighed by induction of petites and in four days the population was approximately 3% parent type and 96% petite.

Both selection for the parent type and the orange variant by virtue of their faster growth rates, and selection against petites by the cultural conditions, were outweighed by the induction of petites in strain X 190 by low temperature. This evidence, therefore, again emphasises the very high rate of induction of petites at reduced temperature.

One disquieting feature of the dilution rate experiments on pure cultures of strain X 190 petites was the wide range of cell density observed (10^3 - 10^7). Theoretically the cell density should be approximately the same at each temperature and this phenomenon seems worthy of further investigation.

Reversion of petites to a respiratory deficient intermediate type

The reversion of petites to respiratory sufficiency has been suggested as a possible explanation of the re-establishment of respiratory sufficient parent type cells during continuous cultivation of strain X 190 at low temperatures. In general, reversion of petites in Saccharomyces has not been observed, and the petite mutation has been regarded as very stable. During experiments to determine the wash-out rates of petite cultures, two red colonies resulted from plating samples. Unfortunately this sample was the last of a particular experiment and so confirmatory evidence of reversion was not obtained. Kraepelin (1964), however, has reported the reversion of petites to respiratory sufficiency, and it was decided to investigate further the possibility of reversion to respiratory sufficiency of petite mutants of strain X 190.

It had been assumed from previous experiments that petites which occurred spontaneously at normal growth temperatures in strain X 190 were identical with petites of strain X 190 induced by low temperatures. Although there was no evidence to the contrary, two stock cultures were prepared, one from a petite colony arising spontaneously from strain X 190 at 30°C, and the other from a petite colony induced in strain X 190 by a temperature of 18°C. Samples of these stock cultures were plated out and over 2000 colonies were counted for each stock culture. As all were petite it was assumed that the entire population was petite.

Temperature induced petite cultures were inoculated into duplicate continuous cultures and cultivated at 18°C, with V = 300 ml., F = 75 ml. per hour. The results (fig 36) show that after 3 days growth in one culture and after 6 days in the other, orange colonies were observed in the sample populations (plate 8). Moreover, these orange variants apparently

Figure 36. Population changes during continuous cultivation of temperature-induced petites of strain X 190 in MAD medium at 18°C.

V = 300 ml. F = 75 ml./hour

Dotted lines indicate duplicate culture.

Δ = petite

▲ = orange

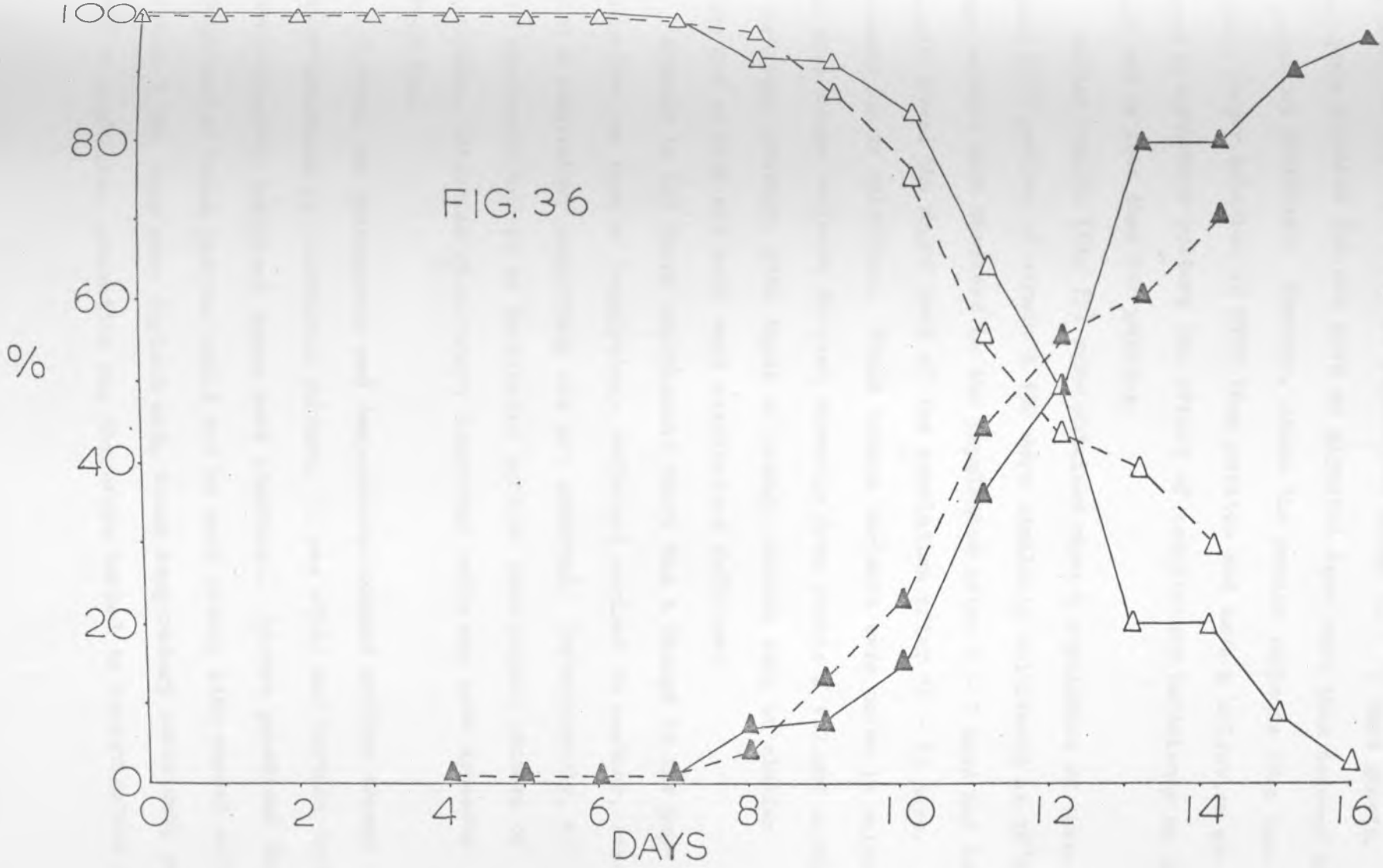


FIG. 36

had a selective advantage over the petites under these cultural conditions and formed the major part of the population after 12 - 13 days growth. These orange variants did not grow on glycerol agar were thus deduced to be respiratory deficient. However, since the orange variants form considerably larger colonies on MYGP than petites and have a selective advantage in continuous culture the effect of respiratory deficiency on their growth rate is less than for petites.

Similar results (fig 37) were obtained when a population of spontaneous (30^{o}) petites of strain X 190 were similarly cultivated at $18^{o}C$. Orange variants were observed in the population after 6 - 7 days and subsequently formed the major part of the population after 12 - 13 days, presumably due to selection. These orange variants were paler in colour than those orange variants derived directly from strain X 190 and showed no sectoring. However, both types of orange variant were of similar colony size on MYGP and both were respiratory deficient.

Although in all these experiments there was a change in the population from one type of respiratory deficient variant to another, reversion to respiratory competency was not observed. Unfortunately, all these experiments had to be terminated earlier than wished because of flocculation, otherwise respiratory competent cells may have appeared through time.

Although the spontaneous and temperature-induced petites showed similar variations in continuous culture, it was still not certain that these respiratory deficient types were identical. Direct genetical investigations of these petites could not be made since, like normal cells of strain X 190, they were diploid and, being respiratory deficient, were unable to sporulate. Attention was therefore turned to investigation of

Figure 37. Population changes during continuous cultivation of spontaneous petites of strain X 190 in MAD medium at 18°C.

V = 300 ml. F = 75 ml./hour

Dotted lines indicate duplicate culture

Δ = petite

▲ = orange

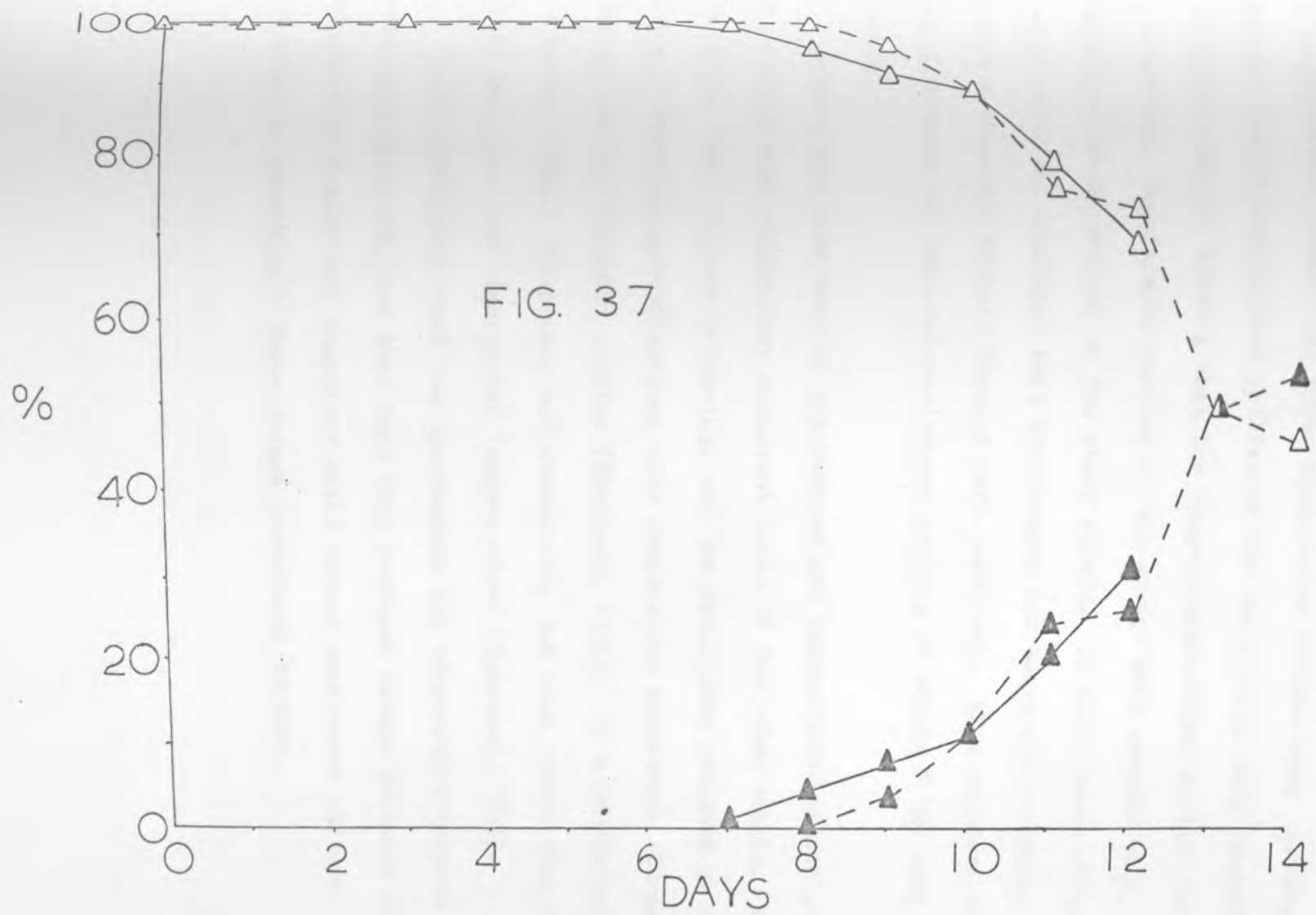


FIG. 37

haploid respiratory deficient strains. Segregants S91 and S110 of strain X 190 were used in this investigation since they were of opposite mating type. Spontaneous petites of these two strains were crossed together and the resulting diploid offspring were all petite. This lack of complementation showed that these spontaneous petites were identical. Similarly, temperature-induced petites of the two strains were crossed with similar results, showing that the temperature-induced petites were also identical. Spontaneous petites of one strain were crossed with temperature-induced petites of the other strain. As all diploid offspring were petite it was concluded that spontaneous and temperature-induced petites of these two haploid strains were identical. This evidence suggested that spontaneous and temperature-induced petites of strain X 190 were also identical.

Crosses were also made of spontaneous and temperature-induced petites of one strain with respiratory competent cells of the other strain. All the zygotes, their diploid offspring, and the ascospores produced on sporulation of these diploid offspring, were respiratory competent. Consequently these petites are 'neutral' petites (Ephrussi, 1953). If a proportion of the zygotes, diploid offspring, and ascospores, had been petite then these petites would have been designated 'suppressive' (Ephrussi, 1953).

Having established that the spontaneous and temperature-induced petites were identical, and that they both produced orange variants but not apparently respiratory competent cells during continuous culture, it was decided to investigate these orange revertants further.

Investigation of the orange variants derived from petites

The orange variants which arise spontaneously from strain X 190 were found to be very unstable, producing parent type cells, adenine recombinants and revertants, and petites. To examine whether or not the orange variants derived from petites showed similar instability, these orange variants were grown in MAD medium for 8 days by 24 hour serial transfers at 30°C. The results (table 7) showed that these orange variants were extremely stable. The only variant from orange which was observed was petite, and this, infrequently.

TABLE 7

Populations of orange variants isolated from a petite strain and grown by serial transfers at 30°C

O = orange P = petite

	NUMBER OF COLONIES							
CULTURE	1		2		3		4	
DAY	O	P	O	P	O	P	O	P
1	703	1	613	0	583	6	582	2
2	315	6	359	4	368	3	195	0
3	227	1	166	2	291	2	182	0
4	232	0	262	0	308	0	204	2
5	268	0	216	2	370	0	314	2
6	280	0	272	0	426	0	344	0
7	376	0	248	0	252	0	206	0
8	404	0	302	0	403	0	274	0

These results are in direct contrast to those obtained from the orange variants which arose directly from strain X 190 (table 2). This

orange variant did not produce any respiratory competant types whereas the orange variant described earlier produced three kinds of respiratory competent variant. On this evidence the two kinds of orange variant are not identical. This second type of orange variant is, however, respiratory deficient as estimation of respiratory quotient by the Warburg technique showed that as no oxygen was absorbed and therefore no aerobic respiratory process was taking place.

The orange revertant from petite was continuously cultivated at 18°C with V = 300 ml., and F = 75 ml. per hour. Samples were taken on ten successive days and with two exceptions all the colonies observed were orange variant type. A colony which was half orange and half red was observed in the sample taken after 2 days cultivation. In the sample taken after 3 days cultivation, two red colonies were observed. In a total of approximately 6000 colonies, only $2\frac{1}{2}$ red colonies were therefore detected.

These two red colonies were subcultured and found to grow on glycerol medium and hence were respiratory competent. Upon sporulation and tetrad analysis, segregations typical of strain X 190 were observed. On this evidence it was evident that reversion of respiratory deficient petite to respiratory competent parent type had taken place via the respiratory deficient orange variant, but at an extremely low rate. Surprisingly there was no evidence of selection of the red revertant since further red colonies were not detected after the third day of continuous cultivation of the orange revertant.

These red respiratory-competent revertants were grown in continuous culture, V = 300 ml., F = 75 ml., per hour, at 18°C. The results (fig 38) showed that induction of petites by temperature did not occur, since they never reached a level greater than 2%. Orange variants were also observed

Figure 38. Population changes during continuous cultivation of the strain X 190 parent type revertant (isolated from the stable orange variant) in MAD medium at 18°C.

V = 300 ml. F = 75 ml./hour

O = parent type

● = adenine recombinants and revertants

Δ = petite

▲ = orange

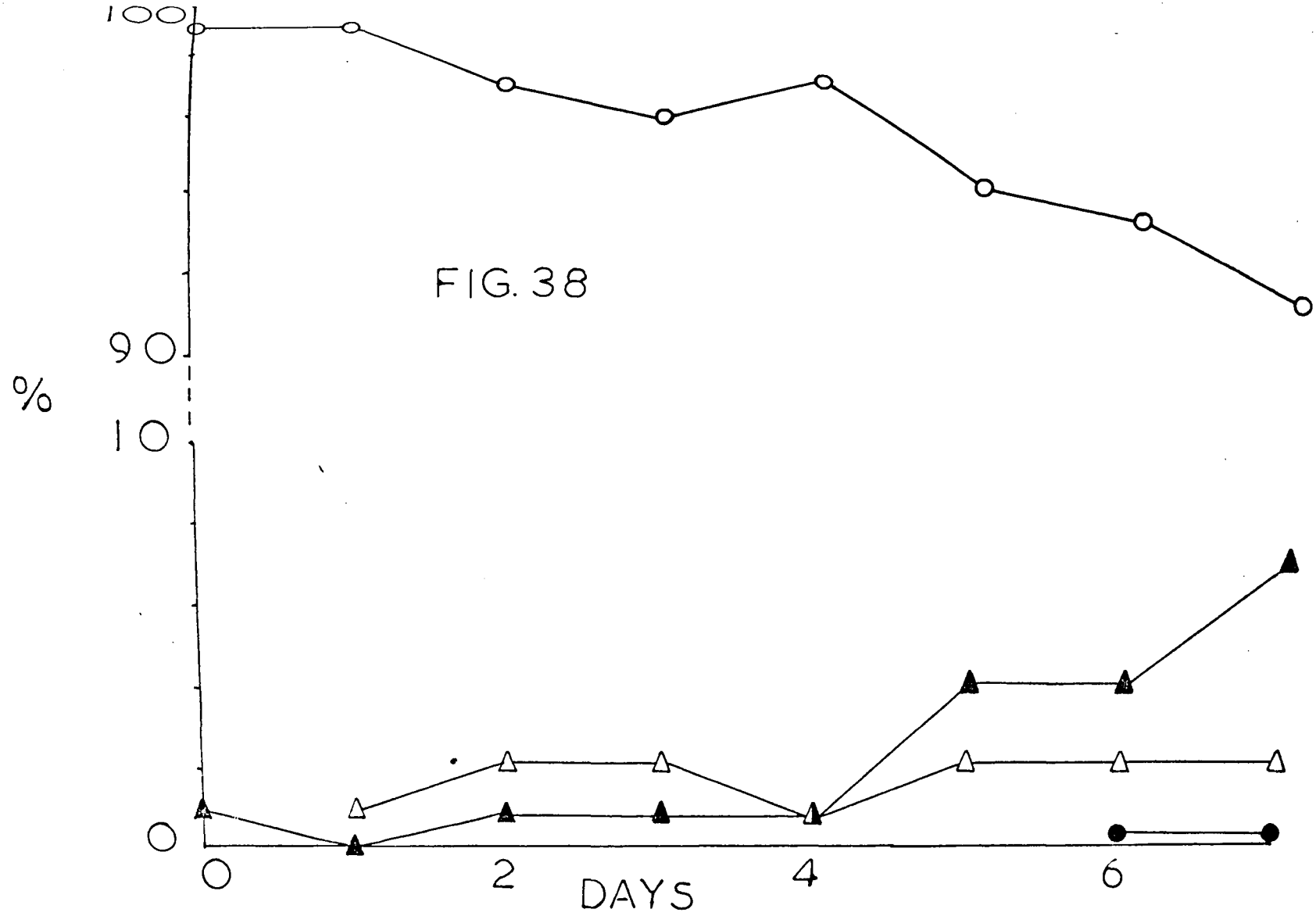


FIG. 38

and colonies of these orange variants contained red sectors, and were therefore of the type which arise directly from strain X 190. White adenine revertant and recombinant colonies were also observed. From these results it was concluded that the red revertant was identical with parent type cells of strain X 190 in every respect except the susceptibility to induction of petites by low temperatures.

Reversion of strain X 190 petites is in agreement with the observations of Kraepelin (1964). The media and cultural conditions in both cases were very similar. The temperature in both cases was below the optimum growth temperature, 18°C and 23°C respectively. Kraepelin employed 'conditions of restricted proliferation' though these were not specified. The continuous culture conditions of this work were also restrictive to the extent that the dilution rates used did not permit optimal growth.

Reversion of petites to respiratory competency has several implications. Mitochondrial DNA might rarely be synthesized 'de novo', but, if so, it would be controlled by the nuclear genetic material, which is contrary to much other evidence. Alternatively, mitochondrial DNA could be present in petites in a 'missense' form and reversion could result from either a repair mechanism or by rare mistranslation of 'missense' DNA resulting in functional mitochondrial DNA.

The effect of temperature on other strains of yeast

Ogur, Ogur and St. John (1960) reported that the induction of petites by low temperatures of 15°C and 18°C was strain dependent. To investigate whether or not petite induction in continuous culture by low temperature was strain dependent, three more strains of yeast were examined. Diploid strain X T5 was bred in the laboratory at 30°C and was homozygous for the ad_2 mutation and heterozygous for the ad_3, ad_6 and ad_8 mutations and consequently formed red colonies on MYGP agar. Strains A and B of Saccharomyces cerevisiae were both non-flocculent commercial strains of yeast received from Allied Breweries.

Strain X T5 was grown in continuous culture, with V = 200 ml. and F = 25 ml. per hour, at 15°C. The results (fig 39, 40) showed that petites were induced in the population of strain X T5 during continuous cultivation at 15°C. In one experiment (fig 39) the rate of increase in the level of petites was slow in comparison with strain X 190, taking 7 days to reach a level of 97%. The proportion of the orange variant rose slowly during the first 3 days to a level of 55% and subsequently fell to a level of 2% in the next 6 days. The proportion of petites reached a maximum after 7 days and subsequently fell to 30% after 9 days. The proportion of the parent type fell rapidly during the first 3 days to 10%, and no parent types were observed on the fourth and fifth days. The proportion, however, rose to 68% after 9 days. In the other experiment (fig 40) the results were similar to those obtained with strain X 190. The level of petites reached 60% after 24 hours and 93% after 48 hours and thereafter remained at a level in excess of 90% for the remaining 6 days. The level of orange variants rose to a maximum of 18% after 24 hours and fell to a level of less than 1% during the remainder of the experiment.

Figure 39. Population changes during continuous cultivation of strain X T5 in MAD medium at 15°C.

V = 200 ml. F = 25 ml./hour

O = parent type

Δ = petite

▲ = orange

FIG. 39

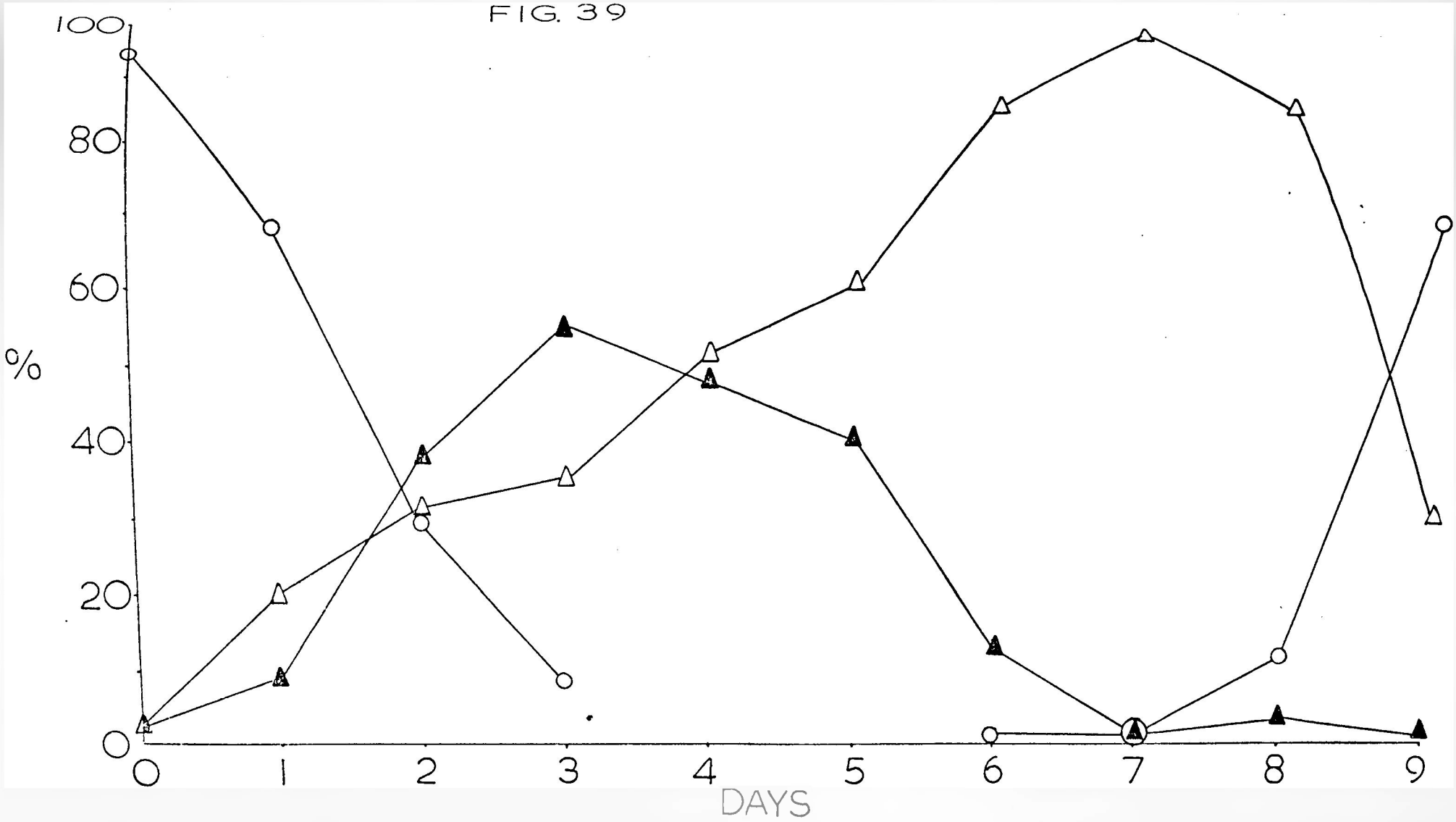


Figure 40. Population changes during continuous cultivation of strain X T5 in MAD medium at 15°C.

V = 200 ml. F = 25 ml./hour

O = parent type

Δ = petite

▲ = orange

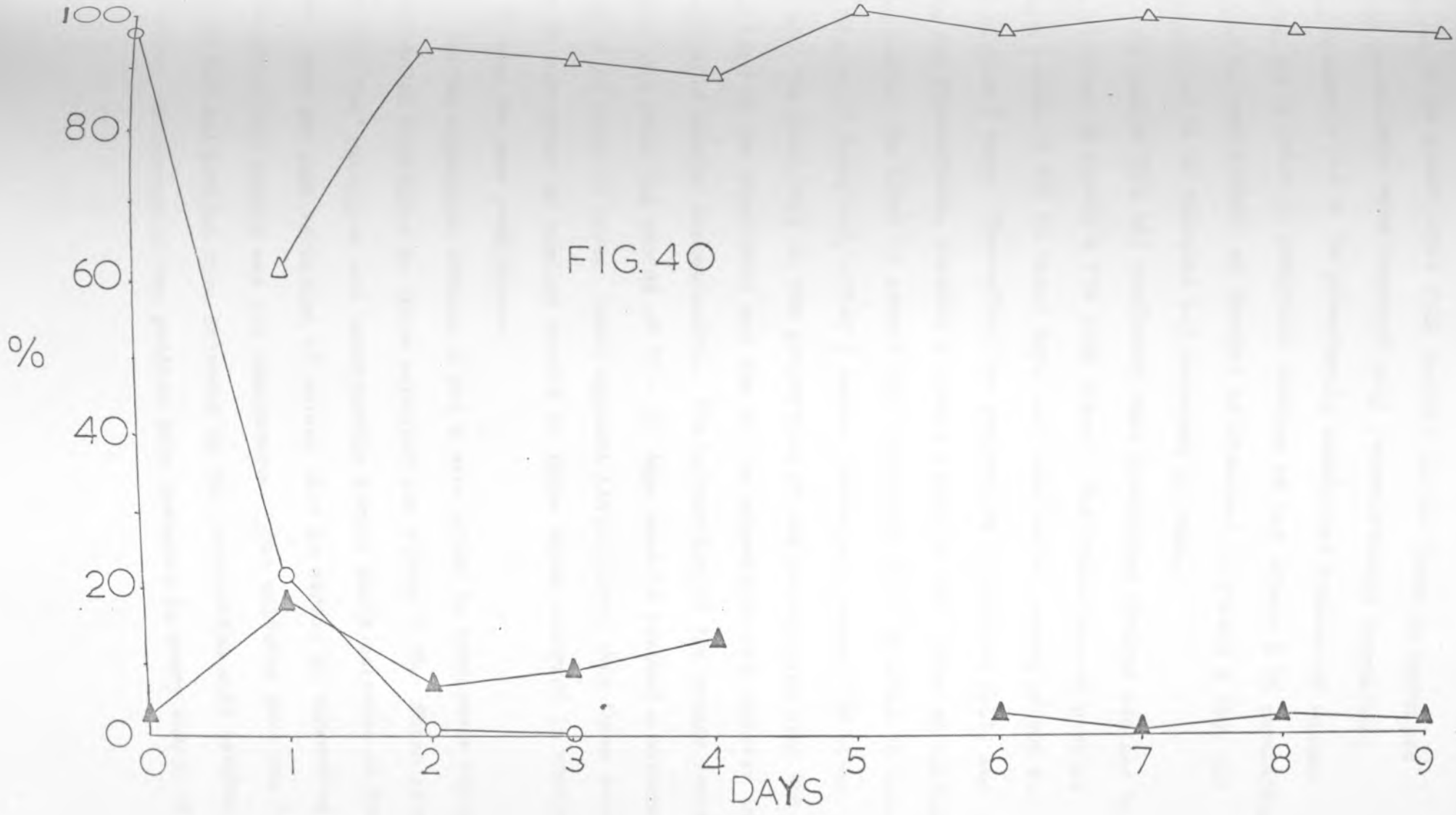


FIG. 40

The level of the parent type fell rapidly in the first 24 hours and parent type colonies were observed only intermittently thereafter. These experiments had to be prematurely terminated because of contamination, and in order to ascertain whether or not strain X T5 populations followed the same pattern of changes as observed in strain X 190, the experiment had to be repeated and extended in time.

The results (fig 41) confirmed that population changes similar to those observed in strain X 190 took place. The proportion of petites rose to a level of 93% in three days and remained in excess of 90% for the following 7 days. Thereafter the proportion of petites fell, and after some fluctuations, reached a steady level of 5%. After an initial sharp decline, the level of parent type increased from 4% after 10 days to 75% after 18 days, and, after a second decrease, reached 93% after 24 days. The final fall in the proportion of the parent type over the last 3 days of the experiment was due to the appearance and increase in proportion of adenine recombinants. The proportion of the orange variant did not rise until the period of 12 - 24 days when it reached a maximum level of 32% after 17 days. There appears little doubt that these results are the consequence of similar events to those which occurred in strain X 190 under the same conditions.

The two commercial strains A and B were grown in continuous culture under similar conditions to those employed for strain X T5. These strains had 'wild-type' genotypes and consequently formed white colonies on MYGP agar. There was some variation of colony size in strain B, suggesting that perhaps this strain was not homogeneous. The cultures were run for 10 days each and petites were detected by the tetrazolium salt overlay technique. An average of two petites were detected in every sample of

Figure 41. Population changes during continuous cultivation of strain X T5 in MAD medium at 15°C.

V = 200 ml. F = 25 ml./hour

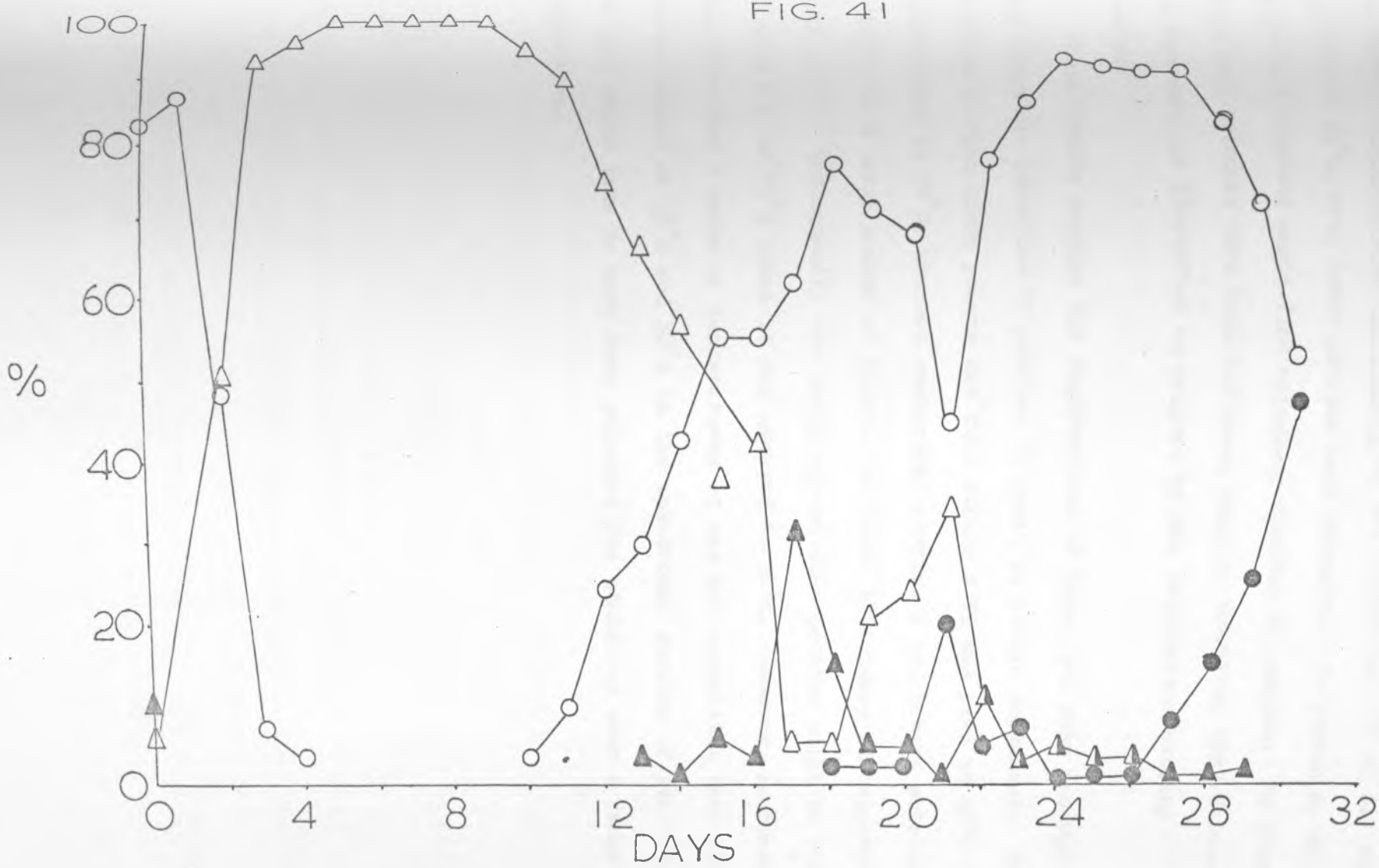
0 = parent type

● = adenine recombinants and revertants

Δ = petite

▲ = orange

FIG. 41



400 colonies, and it was concluded that petites were not induced in these two strains of Saccharomyces cerevisiae by the temperature of 15°C. When cultivated at 30°C, even fewer petites were detected. In strain A, no petites were detected among 7280 colonies, counted in samples. In strain B some small colonies were detected among samples totalling 4882 colonies but these were not identified as petites by the tetrazoluim overlay technique.

These results confirm the observations of Ogur, St. John and Ogur that temperature induction of petites in yeast is strain dependent. One difference between these yeasts was that strain X T5 was bred at 30°C for investigations at 30°C, whereas commercial strains A and B were selected, probably over a large number of years, for their efficiency at temperatures close to 18°C. Consequently one would expect that petites might be induced in strain X T5 at 15°C since it was adapted to 30°C. However, as brewing takes place over a range of temperatures it was not surprising that petites were not induced at 18°C and 30°C in the commercial strains of yeast as one would expect them to have been selected for stability over a range of temperatures.

Flocculation of strain X 190 in continuous culture.

Flocculence, that ability of yeast to form aggregates of cells called 'flocs', is of considerable importance in the investigation of population stability and in the brewing industry. The population of strain X 190, which is non-flocculent, is in effect a mixed culture at low temperature, with several types of cell present. Spontaneous flocculation of one or more types in the population renders it useless for further studies of population stability since one cell type is maintained at an artificial level in the population. Flocculation is very important in brewing, where non-flocculent yeasts are generally used in lager fermentation and flocculent yeasts are used in the production of ales (Rainbow, 1966). Flocculent strains of yeast are also important in the continuous fermentation of beer.

Flocculation is a genetically controlled characteristic in Saccharomyces cerevisiae. Thorne (1951) concluded that flocculence in several yeast strains was under the control of 3 chromosomal genes, and that flocculence was dominant to non-flocculence. Gilliland (1951), however, showed that flocculence was under the control of one recessive gene in the strain examined. Flocculation can also be affected by a number of environmental factors which are reviewed by Rainbow (1966).

Under normal cultural conditions at 30°C, strain X 190 is non-flocculent, and usually remained non-flocculent in continuous culture at this temperature. However during continuous culture experiments on strain X 190 at lower temperatures, flocculation was a recurring problem. Once they reached a certain size, flocs settled out and were selectively retained in the continuous culture vessel. A simple way to distinguish morphologically between flocculent and non-flocculent yeast colonies on

MMP agar was discovered. Non-flocculent cells of strain X 190 formed flat smooth colonies 4 - 5 mm. in diameter after 3 days incubation at 30°C. Clumps of flocculent cells of strain X 190 formed rough peaked colonies of 2 - 3 mm. diameter and 1 - 2 mm. high. These two types of colony were easy to identify and made an excellent diagnostic guide to flocculence in strain X 190. Samples from a continuous culture of strain X 190 above the layer of flocculent yeast , consisted mainly of non-flocculent yeast.

Flocculence was not confined to parent type cells of strain X 190. Flocculent petites were also distinguished by their colony morphology. Spontaneous orange variants of strain X 190 did not give flocculent type colonies, but orange revertants from petites did. Flocculation was confirmed by culturing colonies in MAD medium at 30°C on an orbital shaker for 24 hours. By the end of this period large pellets of cells were formed in cultures from flocculent rough colonies whereas smooth colonies formed a uniform suspension of cells.

In view of the importance of flocculation to the brewing industry and its relevance in continuous culture, a preliminary investigation of its genetical control in strain X 190 was made. Red, respiratory competent, rough colonies of strain X 190 were sporulated. Sporulation was good but the spore viability of most colonies was poor. The best gave 7 complete tetrads from the dissection of 12 asci plus 5 single ascospore colonies, a total of 33 segregants. All segregant colonies were red and rough, and growth in liquid media showed them to be flocculent. All segregant strains grew in the absence of tryptophan, threonine, tyrosine, uracil, and lysine, but not in the absence of adenine. These results suggested that the majority of the segregants were not haploid. Thus it appeared that flocculent

strains of X 190 might be polyploid.

Four of these segregant strains were sporulated and the asci dissected. In all cases, however, these ascospores had a low level of viability. Twelve asci were dissected for each segregant strain and a total of only 7 ascospores formed colonies. Of these 7 colonies, three, each from different strains, were adenine revertants. The remaining four segregants were single segregants from different asci and exhibited genotypes consistent with haploid segregants of strain X 190, moreover these segregants did not sporulate after growth on sporulation media. None of the seven segregants were flocculent.

These results suggest that flocculent variants of strain X 190 were not diploid but polyploid or aneuploid. A tetraploid strain of X 190, X 190T (Johnston and Mackinnon, 1966) is, however, non-flocculent, which could be taken as an indication that flocculent variants of strain X 190 are aneuploid.

Genetic variation in strain X T5 during growth by serial transfer

Genetic variation in populations of Saccharomyces strains during extended growth can be due to a variety of causes, the most likely being mutation. Variation in diploid heterozygous yeasts due to mitotic recombination of genes might be regarded as negligible when compared with the large number of variations due to mutation. However, the rate of mitotic recombination is probably much greater than the mutation rate for many loci, and consequently may be an important mechanism in causing particular variations in populations.

The adenine synthetic pathway of Saccharomyces cerevisiae has proved a fruitful system for the examination of meiotic, and to a lesser extent, mitotic recombination (Roman, 1956; Mortimer and Hawthorne, 1960; Johnston and Mackinnon, 1966). Diploid strain X T5 of Saccharomyces cerevisiae was heterozygous for the ad_3, ad_6 and ad_8 mutations and homozygous for the ad_2 mutation. Consequently strain X T5 formed red colonies after 3 days growth at 30°C on MYGP agar. Recombination at any of the ad_3, ad_6, and ad_8 loci, producing homozygosis for the mutant alleles, results in cells forming white colonies on MYGP which were easily distinguished from the parent type (plate 1).

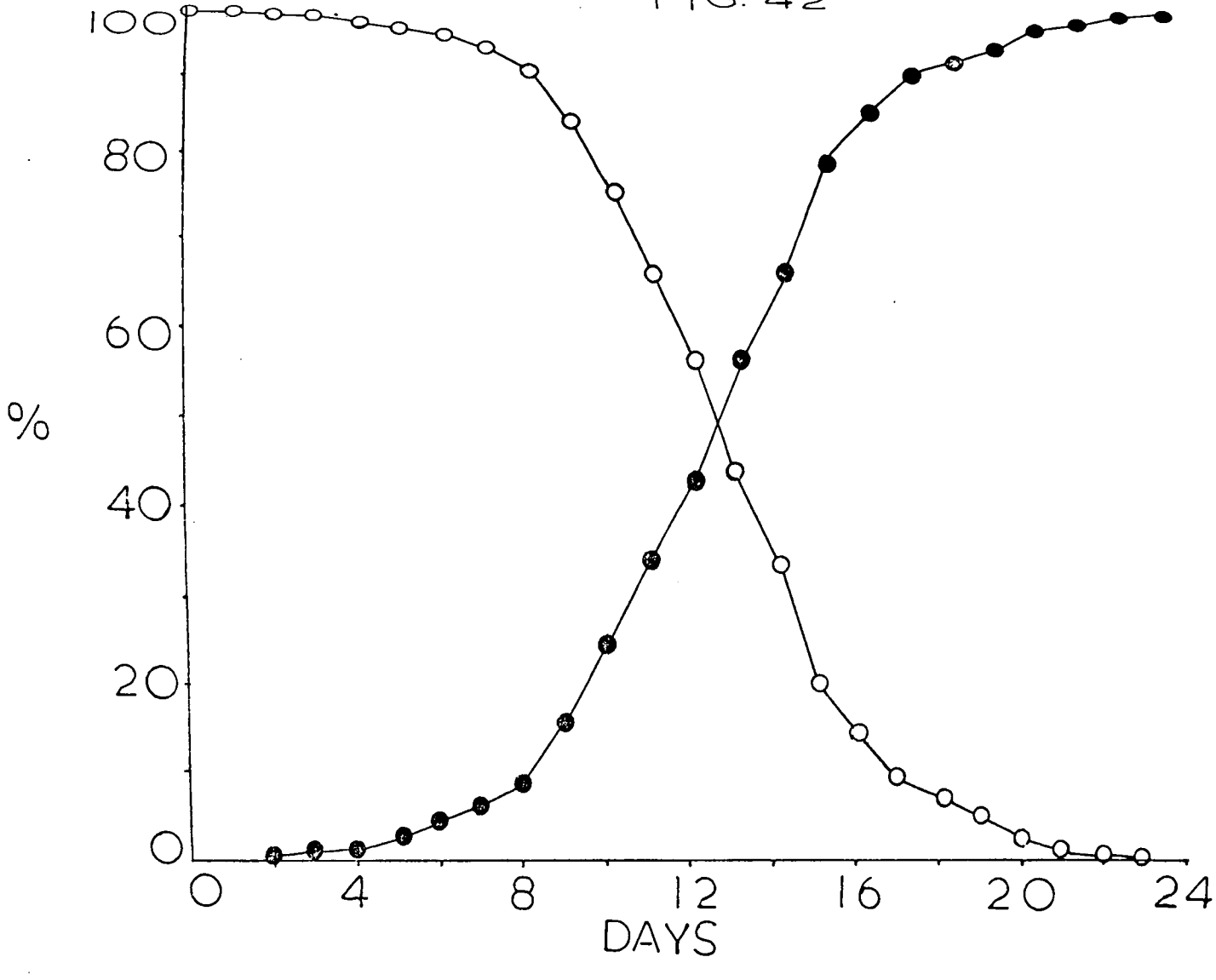
Six cultures of strain X T5 derived from a common stock culture were cultivated by 24 hour serial transfers at 30°C. Samples were plated out on MYGP agar every 24 hours, incubated at 30°C and examined after 3 days. The numbers of red and white colonies observed in each daily sample from all the cultures were pooled. The results (fig 42) showed that white colonies were observed after 48 hours cultivation, and increased to 99.5% of the population in 22 days. These white colonies were confirmed as recombinants by replica plating onto AD omission medium and glycerol medium.

Figure 42. Population changes during serial transfer of shaking cultures of strain X T5 at 30°C.

O = parent type

● = adenine recombinants

FIG. 42



Absence of growth on AD omission medium indicated that the cells required adenine for growth and therefore that the white phenotype was due to recombination of one, or more, of the ad_3, ad_6 and ad_8 mutations. Growth would have indicated reversion of the ad_2 mutation resulting in adenine independence. Growth on glycerol medium showed that the white colonies were not respiratory deficient. These results (fig 42) also show that selection for recombinants took place since the rate of increase in the proportion of recombinants was greater than linear, the graph curving steeply upwards. There would have been an initial linear increase in the proportion of recombinants with the increase in generations and a subsequent concave curve if no selection of recombinants had taken place.

The relationship between the proportion of mutants and the number of generations was approximately linear for 5 days (65 generations) (fig 43), and from the slope of the line of this graph an overall rate of recombination at the three loci was calculated. This combined rate was estimated as $4.8 \pm 1.4 \times 10^{-4}$ per cell per generation.

The white colonies were also tested on TRY omission medium since it was found that the try_4 mutation was linked to the ad_8 mutation (Hawthorne and Mortimer, 1960). Recombination of the ad_8 mutation was frequently accompanied by recombination of the try_4 mutation (Johnston and Mackinnon, 1966), the resulting daughter cells being unable to grow on either AD or TRY omission media. Approximately half the white colonies tested from platings of strain X T5 did not grow on TRY omission medium (fig 44) and were therefore classified as ad_8 recombinants. Similar tests for the ad_3 and ad_6 mutations could not be made since there were no linked markers for these mutations in this strain of yeast. Twenty white colonies which did not grow on TRY omission medium were genetically analysed by the cross-

Figure 43. Estimation of the rate of recombination of adenine genes in strain X T5 at 30°C by the 'line of regression' method.

R = line of regression

FIG. 43

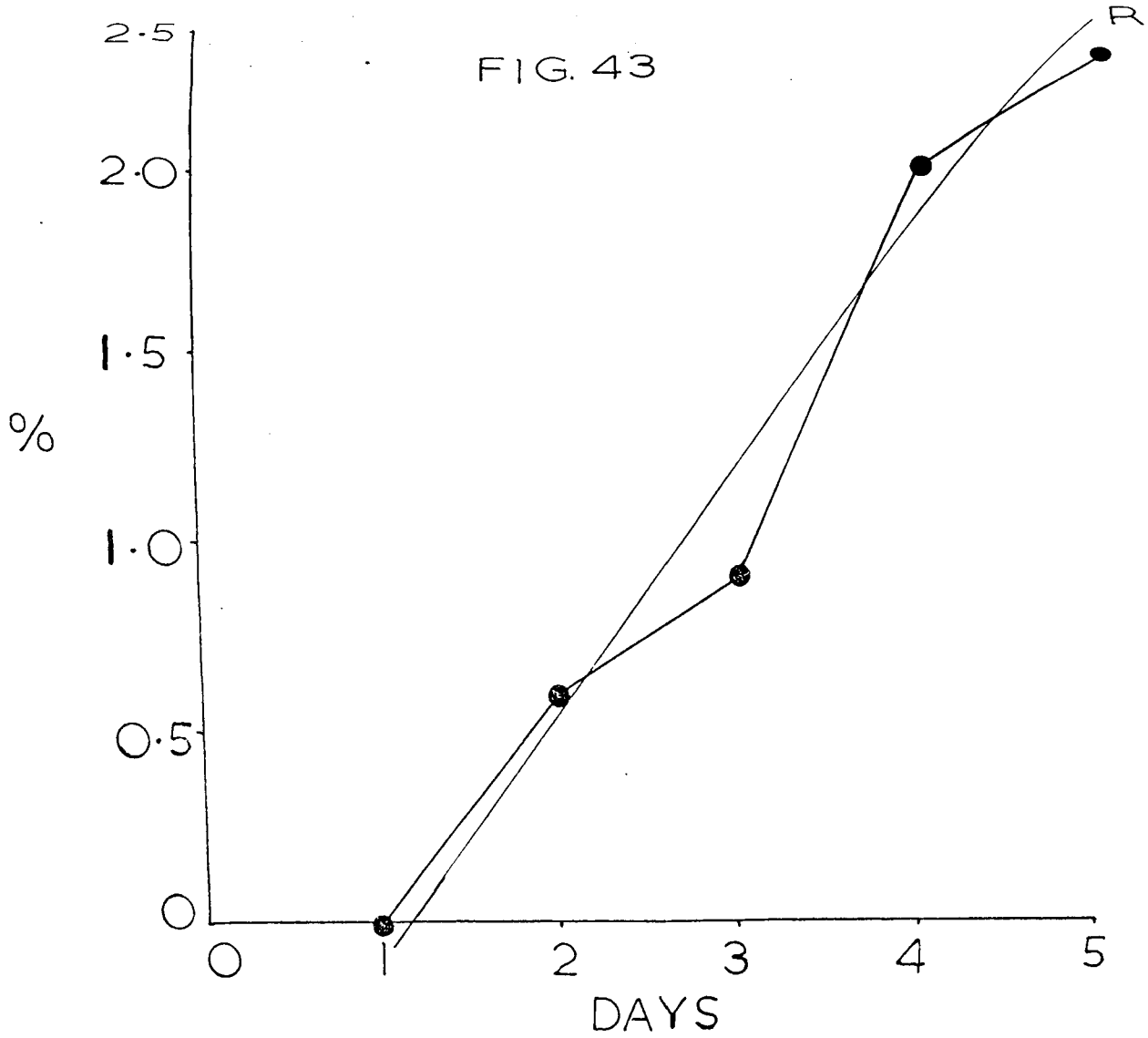
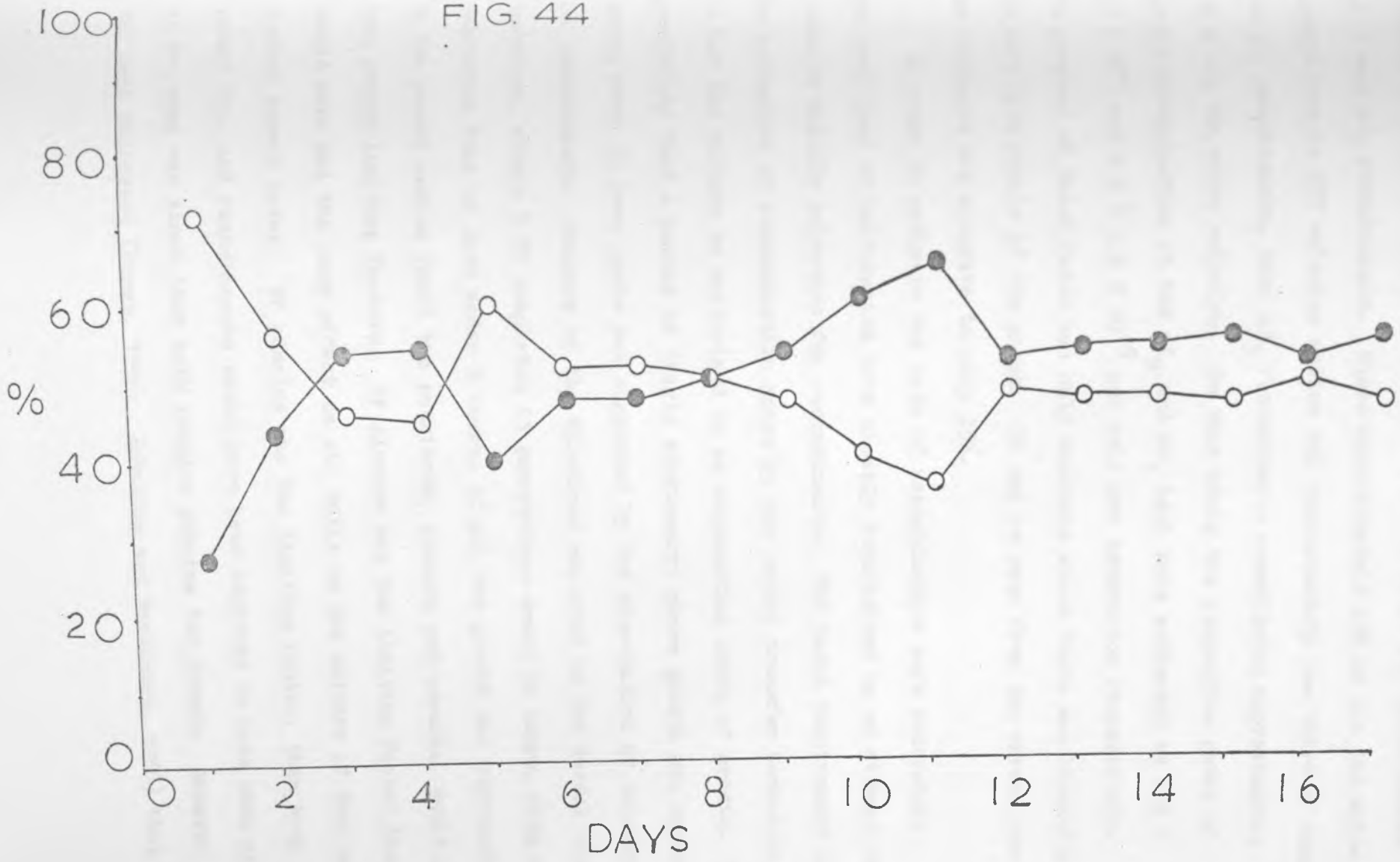


Figure 44. Adenine recombinants of strain X T5 tested on Tryptophan omission medium.

O = growth

● = no growth

FIG. 44



streaking technique. Seven of these were found to be ad_3 recombinants and 13 were ad_8 recombinants. Since approximately 45% of all the white colonies grew on TRY omission medium and approximately one third of these were ad_3 recombinants, then ad_3 recombinants constituted approximately 15% of all the white colonies. On this basis the respective rates of mitotic recombination at the ad_8 and ad_3 loci were estimated as $4.0 \pm 1.1 \times 10^{-4}$ and $7.2 \pm 1.8 \times 10^{-5}$ per cell per generation respectively. The accuracy of these rates was only moderate since there was linearity for only five points of the graph. It can be seen from the errors that the estimates are accurate to only 25%.

In order to estimate the rate of recombination more accurately, the conditions of cultivation were closely scrutinised in an attempt to reduce or nullify selection for recombinants. The basic requirement in the estimation of recombination rates by the serial transfer technique is that the culture be maintained in an exponential state of growth. The possibility that a period of 'early stationary' phase growth was occurring during every 24 hour cycle was suggested by the observation of selection for recombinants. Because of the dilutions employed in the serial transfer technique, strain X T5 completed 13 generations every 24 hours, with a generation time of just under 2 hours, if all the growth was exponential. In the growth medium (MAD) two nutrients, glucose and adenine, could have been growth limiting factors. If glucose was the limiting factor then it should have had the same effect on all cells in the culture if they had similar growth rates. If adenine was the limiting factor, then both parent type and recombinants would have been expected to have been affected in the same way since they both require adenine for growth. However it has been suggested (Roman, 1956; Johnston and Mackinnon, 1966) that the

formation of the red pigment in the parent type cells has an inhibitory effect on the growth of these cells, in which case the selection of recombinants would take place.

To ensure that the cultures were always in an exponential state of growth, the growth medium and the serial transfer technique were altered. The adenine supplement was doubled from 20 mg./litre to 40 mg./litre, and the size of the daily inoculum/transfer was reduced from 6×10^4 cells to 1×10^3 cells. Under these conditions strain X T5 completed 19 generations every 24 hours, with a generation time of just over $1\frac{1}{4}$ hours.

The pooled results of five cultures of strain X T5 (fig 45) grown under these modified cultural conditions showed that although selection for recombinants still operated, it was much less than that previously observed, and so, departure from linearity occurred after many more generations of growth. The relationship between the proportion of recombinants and the number of generations gave a good approximation to a straight line for 14 days (fig 46), three times as long as in the original experiments. The 'line of regression' for these observations gave an overall rate of recombination for ad_3, ad_6 and ad_8 mutations in strain X T5 of 3.9×10^{-4} per cell per generation. This estimate was 20% less than the original estimate for the overall rate of recombination. The white colonies were again replica plated onto AD and TRY omission media and glycerol medium. The relative proportions of growth to non-growth on TRY omission medium was 35 : 65 (fig 47). Forty colonies which did not require tryptophan for growth were analysed by sporulation, tetrad analysis and cross streaking, and thirteen were found to be recombinants for the ad_3 mutation. None of these 40 were recombinants for the ad_6 mutation. Consequently 10% of the overall rate of recombination was due to recombination of the ad_3 mutation

Figure 45. Population changes during serial transfer of shaking cultures of strain X T5 at 30°C.

O = parent type

● = adenine recombinants

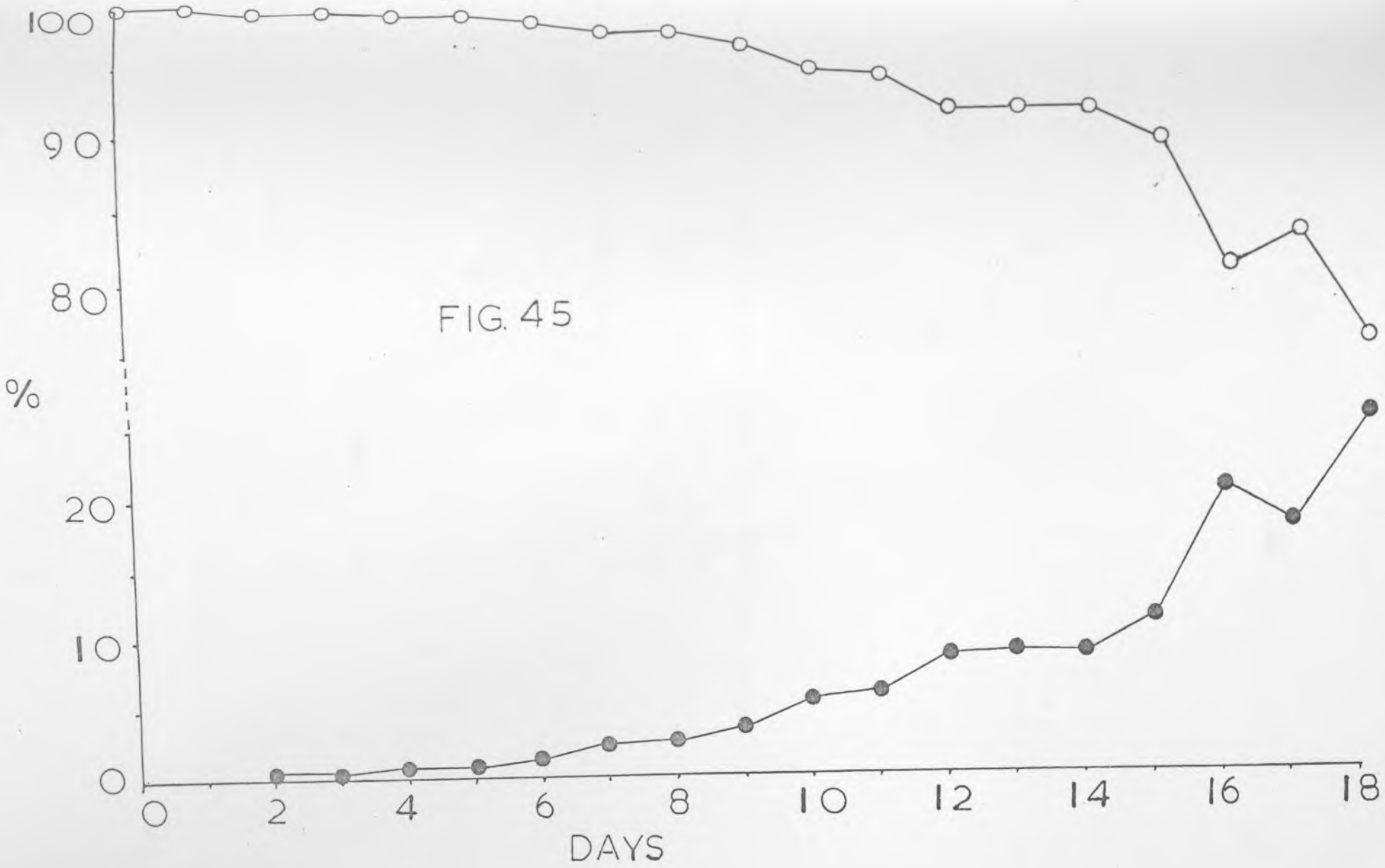


FIG. 45

Figure 46. Estimation of the rate of recombination of adenine genes in strain X T5 at 30°C by the 'line of regression' method.

R = line of regression

FIG. 46

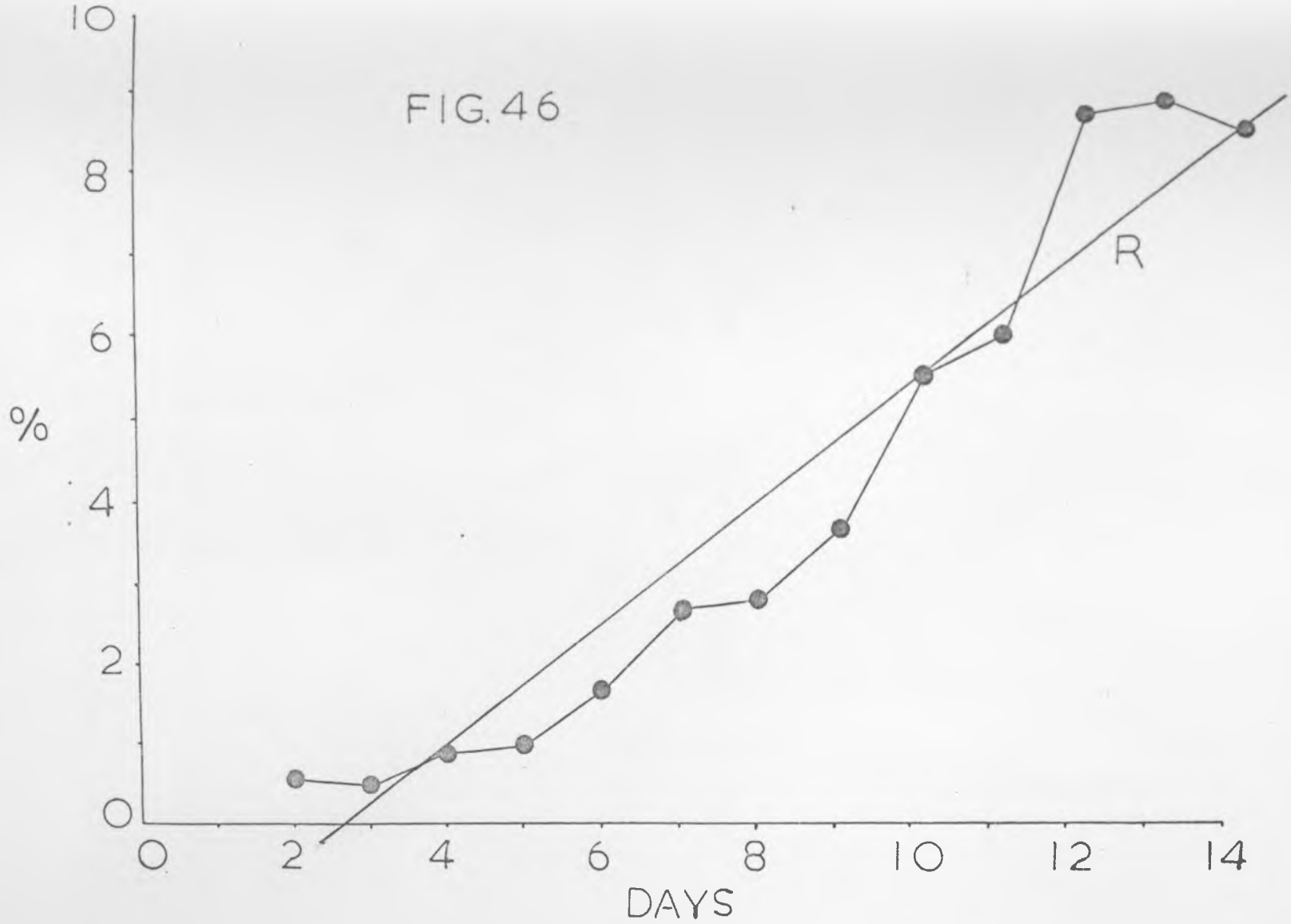
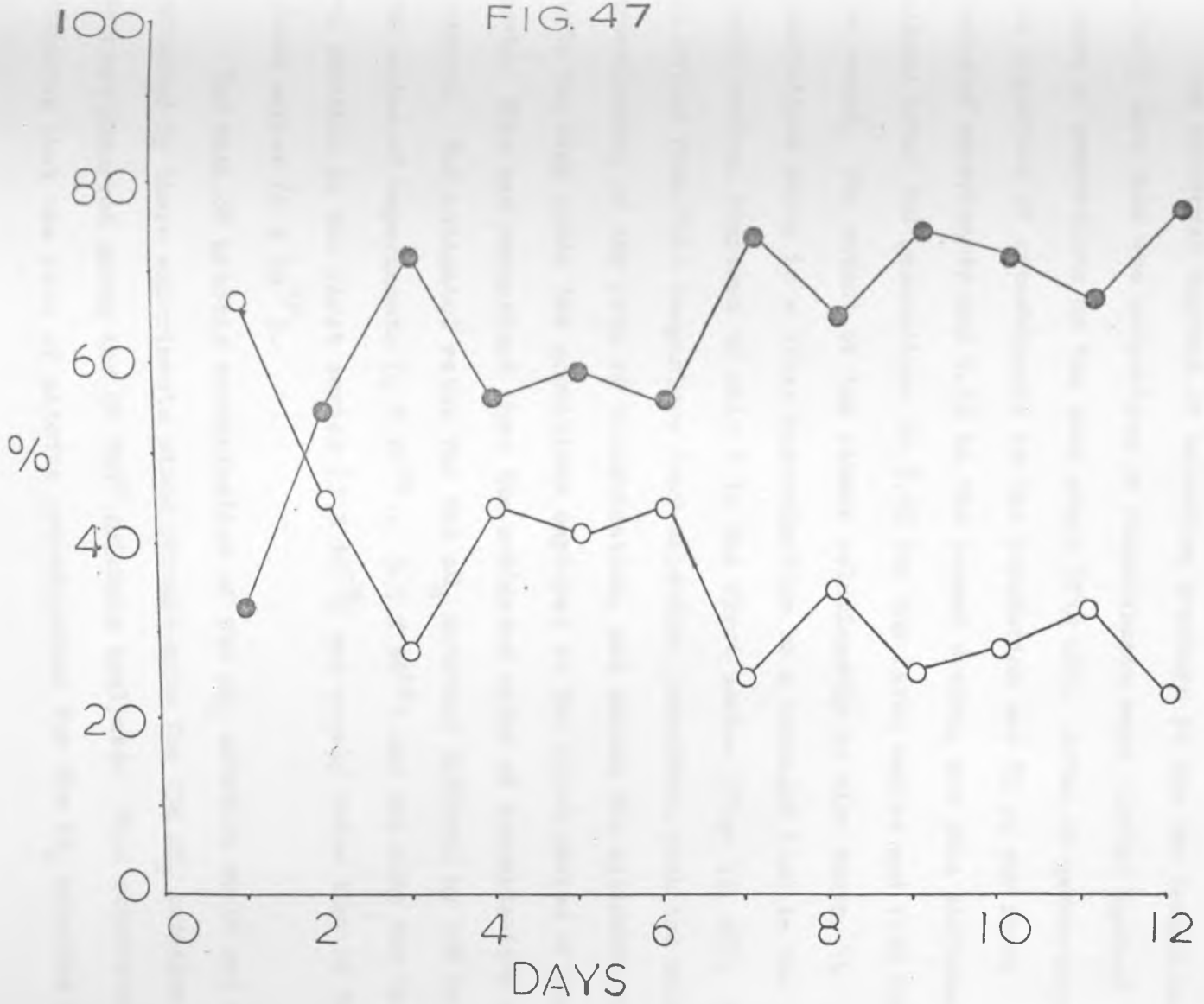


Figure 47. Adenine recombinants of strain X T5 tested on Tryptophan omission medium.

0 = growth

● = no growth

FIG. 47



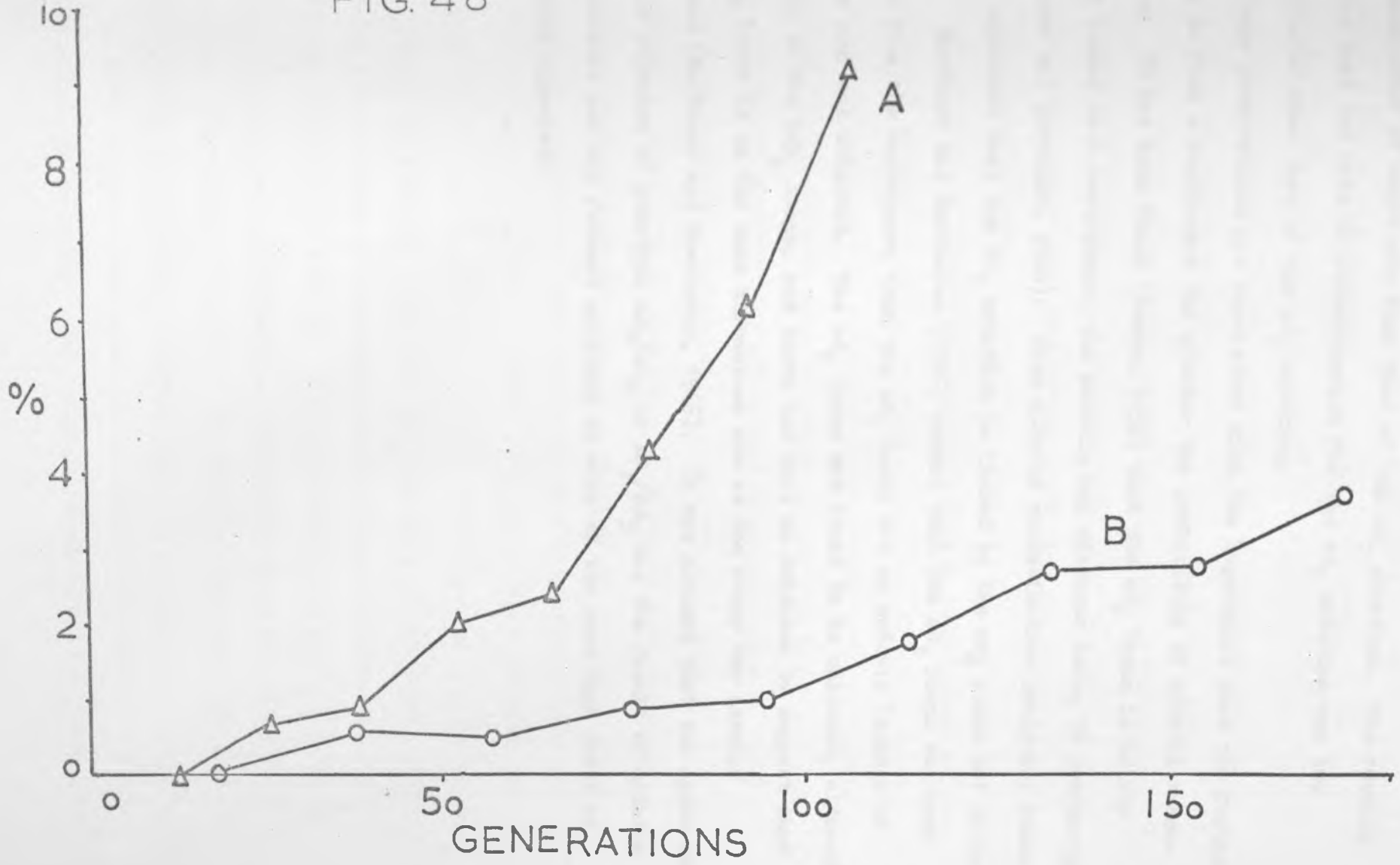
and the remaining 90% was due to recombination of the ad_8 mutation. The rates of recombination of the ad_8 and ad_3 mutations respectively in strain X T5 were estimated as $3.5 \pm 0.7 \times 10^{-4}$ and $4.0 \pm 0.8 \times 10^{-5}$ per cell per generation. It can be seen from the errors that the estimates are accurate to only 20% an improvement in accuracy over the previous estimates of only 5%.

The different degrees of selection pressure in the two series was clearly seen when the proportion of recombinants were plotted against number of generations on the same graph (fig 48). After 40 generations the proportion of recombinants in the population was 1% in the first series of experiments and 0.6% in the second series, but this difference widened after 100 generations to 7.9% for the first series and 1.1% for the second. The extent of the linear relationship is also shown, 14 observations being in a close approximation to a straight line in the second series, compared to only 5 in the first series (figs 43, 46). It is obvious from this comparison that selection interfered with the accurate determination of the rate of recombination, and caused the estimated value to be too high under the conditions employed in the first series of experiments. This was emphasized when the estimated rates of recombination are compared. The estimated rates for the ad_8 mutation differed by 12% in the two series of experiments (4×10^{-4} : 3.5×10^{-4}) and the rate for the ad_3 mutation in the first series (7×10^{-5}) was nearly twice that of the second series (4×10^{-5}).

The rate of mitotic recombination of the ad_6 mutation could not be estimated by these experiments since recombinants for the ad_6 mutation were not observed among the 60 TRY^+ colonies analysed. This observation indicates that the rate of mitotic recombination for the ad_6 mutation is

Figure 48. Comparison of the proportion of adenine recombinants observed in the two series of strain X T5 experiments.

FIG. 48



of a lower order of magnitude than that of the ad_3 mutation. The results also show that the rate of recombination for the ad_8 mutation was ten times greater than that of the ad_3 mutation.

These observations are consistent with the hypothesis that the further a locus is from a centromere the greater the probability of mitotic recombination. It has been found (Roman, 1956) that the ad_6 locus is fairly closely linked to a centromere, the meiotic map distance being 30 centimorgans (Hawthorne and Mortimer, 1966). From mitotic recombination analysis, Roman (1956) concluded that the ad_3 mutation is linked to the ad_6 locus but distal to it. Mortimer and Hawthorne (1966) showed that the ad_3 locus was much further from the centromere than the ad_6 locus but no meiotic linkage of the two loci was detected. The ad_8 locus was found to be unlinked, although loosely, to the try_4 locus, and there has been no evidence to suggest that the ad_8 locus is on the same chromosome arm as the other two adenine mutations (Mortimer and Hawthorne, 1966). It was assumed that the appearance of white colonies of genotype ad_8/ad_8 or ad_3/ad_3 was the result of mitotic recombination and not forward mutation in view of the very high rates at which they appeared.

Rate of mitotic recombination for the ad_{5-7} mutation

Strain X T14 of Saccharomyces cerevisiae was bred in order to determine the mitotic recombination rate of the ad_{5-7} mutation. This strain was homozygous for the ad_2 mutation and heterozygous for the ad_{5-7} mutation and linked markers ly_5, ty_3, met_{13}, and ac^r_2. Strain X T14 normally formed red colonies after 4 days growth on MYGP agar at 30^oC. Recombinants for the ad_{5-7} mutation gave white colonies, and these recombinants would also be expected to be homozygous for one or more of the linked markers.

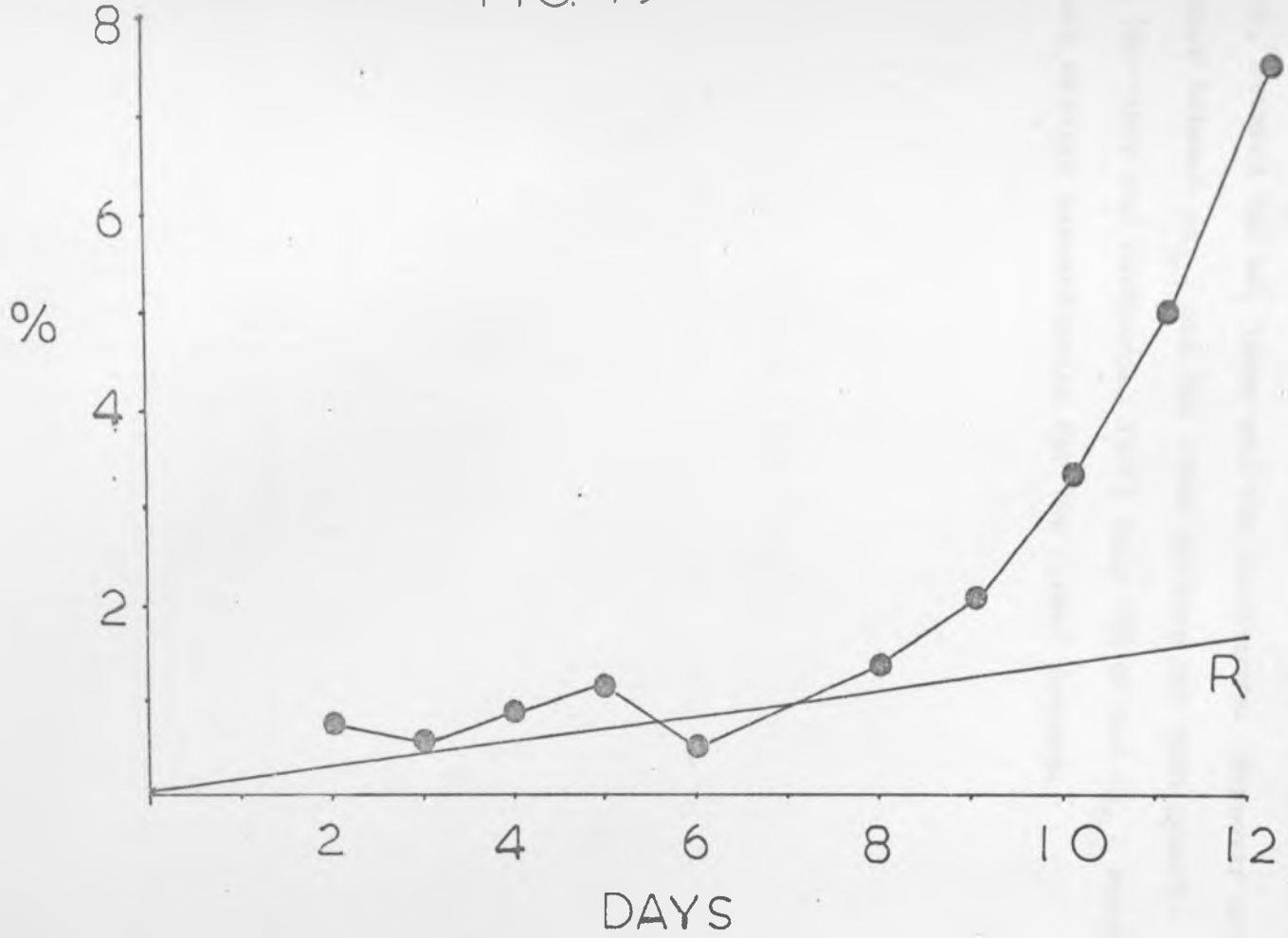
Three cultures of strain X T14 derived from a common stock culture, were cultivated by 24 hour serial transfers at 30^oC. Strain X T14 was found to have a slower growth rate than either strain X 190 or strain X T5 and only 13 generations were completed in every 24 hour cycle of gorwth. The pooled results (fig 49) showed that white colonies were observed after 48 hours cultivation and increased to a level of 7.5% after 12 days. Testing on AD omission medium showed that the white colonies were recombinants for the ad_{5-7} mutation as they could not grow in the absence of adenine. The rate of mitotic recombination for the ad_{5-7} mutation was estimated by the 'line of regression' method over the first nine days' results. After nine days the proportion of recombinants curved steeply upwards and selection was suspected as the cause, although the reason for this was not apparent since the growth medium was supplemented with 40 mg./litre adenine. The rate of mitotic recombination of the ad_{5-7} mutation was estimated from these results as $1.4 \pm 0.8 \times 10^{-4}$ per cell per generation, although it can be seen from the error that the accuracy of this estimate was only moderate.

The white colonies were also replica plated onto LY, TY, and ME omission media and Actidione agar, to test for the linked markers. The

Figure 49. Estimation of the rate of recombination of the ad_{5-7} mutation observed in strain X T14 at 30°C by the 'line of regression' method.

R = line of regression

FIG. 49



results were surprising since it was found that recombination for all the linked markers either did, or did not, occur. In no case was the recombination of only part of the linkage group observed in conjunction with the recombination for the ad_{5-7} mutation. These results were rather puzzling and one would wish to confirm them with more data before drawing any firm conclusions. However they suggest that crossover followed by segregation was only occurring between either the ad_{5-7} mutation and the ly_5 locus, or, between the ac^r_2 locus and the centromere. Moreover although meiotic linkage between ad_{5-7} and the other markers has been clearly established (Mortimer and Hawthorne, 1966) only 10% of the ad_{5-7} recombinants showed mitotic recombination for the linked markers.

D I S C U S S I O N

The stability of strain X 190 of Saccharomyces cerevisiae during growth at 15°C was investigated. Respiratory deficient petite mutants were found to be induced at a high rate in this strain at this temperature. Respiratory deficient orange variants were also induced, but at a much lower rate than petites. The increased frequency of both variants was shown to be due to induction and not to selection when these variants were observed at increased frequencies during cultivation in glycerol medium, which is selective against respiratory deficient types.

Temperature-induction of respiratory deficient variants and longer term effects during continuous cultivation of strain X 190 were investigated over a range of temperatures. The results showed that temperatures of 15°C, 18°C and 21°C, induced both petites and orange variants in strain X 190, and it may be assumed that all temperatures intermediate between 15°C and 21°C would do likewise. Moreover, a similar pattern of changes in the population of strain X 190 were observed at all three temperatures. Petites were induced at a high rate in the first three days of continuous culture, and after four days composed about 90% of the population. Orange variants were induced during the first two days of cultivation, reaching a level between 30% and 60%. Subsequently the level of orange variants fell, but still remained at a relatively high level between 5 - 10%. The population, composed of approximately 90% petite and 5 - 10% orange variant, remained stable for a few days, between 4 and 10 days depending on the temperature, after which the proportion of petites decreased over a number of days with a concurrent increase in the proportion of parent type. At the time when the proportion of petites began to fall, a small, but temporary, rise in the proportion of orange variants to 10 - 25% was observed. Eventually the proportion of petites was 1 - 2% and

that of the orange variants 4 - 5% and the remainder of the population was parent type. This latter population is very similar to that observed in strain X 190 during early stages of continuous cultivation at the optimum growth temperature of 30°C. Induction of petites was not observed at 25°C although the orange variant was induced at a high rate at this temperature.

The orange variant was grown at 30°C and was found to be very unstable. This instability was due principally to a high rate of reversion to respiratory competent parent type, although a relatively high frequency (4 - 5%) of petites was also produced. An approximately normal rate of mitotic recombination for the ad_8 mutation was observed.

Reversion of respiratory deficient petites to respiratory competent parent types was observed, although indirectly. A stable type of respiratory deficient orange variant appeared when temperature-induced and spontaneous (30°) petites were grown in continuous culture at 18°C. This orange variant had a greater growth rate than the petite and consequently, in time, formed the major part of the population. When this orange variant was, in turn, continuously cultivated at 18°C, a few respiratory competent types were isolated. However, the proportion of respiratory competent types did not increase in the population, and it appears that there must be selection against them in competition with the stable orange variant. Genetic analysis of the respiratory competent colonies showed them to be similar to the parent type of strain X 190. Unlike stock cultures of strain X 190, however, respiratory deficient variants were not induced when this 'revertant' parent type was grown in continuous culture at 18°C. Therefore this 'revertant' was a temperature-stable type of strain X 190 with regard to petite induction. The orange variant isolated from petite

cultures was found to be very stable when grown at 30°C in that, unlike the orange variant derived directly from strain X 190, significant proportions of other types were not observed.

Ephrussi (1953) suggested that petites arose by the loss, inactivation, or mutation of a cytoplasmic determinant which controls the formation of cytochromes a + a_3 and b. These cytochromes are associated with the mitochondria, which are the centres of respiratory activity in the cell. Since it has also been shown that mitochondria of petite cells have malformed inner membranes (Yotsuyanagi, 1962; Smith et al., 1969), it has been concluded that the cytoplasmic determinant also controls the formation of the inner membranes of mitochondria. It has also been suggested that the cytoplasmic determinant, referred to as the rho (ρ) factor (Sherman, 1964), might be mitochondrial DNA (Raut and Simpson, 1955). Subsequent studies have revealed that mitochondrial DNA is present in normal yeast cells (Corneo et al., 1966; Moustacchi and Williamson, 1966; Tewari et al., 1965). Although some workers (Corneo et al., 1966; Moustacchi and Williamson, 1966) have reported that petite cells do not contain mitochondrial DNA, there is an increasing amount of evidence which supports the arguement that mitochondrial DNA is present in petite cells in an altered form (Mounolou et al., 1966; Tewari et al., 1966; Smith et al., 1968). Despite this dispute it has now been generally accepted that mitochondrial DNA is the rho (ρ) factor.

There has been no evidence that replication of mitochondrial DNA is controlled directly be chromosomal DNA, and it has therefore been suggested that mitochondrial DNA replicates autonomously. Once a cell has lost its respiratory capability, the chormosomal DNA appears to be incapable of initiating the synthesis of new mitochondrial DNA, thereby

restoring its respiratory ability and consequently, the cell remains respiratory deficient. However, respiratory deficiency in a yeast cell cannot be equated with absence of mitochondrial DNA, which is commonly found in respiratory deficient petites and which, although remaining non-functional, has been shown to replicate in a normal manner (Rabinowitz et al., 1969). The effects of temperatures less than the optimum growth temperature of 30°C, and the changes in the population during continuous cultivation of strain X 190, can be considered in the light of three possible mechanisms, each connected with mitochondrial DNA.

(1) It has been proposed that mutation or loss of yeast mitochondrial DNA results in the formation of respiratory dificient petites. A large number of agents can induce petites, although the precise mutagenic action of each has not been fully explained. In these experiments, reduced temperature in the range 15 - 21°C has been established as the inducing agent. Temperature, unlike acriflavine (Ephrussi, 1953), induced petites in only a proportion of the progeny, consequently some respiratory competent parent types should always be present in the population. In these circumstances, an equilibrium, with fixed proportions of each type being present in the population, and based upon the mutation rate to petite and the degree of selection in favour of normal cells, should occur during continuous cultivation. Such an equilibrium apparently occurred in all of these experiments, although it was temporary and applied for periods of time. In view of the high rate of induction of petites observed initially it would be expected that the petites would form a considerable proportion of the population, even in the selective conditons employed. However, the level of petites fell to 1 - 2% which is the same as the rate at which petites occur spontaneously at the

optimum growth temperature.

Originally, it was assumed that all vegetative petites were identical, but analysis of acriflavine-induced petites revealed that there were biochemical differences between petites, and at least four classes were established (Avers, Pfeffer, and Rancourt, 1965). These different classes could result from the mode of action of acriflavine. It was found that acriflavine binds preferentially with mitochondrial DNA rather than chromosomal DNA. The acriflavine could either prevent replication of mitochondrial DNA or alter the replica (Lerman, 1963; Tewari et al., 1966), or it could interfere with the function of specific RNA polymerase (Richardson, 1966). Unlike vertebrates in which the length of mitochondrial DNA is uniform, the length of yeast mitochondrial DNA was found to be variable (Avers, 1967). Yeast mitochondrial DNA consisted of both linear and circular forms in normal cells (Avers et al., 1968). Mitochondrial DNA from petites consisted mainly of double ended rods (Avers, 1967) although some circular forms were also observed (Avers et al., 1968). Acriflavine-induced haploid petites, containing morphologically different mitochondria, were crossed to form a diploid petite. When the mitochondria of this diploid were examined after many generations, they were identical with the mitochondria of the two parents (Avers et al., 1965; Federman and Avers, 1967). The evidence suggest that (a) a mutagen such as acriflavine does not give identical mutants and (b) yeast mitochondrial DNA is heterogeneous. If yeast mitochondrial DNA were heterogeneous, then it is possible that it will not have a uniform response to the mutagenic action of temperature. Some of the mitochondrial DNA may be insensitive to the action of temperature. In this case, if only a small proportion of the population of strain X 190 contained non-sensitive mitochondrial DNA, then initially

there would be induction of petites at a high rate. There would also be selection for cells containing non-sensitive mitochondrial DNA since the cultural conditions are selective for respiratory competant types. Eventually, during extended cultivation, the population would consist entirely of cells which would contain non-sensitive mitochondrial DNA. This scheme would explain the changes in the population during continuous cultivation of strain X 190 at temperatures between 15°C and 21°C. The orange variant is also respiratory deficient, but it different to petite, and could also arise from the mutagenic effect of temperature. However, these proposals are not supported by the results of the recycling experiments. When the sample was subcultured in glycerol at 30°C and then recycled at a lower temperature one would not expect to find petites or orange variants induced to any considerable extent. In practice, however, considerable proportions of petite and orange variants were observed. The mitochondrial DNA should be homogeneous in its response to temperature in this culture, since 'de novo' synthesis of mitochondrial DNA has not been observed, and non-sensitive mitochondrial DNA is the only template for replication of functional mitochondrial DNA. Thus, although this scheme does explain the population changes during continuous cultivation of strain X 190 at reduced temperatures, considerable doubt is cast on its validity by the results of the recycling experiments.

(2) The possibility exists that the population changes observed in these continuous cultures of strain X 190 are the result of the forward and back mutation (reversion) of the cytoplasmic determinant, mitochondrial DNA. The temeprature effect could be mutagenic and cause 'nonsense' or 'missense' mitochondrial DNA, and consequently petites, to be formed. The ultimate fall in the level of petites in the population, with the concurrent

rise in the level of respiratory competent parent type, could be the result of reversion to functional mitochondrial DNA and respiratory competency. Many attempts have been made to detect reversion in respiratory deficient diploid (Ephrussi, 1953) and haploid petites (Raut, 1954) but all but one have failed. Kraepelin (1964) reported reversion of stable petites of Saccharomyces cerevisiae to respiratory competency. Reversion of some petites of other Saccharomyces species has also been reported (Nagai, 1969), but apparently these petites were unstable anyway.

It was found that both spontaneous and temperature-induced petites of strain X 190 gave rise to respiratory deficient orange variants when cultivated for 8 - 10 days in a Chemostat at 18°C. This orange variant, when similarly cultivated at 18°C, gave rise to a few red colonies. However, only one half-sectored and two complete red colonies were detected in a total of 600 colonies from over 10 days' samples. These colonies, when subcultured, were found to be respiratory competent, and on sporulation and dissection gave typical segregation of marker genes of strain X 190. Thus, there appears little doubt that reversion of petite to respiratory competent parent type took place, for even in the unlikely event that the two red X 190 type colonies appeared on the plates by contamination, firm evidence of reversion was provided by the half-sectored colony. Moreover, the process seems to take place in two steps, reversion from petite to a stable orange variant, and reversion from the stable orange variant to parent type. Assuming that reduced temperature has a progressive mutagenic effect on mitochondrial DNA the lower the temperature, mutagenesis and reversion of mitochondrial DNA would explain the population changes during continuous cultivation of strain X 190 at temperatures between 15°C and 25°C, and also possibly the origin of the orange variant. Mutation of

of mitochondrial DNA could result in the formation of the unstable type of orange variant which, although respiratory deficient, has growth rate much greater than that of petite and only slightly less than that of the respiratory competent parent type. It was not possible to obtain this orange variant in pure culture as, on cultivation at 30°C, parent type, petites, ad_8 recombinants and ad_2 revertants were all isolated. The orange variant isolated from petite strains of X 190 was very stable when cultivated at 30°C and the only variants isolated were a few petites. This stable orange variant could have arisen as the result of the partial repair of mitochondrial DNA which gave a variant which, although respiratory deficient, had a greater growth rate than the petite and was stable in the same manner as the petite. The characteristics of colour, colony size, and respiratory deficiency were common to both types of orange variant. The results suggest that both types of orange variant are intermediate, although different so, between, parent type and petite. If the mutation which results in petite mitochonrial DNA is 'missense' rather than 'nonsense' then there seems to be no reason why reversion of mitochondrial DNA, similar to reversion of chromosomal DNA, should not occur. A 'missense' mutation is the result of the replacement of a codon, specifying one amino acid, with a codon specifying another amino acid. The result of a 'missense' mutation is the synthesis of an altered protein which may retain some biological activity. A 'nonsense' mutation is the result of a change to a codon which does not specify any amino acid. A 'nonsense' mutation caused the reading of mRNA to cease and results in an incomplete polypetide being released. It has been assumed that the mutation to the petite state is the result of a 'nonsense' mutation, because petites are so stable and because the buoyant density of mitochondrial DNA is altered (Mounolou et al., 1966). However,

it has been suggested that only part of yeast mitochondrial DNA is concerned with respiration (Slonimski et al., 1968) and an analysis of similar respiratory deficiency (mi mutants(in Neurospera species has shown that the ultimate effect is the replacement of a single amino acid in a structural protein (named MSP) produced by mitochondrial DNA (Woodward and Munkres, 1966). Hence the evidence of Kraepelin (1964) and these results, suggest that the petite mutation may be a 'missense' mutation and that reversion to a respiratory competent state, although rare, is possible.

Reversion of petites, coupled with selection, could explain the ultimate rise in the level of parent type, and would also explain the rise in the proportion of orange variants when these changes were taking place. Since the 'revertants' arose at 18°C they were stable at this temperature and hence stability of the populations recycled at one temperature (18°C) would be explained. But the instability of recycling populations at 30°C and then at 18°C is not explained. Further doubt is cast on these proposals by the population changes observed in strain X 190 when grown in glycerol medium at 18°C. One would expect to find an equilibrium formed between parent type, petite, and orange variants in this medium. However, no such equilibrium was observed and, moreover, the rise in the level of parent type occurred after only 4 days. Since reversion from petite via orange variant to parent type was found to be a rare event and occurred only after a considerable period of cultivation, it seems unlikely that the rise in the level of strain X 190 parent type in glycerol medium can be attributed to reversion alone. Moreover, selection for parent type 'revertants' against stable orange variants was not observed during continuous cultivation. This evidence provides strong arguement against

reversion of petites as the explanation of the population changes continuous cultivation of strain X 190.

(3) The third possible explanation of the population changes in continuous cultivation of strain X 190 is concerned with the rate of replication of mitochondrial DNA. Studies on a number of organisms have suggested that replication of mitochondrial DNA does not occur synchronously with replication of nuclear DNA. This view has been supported by similar investigations in Saccharomyces lactis (Smith et al., 1968) and Saccharomyces cerevisiae (Williamson and Moustacchi, unpublished). In a synchronised culture of Saccharomyces lactis it was found that replication of mitochondrial DNA was periodic but was not concurrent with the replication of chromosomal DNA. The rate of cell division and thus chromosomal DNA decreases in strain X 190 as the temperature falls below the optimum growth temperature of 30°C. Similarly one would expect the rate of replication of mitochondrial DNA to fall. However, the temperature may act differentially on the two rates of replication, and the rate of replication of mitochondrial DNA could be reduced more than the rate of replication of chromosomal DNA. If this were true, then one would expect to find that an increasing proportion of the population would be respiratory deficient the lower the temperature because fewer of the daughter cells would contain functional mitochondria and mitochondrial DNA. The precise relationship between structure and function of mitochondria and mitochondrial DNA has not yet been clearly extablished. Although it has not been clearly demonstrated that mitochondria cannot function in the absence of mitochondrial DNA, it seems unlikely as mitochondrial DNA carries information for the formation of cytochromes a + a_3 and b, which are an integral part of the inner membranes of the mitochonrion. (Prezbindowski et al., 1968).

However, there may not be a one mitochondrion to one mitochonrial DNA molecule relationship and, indeed, there is evidence to suggest that the number of mitochondria in a cell is considerably greater than the number of molecules of mitochondrial DNA (Maroudas and Wilkie, 1968; Allen and MacQuillan, 1969).

If the rate of replication of mitochondrial DNA subsequently adapted to the rate of cell division at the lower temperatures, then the proportion of petite daughter cells would decrease. This scheme would explain the population changes in strain X 190 during continuous cultivation at temperatures between 15°C and 21°C. It is similar to that proposed by Sherman (1959) to explain somewhat similar observations at elevated temperatures. Sherman suggested that at supraoptimum temperatures the self-replicating particles, now considered to be mitochondrial DNA, either cease to reproduce, or, reproduce at a slower rate in relation to cell division. The studies at elevated temperatures were not pursued beyond 5 days cultivation, however, and consequently it was not observed whether the rate of replication of mitochondrial DNA adapted to the higher temperature.

This relatively simple scheme appears to offer the best explanation for the population changes in strain X 190 during continuous cultivation at temperatures between 15°C and 21°C. With the rate of replication of mitochondrial DNA slower than cell division at these temperatures a proportion of petites would be induced in the population initially. Subsequently replication of mitochondrial DNA increases as the result of some adaption process and the proportion of petites induced decreases. Moreover, selection for respiratory competent cells is taking place and consequently the proportion of petites already present in the population decreases. The results of the recycling experiments are also explained,

as adaptation to different temperatures was not necessary in one case and petites were not induced. In the other case, where subculturing took place at 30°C followed by recycling at 18°C, replication of mitochondrial DNA had to adapt to two temperatures, and consequently petites were induced in the population on recycling at 18°C. These proposals do not, however, explain the origin of the two types of orange variant, nor do they explain the population changes observed at 25°C.

The complete explanation of these results could involve a combination of these three schemes. The effect of infra-optimum temperatures may be two-fold, (1) a mutagenic effect which results in 'missense' mutation of mitochondrial DNA, resulting in the production of the unstable orange variant and the petite mutant, and (2) a differential effect on the rates of replication of mitochondrial DNA and chromosomal DNA resulting in a proportion of the progeny being respiratory deficient, the rate of replication of mitochondrial DNA subsequently adapting to the temperature and the rate of cell division.

The recycling experiments showed that cultures of strain X 190 could be stabilised with respect to petite production at a specific temperature if they were always maintained at that temperature. If a culture was subcultured at another (higher) temperature during a recycling experiment and was subsequently recycled at a lower temperature, it again became unstable. This was supported by the observations that temperatures of 18°C and 30°C did not induce petites in cultures of commercial strains of yeast. This result, however, is not surprising since these yeasts would have been selected for their stability at brewing temperatures close to 18°C, while still having an optimum growth temperature about 30°C.

A recurring problem encountered during continuous cultivation of

strain X 190 was flocculation. Strain X 190 is a non-flocculent strain of yeast but on many occasions, particularly at 25°C, continuous cultures of strain X 190 became flocculent. At all temperatures cultures of strain X 190 were, in effect, mixed cultures with parent type, orange variant, and petites, present in the population. Both parent type and petite became flocculent spontaneously, although simultaneous flocculation of both types was not observed. The unstable orange variant arising directly from parent type did not flocculate, but spontaneous flocculation of the stable orange variant arising from petite was observed. The direct consequence of flocculation in strain X 190 was that population studies could no longer be performed, since selection occurred for the flocculent type in the population and since the proportion of cells in the population involved in the flocs could not be determined. The whole basis of these population studies was that the sample plated out reflected accurately the population composition of the culture. Once flocculation occurred this was no longer true. Although flocculence is genetically controlled (Thorne, 1951; Gilliland, 1951), changes in the environmental conditions can also cause flocculation (Rainbow, 1966). In a preliminary genetic investigation of flocculence in strain X 190, sporulation and dissection gave tetrads, in which, all segregants were red, flocculent, and adenine-requiring, and had no other growth requirements. These segregants were, in turn, sporulated and dissected and, although spore viability was poor and no complete tetrads were obtained, all the viable segregants were non-flocculent. Since simultaneous flocculation of all types in the population was not observed, and since flocculation did not occur in all the experiments, flocculation due to environmental conditions, although they were constant, was unlikely. The results of tetrad analysis suggest that flocculence in strain X 190 is

associated with aneuploidy although any firm conclusions would require further data for confirmation.

Genetical stability of microorganisms is desirable in the vast majority of experiments and processes, especially in those which are extended over considerable periods of vegetative growth. Although gene mutation is the main source of genetic variation in microorganisms, in some fungi such as Saccharomyces cerevisiae, Aspergillus nidulans, and Ustilago maydis, there is another process which gives rise to comparable or greater variation during vegetative growth. Mitotic segregation of genes has been observed in these organisms and several mechanisms have been proposed to explain a variety of results (James and Lee-Whiting, 1955; Pontecorvo et al., 1954; Wilkie and Lewis, 1963; Hurst and Fogel, 1964; Holliday, 1964). However, practically all the investigations of mitotic segregation have been studies of induced segregation. The spontaneous rate of mitotic segregation and its importance in genetic stability of cultures during extended growth has been largely neglected. The spontaneous rate of mitotic recombination was estimated for the ad_3, ad_6 and ad_8 mutations in strain X T5, and for the ad_{5-7} mutation in strain X T14. These rates were, for ad_3, $4.0 \pm 0.8 \times 10^{-5}$, for ad_{5-7} $1.4 \pm 0.8 \times 10^{-4}$, and for ad_8 $3.5 \pm 0.7 \times 10^{-4}$ per cell per generation. The spontaneous rate for the ad_6 mutation was apparently in the order of less than 10^{-5} per cell per generation. There are no published forward mutation rates (Ad ad) for these genes, but some evidence from other studies (Roman, 1956) suggest that the mutation rates are much lower than the mitotic recombination rates given above. These ralatively high rates means that the probability of genetic variation due to mitotic recombination is much higher than that due to mutation during extended cultivation. It is significant that these relatively high recombination rates

were for non centromere-linked genes and, in the case of the ad_3 and ad_8 mutations especially, genes probably near the extremity of their respective chromosome arms. On the other hand the ad_6 mutation is an example of a centromere-linked gene, and the lower rate of recombination is consistent with the idea that, the rate increases as the distance from the centromere increases.

Selection plays an important role in determining the extent of variation due to mitotic recombination during extended cultivation. It was seen during extended cultivation of strain X T5 that there was selection for adenine recombinants, and ultimately they formed 99% of the population. Selection against these recombinants would have meant that they probably would not have been detected and would have had little effect on the stability of the population. The exact mechanism of spontaneous mitotic segregation of genes has not been elucidated but in some of the suggested mechanisms reciprocal recombinants are produced, e.g. recombination at the heterozygous Ad_8/ad_8 locus would result in the homozygous recombinants Ad_8/Ad_8 and ad_8/ad_8. Without selection, and neglecting effects of mutations resulting in the homozygous condition being converted into the heterozygous condition, each recombinant should ultimately form 50% of the population. However, selection for either recombinant would result in the population becoming increasingly uniform, since only through further mutation would variation due to recombination at the Ad_8/ad_8 locus be possible.

Mitotic chromosomal mechanisms do not result in recombination if the genes are homozygous. However, there may well be a fair degree of heterozygosis in most laboratory and commercial strains of yeast. Moreover, it may be beneficial to hybridise new strains of yeast for laboratory and commercial processes, and since hybridisation is likely to increase the

degree of heterozygosis, the likelihood of mitotic segregation and consequent variation will be greater. The frequency of segregation will depend on the proximity of loci to their centromeres. The variation may be unimportant, but if segregation occurs at a locus controlling a characteristic involved in a commercial process, or an academic investigation then the consequences could be very significant.

S U M M A R Y

(1) The genetic stability of several strains of yeast during extended cultivation at temperatures between 15°C and 30°C was examined.

(2) Respiratory deficient petite mutants were induced in diploid strain X 190 by temperatures between 15°C and 21°C. Similar mutants were induced by a temperature of 15°C in a second diploid strain, X T5, and in two haploid strains, S 91 and S 110. Use of glycerol medium selective against petite mutants showed the effect to be due to induction and not selection. Petite mutants were not induced by temperatures of 18°C and 30°C in two commercial strains of brewers' yeast.

(3) A respiratory deficient (non-petite) orange variant was induced in strain X 190 by temperatures between 15°C and 25°C. This orange variant was found to be very unstable, with a high rate of reversion to respiratory-competent parent type.

(4) During continuous cultivation of strain X 190 at temperatures of 15°C, 18°C, and 21°C, petites were induced at a high rate and quickly formed approximately 90% of the population. Subsequently, however, the proportion of parent type cells increased and ultimately formed over 90% of the population. In recycling this population at the same temperature, e.g. 18°C, it was found to be stable. If, however, the sample was subcultured for a short time at 30°C and then recycled petite mutants and orange variants were again induced in the population.

(5) When spontaneous and temperature-induced petites of strain X 190 were continuously cultivated at 18°C, a stable respiratory deficient orange variant (unlike the unstable orange variant obtained directly from strain X 190) appeared. Selection for this orange variant occurred and it quickly became predominant in the population. Its continuous

cultivation at 18°C led to the isolation of a few respiratory competent colonies of X 190 genotype. This suggested that reversion from respiratory deficient petite mutant to respiratory competent parent type had taken place

(6) Three models were proposed to explain the population changes observed during continuous culture of strain X 190 at temperatures between 15°C and 21°C.

(a) Mitochondrial DNA is heterogeneous and a small fraction is not susceptible to the inductive effect of these temperatures. Consequently there is a high rate of petite induction initially, followed by selection for the fraction of cells which contain temperature-stable mitochondrial DNA. Eventually these respiratory competent cells form the major proportion of the population.

(b) The rate of replication of mitochondrial DNA is decreased more than that of nuclear DNA by reduced temperature. Consequently petites are induced in the population, subsequent adaptation of replication of mitochondrial DNA to the same rate as nuclear DNA, leads to more respiratory competent cells being produced.

(c) After initial induction of petites in the population, reversion to respiratory competency takes place. These respiratory competent revertants are selected for in the culture.

(7) A preliminary genetic investigation of flocculence in strain X 190 in continuous culture suggested that this property was associated with polyploidy or aneuploidy in this strain.

(8) The rates of mitotic recombination for the ad_3, ad_{5-7} and ad_8 loci were estimated in two Saccharomyces hybrid strains during extended serial transfer cultivation at 30°C. The rates were for ad_3 4.0 ±

0.8×10^{-5}, for ad_{5-7} $1.4 \pm 0.8 \times 10^{-4}$, for ad_8 $3.5 \pm 0.7 \times 10^{-4}$ per cell per generation.

REFERENCES

ALLEN, N.E., MACQUILLAN, A.M. 1969. Target analysis of mitochondrial genetic units in yeast. J. Bact. 97 pp. 1142 - 1148

ATWOOD, K.C., SCHNEIDER, L.K., RYAN, F.J. 1951. Selective mechanisms in Bacteria. Cld. Spr. Harb. Symp. Quant. Biol. 16 pp. 345 - 355

AVERS, C.J., RANCOURT, M.W., FU HAI LIN. 1965. Intracellular mitochondrial diversity in various strains of Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. 54 pp. 527 - 535

AVERS, C.J., PFEFFER, C.R., RANCOURT, M.W. 1965. Acriflavine induction of different kinds of petite mitochondrial populations in S. cerevisiae J. Bact. 90 2 pp. 481 - 494

AVERS, C.J., 1967. Heterogeneous length distribution of circular DNA filaments from yeast mitochondria. Proc. Nat. Aca. Sci. 58 pp. 620 - 627

AVERS, C.J., BILLHEIMER, F.E., HOFFMANN, H.P., PAULI, R.M. 1968. Circularity of yeast mitochondrial DNA. Proc. Nat. Acad. Sci. 61 pp. 90 - 97

BECCARI, E., MODIGLIANI, P., MORPURGO, G. 1967. Induction of inter and intragenic mitotic recombination by Fluorodeoxyuridine and Fluorouracil in Aspergillus nidulans. Genetics 56 pp. 7 - 12

BULDER, C.J.E.A. 1964(a). Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts. Ant. van. Lee. 30 pp. 1 - 9

BULDER, C.J.E.A. 1964(b). Lethality of the petite mutation in petite negative yeasts. Ant. van. Lee. 30 pp. 442 - 454

BEN-PORAT, T., REISSI, M., KAPLAN, A.S. 1961. Effect of Mitomycin C on the synthesis of infective virus and deoxyribonucleic acid in Pseudorabies virus-infected Rabbit Kidney cells. Nature 190 pp. 33 - 34

BERNARDI, G., CARNEVALI, F., NICOLAIEFF, A., PIPERNO, G., TECCE, G. 1968. Separation and characterization of a satellite DNA from a yeast cytoplasmic 'petite' mutant. J. Mol. Biol. 37 pp. 493 - 505

BEVAN, E.A., 1960. The Genetics of Fungi. Biology and Human Affairs 25 p. 17

CAPALDO, F., MANNEY, T.R. 1968. Radiation Induced Mitotic Recombination in Yeast: High Negative Interference. Bact. Proc. GP 27 pp. 50 - 51

CHEN, S.Y., EPHRUSSI, B., HOTTINGUER, H. 1950. Nature genetique des mutants a dificience respiratoire de la souche B - 11 de la levure de boulangerie. Heredity 4 pp. 337 - 351

CLARK-WALKER, G.D., LINNANE, A.W. 1967. The Biogenesis of Mitochondria in S. cerevisiae. A comparison between cytoplasmic RD mutant yeast and chloramphenicol inhibited wild type cells. J. Cell Biol. 34 pp. 1 - 14

CORNEO, G., MOORE, C., SANADI, D.R., GROSSMAN, L., MARMUR, J. 1966. Mitochondrial DNA in yeast and some mammalian species. Science 151 pp. 687 - 689

COX, B.S., BEVAN, E.A., 1962. Aneuploidy in Yeast. New Phytol 61 pp. 342 - 355

EMEIS, C.C. 1961. Stability of diploid and polyploid culture yeast strains. Proc. Eur. Brew. Conv. Vienna. p. 205

EMERSON, S. 1956. Notes on the identification of different causes of aberrant tetrad ratios in Saccharomyces. C.R. Lab. Carlsberg. ser Physiol. 26 p. 71

EPHRUSSI, B., HOTTINGUER, H., TAVLITZKI, J. 1949. Action de l'acrivlavine sur les levures. II Etude genetique du mutant 'petite colinie'. Ann. Inst. Pasteur. 76 pp. 419 - 450

EPHRUSSI, B., HOTTINGUER, H. 1951. On an unstable cell state in yeast. Cold Spring Harbour Symposium in Quant. Biol. 16 pp. 75 - 84

EPHRUSSI, B. 1953. Nucleo-Cytoplasmic Relations in Microorganisms. Clarendon Press, Oxford.

EPHRUSSI, B., MARGERIE-HOTTINGUER, H., ROMAN, H. 1955. Suppressiveness: a new factor in the genetic determination of the synthesis of respiratory enzymes in yeast. Proc. Nat. Acad. Sci. U.S. 41 p. 1065

EPHRUSSI, B., JAKOB, H., GRANDCHAMP, S. 1966. Etudes sur la suppressivite des mutants a deficience respiratoire de la levure. Genetics 54 pp. 1 - 29

FEDERMAN, M., AVERS, C.J. 1967. Fine structure analysis of intercellular and intracellular mitochondrial diversity in Saccharomyces cerevisiae J. Bact. 94 pp. 1236 - 1243

FOGEL, S., HURST, D.D., 1963. Coincidence relations between gene conversion and mitotic recombination in Saccharomyces. Genetics 48 pp. 321 - 328

FOWELL, R.R. 1952. Sodium acetate agar as a sporulation medium for yeast. Nature 170 p. 578

FRATELLO, B., MORPURGO, G., SERMONTI, G., 1960. Induced somatic segregation in Aspergillus nidulans. Genetics 45 pp. 785 - 800

HAWTHORNE, D.C., MORTIMER, R.K. 1960. Chromosome mapping in Saccharomyces centromere linked genes. Genetics 45 pp. 1085 - 1110

HOLLIDAY, R. 1961. Induced mitotic crossing over in Ustilago maydis. Genet. Res. 2 pp. 231 - 248

HOLLIDAY, R. 1962. Effect of photoreactivation on ultra-violet-induced segregation of heterozygous diploids. Nature 193 pp. 95 - 96

HOLLIDAY, R. 1964. The induction of mitotic recombination by Mitomycin C in Ustilago. Genetics 50 pp. 325 - 335

HUANG, M., BIGGS, D.R., CLARK-WALKER, G.D., LINNANE, A.W. 1966. Chloramphenicol inhibition of the formation of particulate mitochondrial enzymes of S. cerevisiae. Biochmy. Biophys. Acta. 114 pp. 434 - 436

HURST, D.D.,FOGEL, S. 1964. Mitotic recombination and heteroallelic repair in Saccharomyces cerevisiae. Genetics 50 pp. 435 - 458

JAKOB, H. 1965. Complementation entre mutants a deficience respiratoire de Saccharomyces cerevisiae establissement et relulation dans la zygote et dans leur proche descendenc. Genetics 52 p. 75

JAMES, A.P. 1954. Relative frequencies of sectored and non sectored mutant colonies in yeast as a function of ultraviolet dose. J. Bact. 67 pp. 237 - 242

JAMES, A.P., LEE-WHITING, B. 1955. Radiation induced genetic segregantions, in vegetative cells of diploid yeast. Genetics 40 pp. 826 - 831

JOHNSTON, J.R., MACKINNON, J. 1966. Mitotic segregation of ad_8 in diploid and tetraploid Saccharomyces. Proc. 2nd Symp. Yeasts. Bratislava.

JOHNSTON, J.R., MORTIMER, R.K. 1959. Use of snail juice digestive enzyme in isolation of yeast spore tetrads. J. Bact. 78 p. 292

KAFER, E. 1963. Radiation effects and mitotic recombination in diploids of Aspergillus nidulans. Genetics 48 pp. 27 - 45

KONO, T., ASAI, T. 1969. Kinetics of Continuous Cultivation. Biotechnol Bioeng. 11 pp. 19 - 36

KRAEPELIN, G. 1964. Normalisierung des atmungsdefetes bei hefe ruckfuhring stabilisierter RD - mutanten in voll atmungsfanige normalizellen. Arch. Mikrobiol. 48 pp. 299 - 305

LASKOWSKI, W. 1954. Induction par le enlorure de tetrazolium, de la mutation 'petite colonie' chez la levure. Heredity 8 pp. 79 - 88

LEDERBERG, J., LEDERBERG, E.M. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. 63 pp. 399 - 406

LINDEGREN, C.C. 1949. The Yeast Cell, its Genetics and Cytology. Educational Publishers Inc. St. Louis, Missouri.

LINDEGREN, C., NAGAI, S., NAGAI, H. 1958. Induction of Respiration Deficiency in yeast by manganese, copper, cobalt, and nickel. Nature 182 p. 446

MARCOVITCH, H. 1951. Action de l'acriflavine sur les levres VIII determination du composant actif et etude de l'enflavine. Ann. Inst. Pasteur. 81 p. 452

MAROUDAS, N.G., WILKIE, D. 1968. Ultraviolet irradiation studies on the cytoplasmic determinant of the yeast mitochondrion. Biochim. Biophys. Acta. 166 pp. 681 - 688

MCLARY, D.O., NULTY, W.L., MILLER, G.R. 1959. Effects of potassium versus sodium in the sporulation of Saccharomyces. J. Bacteriol 78 pp. 362 - 368

MODOD, J. 1950. La Technique de Culture Continue. Ann. Inst. Pasteur. 79 pp. 390 - 410

MORTIMER, R.K., HAWTHORNE, D.C. 1966. Genetic Mapping in Saccharomyces. Genetics 53 pp. 165 - 173

MORPURGO, G. 1963. Induction of mitotic crossing over in Aspergillus nidulans by bifunctional alkylating agents. Genetics 48 pp. 1259 - 1263

MOUNOLOU, J.C., JAKOB, H., SLONOMSKI, P.P. 1966. Mitochondrial DNA from yeast 'petite' mutants: specific changes of buoyant density corresponding to different cytoplasmic mutations. Biochem, Biophys. Res. Com. 24 pp. 218 - 224

MOUSTACCHI, E., WILLIAMSON, D.H. 1966. Physiological variations in satellite components of yeast DNA detected by density gradient centrifugation. Biochem. Biophys. Res. Com. 23 pp. 56 - 61

NAGAI, S., NAGAI, H. 1958(b). Demonstration of the induction of respiration deficient yeast by manganese using a selective culture medium. Naturwissenschaften 45 pp. 441 - 442

NAGAI, S., NAGAI, H. 1958(a). Relation of respiratory competence in yeast to colour development and resistance to copper. Exptl. Cell Research 15 pp. 623 - 624

NAGAI, S. 1969. Unstable and reversible forms of hereditary respiration deficiency in Saccharomyces logos and S. rosei. Ant. van Leewen. 35 Supplement: Yeast Symposium.

NASS, M.M., NASS, S., AFZELIUS, B. 1965. The general occurrence of mitochondrial DNA. Exptl. Cell Res. 37 pp. 516 - 539

NEGROTTI, T., WILKIE, D. 1968. Induction of RD by repression of the respiratory system in a mutant of S. cerevisiae. Biochem. Biophys. Acta. 153 pp. 341 - 349

NOVICK, A., SZILARD, L. 1950. Experiments with the Chemostat on Spontaneous Mutations of Bacteria. Proc. Nat. Acad. Sci. 36 pp. 708 - 719

OGUR, M., ST. JOHN, R. 1956. A differential and diagnostic plating method for population studies of respiration deficiency in yeast. J. Bact. 72 pp. 500 - 504

OGUR, M., ST. JOHN, R., NAGAI, S. 1957. Tetrazolium overlay technique for population studies of respiration deficiency in yeast. Science 125 pp. 928 - 929

OGUR, M., ST. JOHN, R., OGUR, S. 1959. Direct experimental observation of cells in phenomic lag. Science 129 pp. 1612 - 1613

OGUR, M., OGUR, S., ST. JOHN, R. 1960. Temperature dependence of the spontaneous mutation rate to respiration deficiency in Saccharomyces. Genetics 45 pp. 189 - 194

PEYNAUD, I.E. 1967. Wine Making by Continuous Fermentation. Proc. Biochem. 2 pp. 44 - 46

PITTMAN, D., PEDIGO, P.R. 1959. Photoreactivation studies on yeast. I Ultra-violet inactivation and photoreactivation of respiration-sufficient and respiration-deficient yeasts. Exptl. Cell Research 17 pp. 359 - 367

PITTMAN, D., RANGANATHAN, B. 1959. Photoreactivation studies on yeast. II Photoreactivation of the ultra-violet damage producing respiratory deficiency in haploid and tetraploid yeasts. Exptl. Cell. Research. 17 pp. 368 - 377

POMPER, S., BURKHOLDER, P.R. 1949. Studies on the biochemical genetics of yeast. Proc. Nat. Acad. Sci. 35 pp. 456 - 464

PONTECORVO, G., TARR-GLOOR, E., FORBES, E. 1954. Analysis of mitotic recombination in Aspergillus nidulans. J. Genet. 52 pp. 226 - 237

PREZBINDOWSKI, K.S., RUZICKA, F.J. SUN, F.F., CRANE, F.L. 1968. A double layer of protein in mitochondrial christae. Biochem. Biophys. Res. Commun. 31 2 pp. 164 - 169

RABINOWITZ, M., GETZ, G., CASEY, J., SWIFT, H. 1969. Synthesis of mitochondrial and nuclear DNA in anaerobically grown yeast during the development of mitochondrial function in response to oxygen. J. Mol. Biol. 41 pp. 381 - 400

RAINBOW, C. 1966. Flocculation of brewer's yeast. Proc. Biochem. 1 pp. 489 - 492

RAUT, C. 1953. A cytochrome deficient mutant of Saccharomyces cerevisiae Exptl. Cell Research 4 pp. 295 - 305

RAUT, C. 1954. Hereditable non-genic changes in yeast by ultra-violet light. J. Cellular, Comp. Physiol 44 pp. 463 - 475

RAUT, C., SIMPSON, W.L. 1955. The effect of X-rays and of U.V. light of different wave-lengths on the production of cytochrome deficient yeasts. Arch. Biochem. Biophys. 57 pp. 218 - 228

RAUT-HEBB, C., SLEBODNIK, J. 1958. The effect of prior growth conditions on the kinetics of adaptive enzyme formation in yeast. Exptl. Cell Res. 14 pp. 286 - 294

REILLY, C., SHERMAN, F. 1965. Glucose repression of cytochrome a synthesis in cytochrome deficient mutants of yeast. Biochem. Biophys. Acta. 95 pp. 640 - 651

ROMAN, H., PHILLIPS, M.M. SANDS, S.M. 1955. Studies of Polyploid Saccharomyces I Tetraploid Segregation. Genetics 40 p. 546

ROMAN, H. 1956. A system selective for mutations affecting the synthesis of Adenine in Yeast. Compt. Rend. Trav. Lab. Carlsberg, Ser, Physiol. 26 pp. 299 - 314

ROODYN, D.B., WILKIE, D. 1967. A characteristic pattern of respiratory enzymes in cytoplasmic petite strains of S. cerevisiae as revealed by multiple enzyme analysis. Biochem. J. 103 pp. 3c - 5c

ROODYN, D.B., WILKIE, D. 1968. The Biogenisis of Mitochondrial. Methuen & Co., Ltd.

SAGER, R., RAMANIS, Z. 1965. Recombination of non chromosomal genes in Chlamydomonas. Proc. Nat. Acad. Sci. 53 pp. 1053 - 1061

SAGER, R., RAMANIS, Z. 1967. Biparental inheritance of non chromosomal genes induced by ultraviolet irradiation. Proc. Nat. Acad. Sci. 58 pp. 931 - 937

SAGER, R., RAMANIS, Z. 1968. The pattern of segregation of cytoplasmic genes in Chlamydomonas. Proc. Nat. Acad. Sci. 61 pp. 324 - 331

SARACHEY, A. 1958. The induction by ultra-violet radiation and the photo-reactivation of hereditable respiratory deficiency in Saccharomyces adapted and unadapted to aerobic respiration. Cytologia 23 pp. 143 - 158.

SCHATZ, G., HALSBRUNNER, E., TUPPY, H. 1964. DNA associated with Yeast Mitochondria. Biochem. Biophys. Res. Com. 15 pp. 127 - 132

SHERMAN, F. 1959. The effects of elevated temperatures on yeast. (II) Induction of respiratory-deficient mutants. Jour. Cell and Comp. Physiol. 54 pp. 37 - 52

SHERMAN, F. 1962. The relationship between respiration deficiency in yeast as determined with segregational mutants. Genetics 47(6) pp. 695 - 700

SHERMAN, F. 1963. Respiration deficient mutants of yeast I Genetics. Genetics 48 pp. 375 - 385

SHERMAN, F., ROMAN, H. 1963. Evidence for two types of allelic recombination in yeast. Genetics 48 pp. 255 - 261

SHERMAN, F., SLONIMSKI, P.P. 1964. Respiration deficient mutants of yeast. II Biochemistry. Biochem. et Biophys. Acta. 90 pp. 1 - 15

SHERMAN, F., STEWART, J.W., MARGOLIASH, E., PARKER, J., CAMPBELL, W. 1966. The structural gene for yeast cytochrome C. Proc. Nat. Acad. Sci. 55 pp. 1498 - 1504

SILVER, J.M., EATON, N.R. 1968. Biosynthetic blocks of eight non allelic adenine requiring mutants of Saccharomyces cerevisiae. Genetics 60 pp. 225 - 226

SLONIMSKI, P.P., PERRODIN, G., CROFT, J.H. 1968. Ethidium bromide induced mutation of yeast mitochondria: complete transformation of cells into respiratory-deficient non-chromosomal 'petites'. Biochem. Biophys. Res. Commun. 30 3 pp. 232 - 239

SMIRNOV, M.N., SMIRNOV, V.N., BUDOWSKY, E.I., IWGE-BECHTOMOV, S.G., SEREBRAJAKOV, N.G. 1967. Red pigment of adenine deficient yeast. Biophys. Biochem. Res. Com. 27 p. 299

SMITH, D., TAURO, P., SCHWEIZER, E., HALVORSON, H.O. 1968. The replication of mitochondrial DNA during the cell cycle in Saccharomyces lactis. Proc. Nat. Acad. Sci. 60 pp. 936 - 942

SMITH, D.G., MARCHANT, R., MAROUDAS, N.G., WILKIE, D. 1969. A comparative study of the mitochonrial structure of petite strains of Saccharomyces cerevisiae. J. Gen. Microbiol. 56 pp. 47 - 54

STERN, C. 1936. Somatic crossing over and segregation in Drosophila melanogaster. Genetics 21 pp. 625 - 730

STOCKER, B.A.D. 1949. Measurements of rate of mutation of flagellar antigenic phase in Salmonella typhi-murium. J. Hyg. 47 pp. 398 - 413

SUGIMURA, T., OKABE, K., NAGAO, M., GUNGE, N. 1966. A respiration deficient mutant of S. cerevisiae which accumulates porphyrins and lacks cytochromes. Biochem, Biophys. Acta. 115 pp. 267 - 275

SUGIMURA, T., OKABE, K., KODAMA, M. 1969. Induction of respiration-deficient mutant of Saccharomyces cerevisiae by pinacyanol. J. Bact. 97 pp. 964 - 965

TECTOR, M.A., KAFER, E. 1962. Radiation-induced chromosomal aberrations and lethals in Aspergillus nidulans. Science 136 pp. 1056 - 1057

TEWARI, K.K., JAYARAMAN, J., MAHLER, M.R. 1965. Separation and characterisation of mitochondrial DNA from yeast. Biochem. Biophys, Res. Commun. 21 pp. 141

TEWARI, K.K., VOTSCH, W., MAHLER, H.R., MACKLER, B. 1966. Biochemical correlates of respiratory deficiency: VI Mitochondrial DNA. J. Mol. Biol. 20 pp. 453 - 481

THOMAS, D.Y, WILKIE, D. 1968. Inhibition of mitochondrial synthesis in yeast by erythromycin: cytoplasmic and nuclear factors controlling resistance. Genet. Res. 11 1 pp. 33 - 41

THORNE, R.S.W. 1951. The Genetics of Flocculence in Saccharomyces cerevisiae. Proc. Eur. Brew. Conv., Brighton. p 21

WILKIE, D. 1963. The induction by monochromatic U.V. light of respiratory deficient mutants in aerobic cultures of yeast. J. Mol. Biol. 7 pp. 527 - 533

WILKIE, D., LEWIS, D. 1963. The effect of U.V. light on recombination in yeast. Genetics 48 pp. 1701 - 1716

WILKIE, D., SAUNDERS, G., LINNANE, A.W. 1967. Inhibition of respiratory enzyme synthesis in yeast by chloramphenicol: relationship between chloramph. tolerance and resistance to other antibacterial antibiotics. Genet. Res. 10 pp. 199 - 203

WILLIAMSON, D.H., SCOPES, A.W. 1962. Cell division and synthesis of macromolecules in synchronously dividing cultures of Saccharomyces cerevisiae. Proc. 22nd Intern. Congr. Intern. Union Physiol. Sci, Leiden 1 pp. 759 - 765

WINTERSBERGER, E. 1966. Occurrence of a DNA-polymerase in isolated yeast mitochondria. Biochem. Biophys. Res. Com. 25 pp. 1 - 7

WOODWARD, D.O., MUNKRES, K.D. 1966. Alterations of a maternally inherited mitochondrial structural protein in respiratory deficient strains of Neurospora. Proc. Nat. Acad. Sci. 55 pp. 872 - 880

YCAS, M. 1956. A hereditary cytochrome deficiency appearing in yeast grown at elevated temperature. Exp. Cell Res. 10 p. 746

YOTSUYANAGI, Y. 1962. Etudes sur la chondriome de la levure: Chondriomes des mutants a deficience respiratoire. J. Ultrastruct. Res. 7 pp. 141 - 158

A C K N O W L E D G E M E N T S

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PLATES

Plate 1. Strain X 190 population; showing red, parent type colonies, and white, adenine recombinant colonies, after 4 days growth at 30°C on MYGP agar.

Plate 2. Tetrazolium salt overlay technique for detecting respiratory deficient colonies. Respiratory sufficient colonies are red, and the single respiratory deficient colony shown (far right of plate) is white.

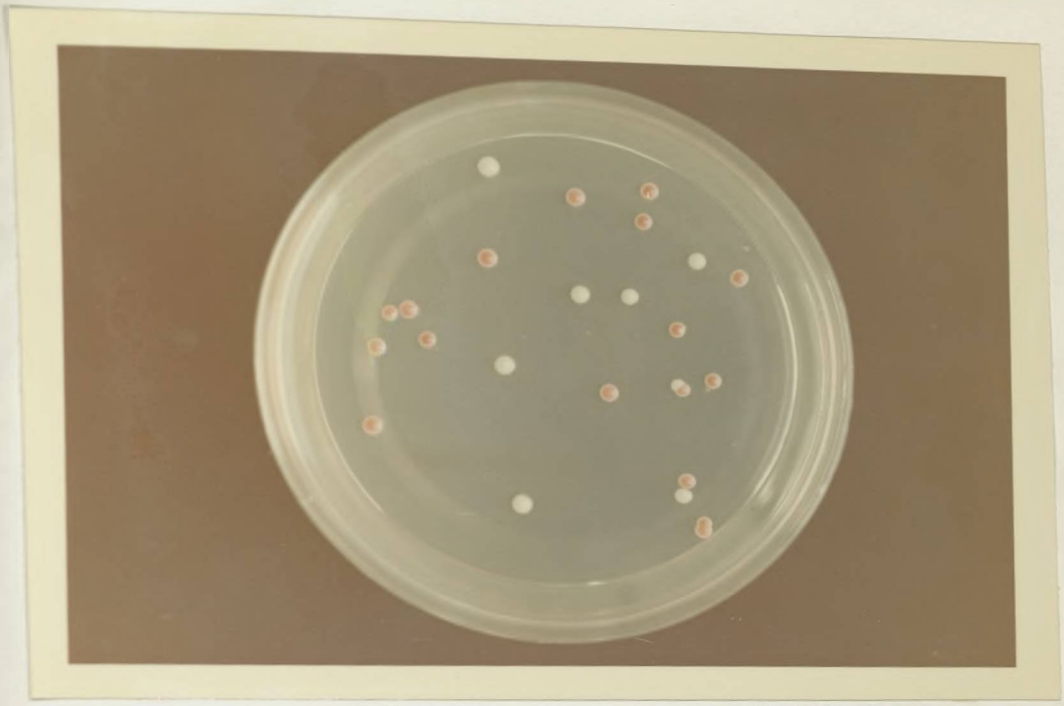

PLATE 1

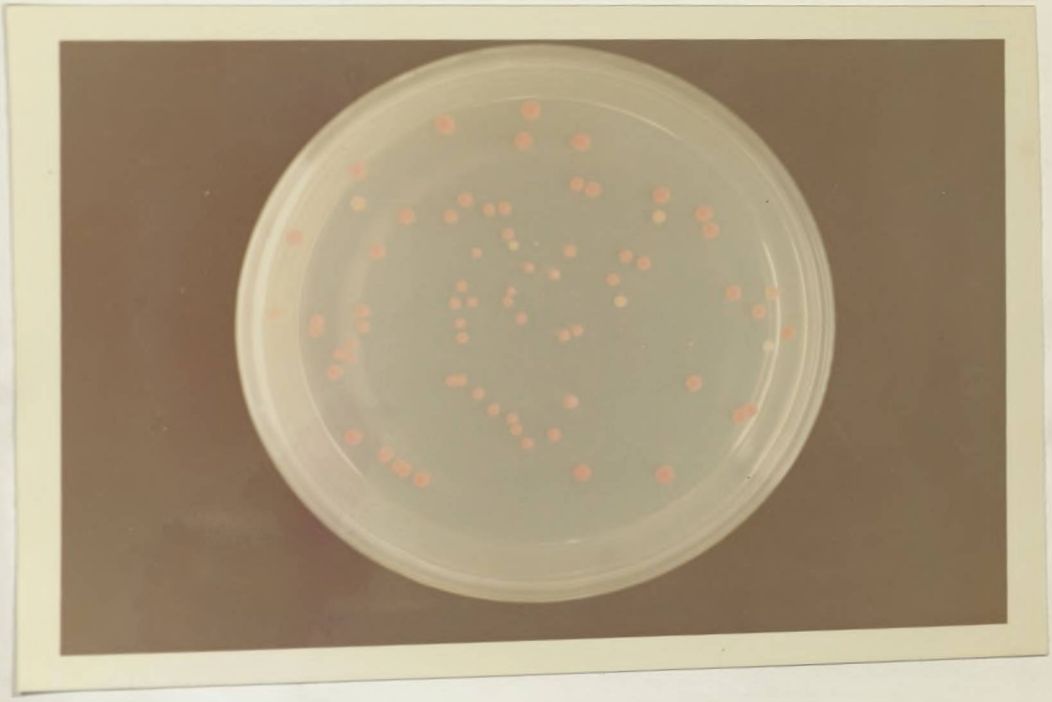

PLATE 2

Plate 3. Continous culture apparatus. From left to right the apparatus consists of: reservoir, peristaltic metering pump, culture vessel, effluent vessel, air pump.

Plate 4. Continuous culture vessel. The sintered glass air inlet, and the end of the overflow tube, can be seen below the clamp. The inlet connections, which are sealed during operation, can be seen above the clamp as can be the spring clip which holds the two parts of the vessel together.

PLATE 3

PLATE 4

Plate 5. Population of strain X 190 after 2 days cultivation at 18°C. Clearly visible are red parent type colonies, orange colonies, sectored red/orange colonies, and petite white colonies.

Plate 6. Normal strain X 190 population on MYGP agar after 4 days growth at 30°C.

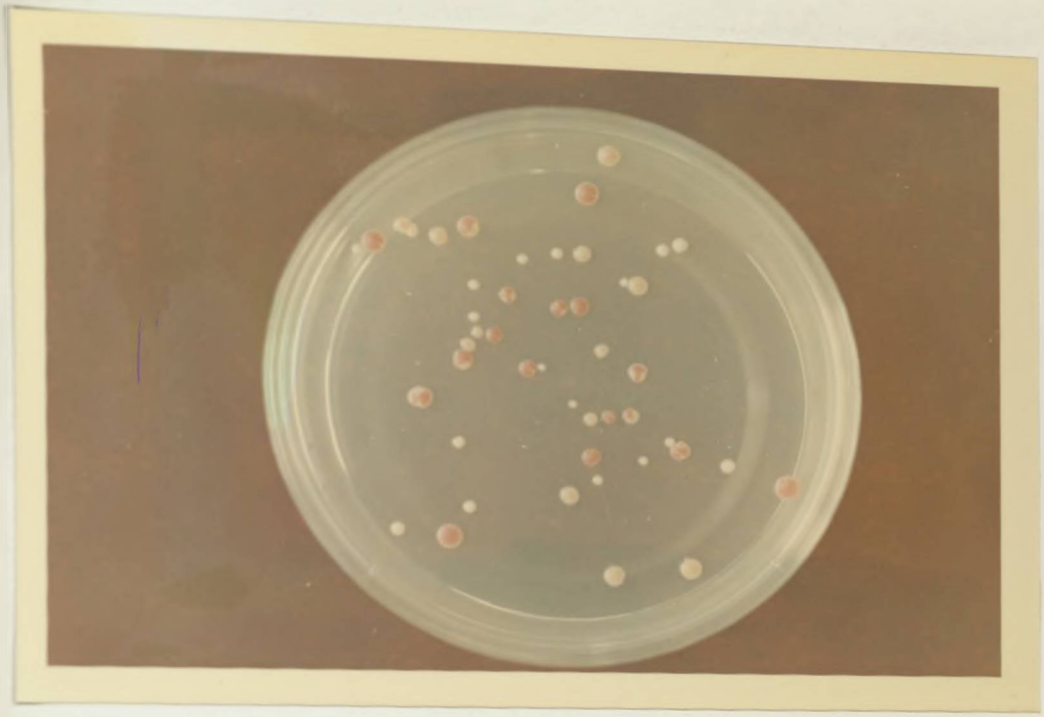

PLATE 5

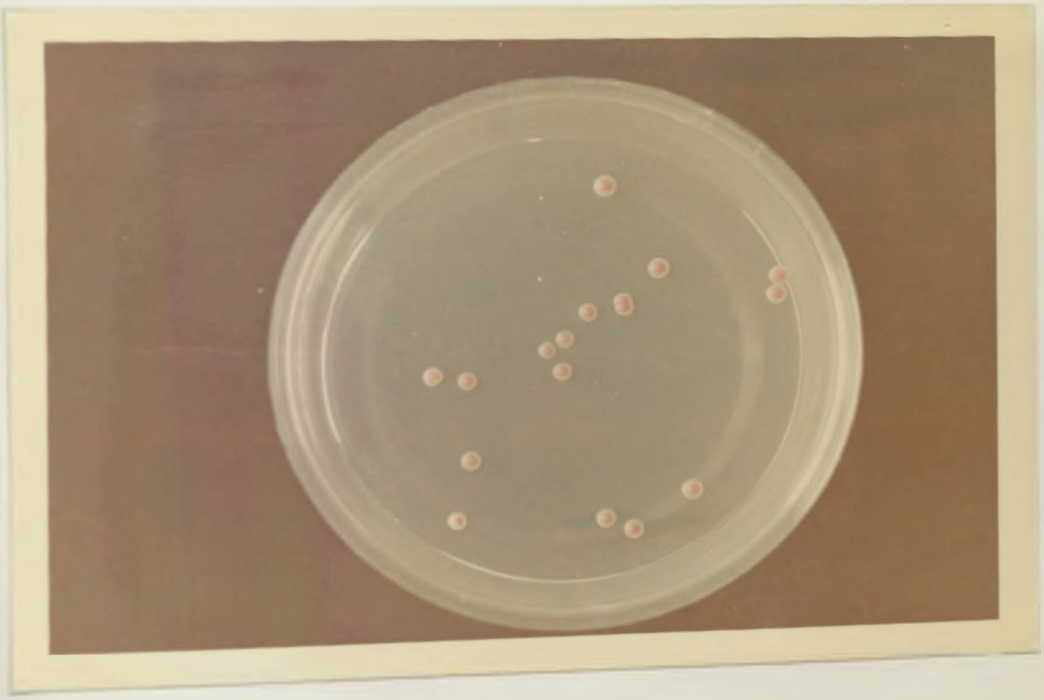

PLATE 6

Plate 7. Tetrazolium salt overlay of a strain X 190 population. Respiratory sufficient colonies and sectors are clearly distinguished by their bright red appearance. Respiratory deficient petite colonies are white. The reaction of orange colonies and sectors is not clear.

Plate 8. Population of strain X 190 temperature induced petites after continuous cultivation at 18°C for 10 days. The stable orange variants can be clearly distinguished, by colour and larger colony size, from the petite colonies. Note also that these orange colonies are paler in colour than those which arise directly from strain X 190 parent type.

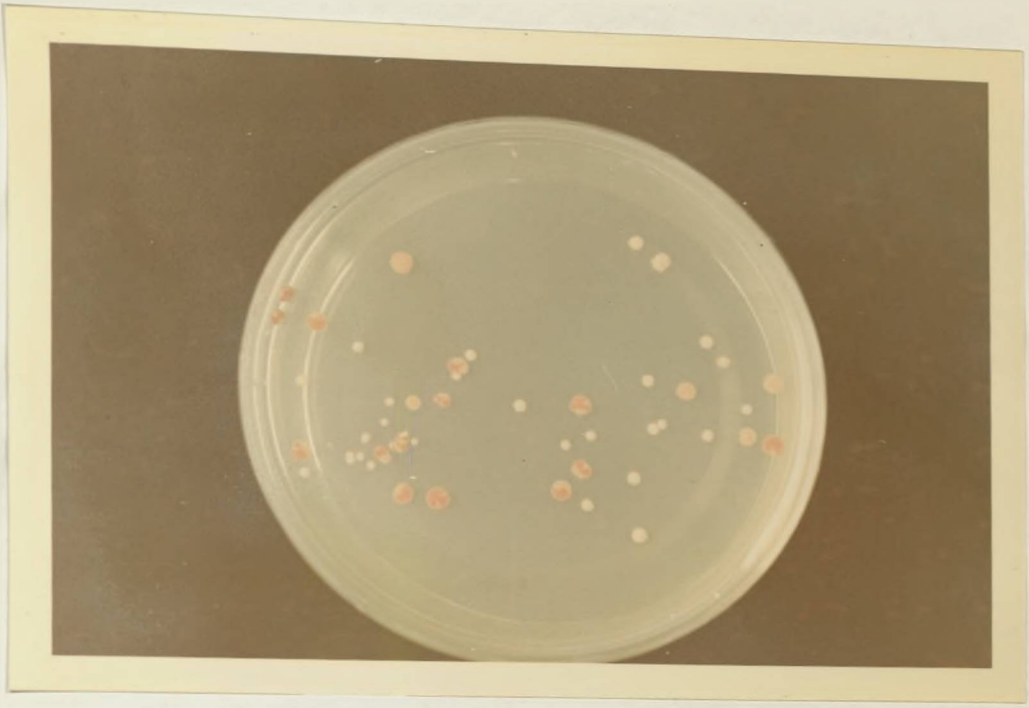

PLATE 7

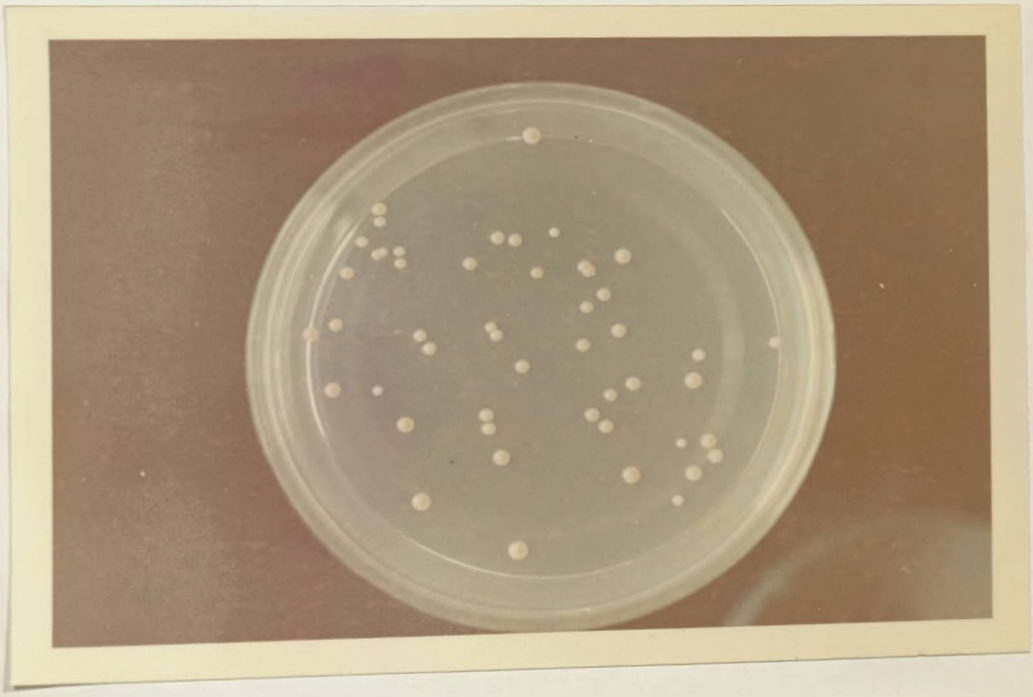

PLATE 8