Genetic control of DNA synthesis in *Bacillus subtilis*

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CONTENTS

Abstract

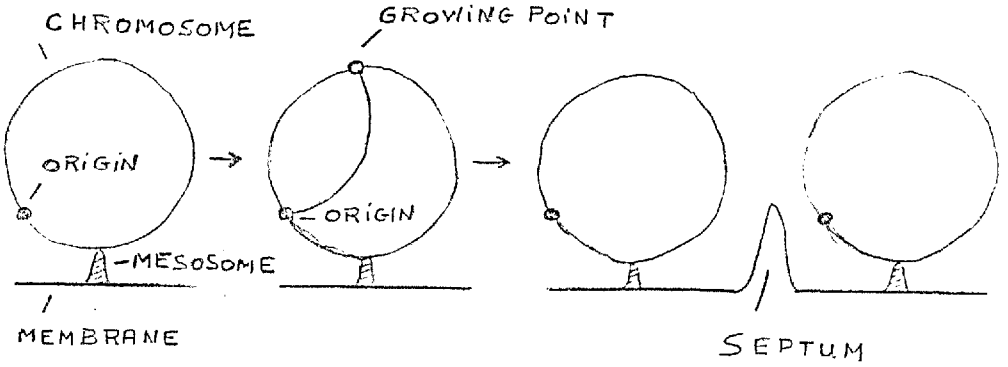
Over 600 temperature-sensitive mutants of B. subtilis were isolated at random from Nitroso-guanidine-treated cells. Mutants defective in DNA synthesis (ts DNA mutants) were identified by determining the ratio of the amount of protein to DNA synthesized at 45°C. Thirty-eight mutants were specifically inhibited in DNA synthesis. Genetic analysis by transformation of 29 of these ts DNA mutants shows that they are distributed in 4 small linkage groups designated A, B, C and D. By transduction it is shown that these groups are located in different regions of the B. subtilis genetic map.

The following physiological properties at 45°C of 27 ts DNA mutants were determined: residual DNA and protein synthesis, viability, PBSX induction and morphology. After cessation of DNA synthesis at 45°C mutant cells of groups A and C elongate but cannot divide, whereas B and D group mutant cells do divide and produce anucleate cells. Preliminary experiments suggest that at 45°C B and D group mutants complete all current rounds of replication but cannot initiate new ones.

1. INTRODUCTION

1.1. DNA replication in bacteria

DNA contains all the hereditary information of the cell. It directs all the activities of the cell. One of its roles is to secure its own replication. It would be interesting to know how DNA synthesis occurs and by what mechanism this process is controlled and co-ordinated with cell division. This problem has been extensively studied in bacteria, in particular in Escherichia coli and Bacillus subtilis. The results can be summarised in the general picture presented and described below.



The bacterial chromosome is a single giant double-stranded molecule of DNA (Cairns, 1963; Dennis and Wake, 1966). In E. coli the two ends are covalently linked (Davern, 1966) and the molecule forms a closed ring. There is no evidence so far that the B. subtilis chromosme is circular. The chromosome is attached to the membrane by the intermediary of a mesosome (Ryter and Jacob, 1966).

Replication starts at a well determined point of the chromosome called the origin (Yoshikawa and Sueoka, 1963; Abe and Tomisawa, 1967; Berg and Caro, 1967). Once initiated, the synthesis of new chains occurs at the growing point (Cairns, 1963; Bonhoeffer and Gierer, 1963) which progresses unidirectionally along the chromosome (Yoshikawa and Sueoka, 1963; Dubnau et al, 1967). The replication is semi-conservative (Meselson and Stahl, 1958). In other words it occurs as if the two complementary strands separated and the complement of each one laid down along it by specific base pairing. The end result is that the two daughter chromosomes are each formed of one parental and one newly synthesized strand. Up to a certain growth rate (see below), the genome replicates as one unit. In other words no new round of replication is initiated before the completion of the previous one (Meselson and Stahl, 1958; Yoshikawa and Sueoka, 1963).

The time needed for one round of replication is constant at a given temperature. It is independent of the growth rate. For E. coli at 37°C one round of replication takes about 40 min. (Helmstetter and Cooper, 1968). If the generation time is longer than the time needed for one round of replication the chromosome replicates as one unit. If it is of the same length, or shorter

than the chromosome replication time, DNA synthesis continues throughout the division cycle. In rich media, where generation times are shorter than the replication time, new rounds of replication are initiated before the completion of those under way (Helmstetter and Cooper, 1968; Oishi et al, 1964). This provides a mechanism by which a cell can double its DNA content in a short generation time and results in multiple forks of replication. It seems that under normal conditions of growth, regardless of the richness of the medium, the maximum number of growing points is three (Cooper and Helmstetter, 1968).

The relationship of the time of initiation to cell division has been studied in E. coli (Clark and Maaløe, 1967). The results show that when the generation time is equal to the replication time, new rounds of replication are initiated half way through the cell division cycle. This picture was confirmed and described in more adequate terms by saying that each round of replication starts about 60 min. before the corresponding cell division (Cooper and Helmstetter, 1968).

On the basis of the picture of DNA replication presented above, one can tentatively divide the enzymes involved in this process into three categories:

a) Intermediary metabolism enzymes, involved in the synthesis of DNA precursors

b) Enzymes involved in replication (acting at the growing point)

c) Enzymes involved in regulation (initiation of new rounds of replication)

In vitro experiments provide some information about precursors and the enzymes involved in replication. If a mixture containing basically all four 5'-deoxyribonucleotide tri-phosphates, purified DNA-polymerase enzyme and a primer DNA, is incubated, new DNA is synthesized (Kornberg, 1962). Irrespective of the origin of the primer the newly synthesized DNA is identical to it in base composition and nearest-neighbour frequencies (Kornberg, 1962). This in vitro synthesis is limited in the amount of product formed, but is significantly increased when denatured DNA is used as primer. The enzyme starts functioning at any free 3'-hydroxy end of primer DNA and proceeds along it, laying down a complementary chain. Electron microscopy reveals that the newly synthesized DNA has a lot of lateral branches. This is interpreted by assuming that the polymerase, when copying one strand, may return and copy the strand which it has just synthesized (Mitra et al, 1967). DNA, thus synthesized, has no biological activity.

Recently the enzyme DNA ligase has been isolated (Gellert, 1967). It is capable of making covalent bonds between breaks in single stranded DNA, carrying 5'-phosphate and 3'-hydroxyl termini. More complex *in vitro* systems using purified DNA polymerase and DNA ligase, with single stranded DNA of phage ϕx-174, as primer, have resulted in the synthesis of biologically active DNA (Goulian *et al*, 1967).

These results indicate that the precursors of DNA are the deoxyribose-nucleoside triphosphates and that at least two enzymes, the DNA polymerase and the DNA ligase, are involved in DNA replication.

The only information concerning the enzymes involved in initiation of DNA synthesis is that protein synthesis is necessary for initiation of new rounds of replication. All experiments which provide this evidence are basically of the same type. They consist in measuring the amount of DNA made, by synchronised population of cells, after specific inhibition of protein synthesis by addition of chloramphenicol, or by withdrawal of an amino acid from amino acid auxotrophs. Germinating spores of *B. subtilis* grow synchronously for several generations. In experiments of Yoshikawa (1965) samples from such synchronised cultures are withdrawn at intervals, chloramphenicol is added to each sample, and the amount of DNA

synthesized during further incubation measured. If chloramphenicol is added at any time during the first two hours no DNA is made. If it is added at any time between two and three hours the amount of DNA synthesized in each case corresponds exactly to one doubling, and if it is added between three and four hours the amount of DNA synthesised represents a four-fold increase. This experiment suggests that inhibition of protein synthesis, by addition of chloramphenicol, allows completion of all the rounds of replication under way but prevents initiation of new rounds of replication. With synchronised populations of E. coli cells Clark and Maaløe (1967) showed that the rate of DNA synthesis doubleshalf-way through the cell division cycle. If chloramphenicol is added at any time before the change of rate, there is no initiation and DNA synthesis stops after completion. If it is added at any time after the change of rate, the rate remains unchanged. This experiment shows that protein required for initiation is made just at the time of initiation. Experiments by Lark and Lark (1964) suggest that two proteins are involved in initiation. They can be distinguished by the different sensitivity to chloramphenicol of the mechanisms by which they are synthesized. A structural protein is synthesized in the presence of low concentration of chloramphenicol, whereas the synthesis of the actual initiator is inhibited by the same concentrations of chloramphenicol.

1.2. Conditional lethal mutants

Mutations which prevent the synthesis of components essential for bacterial or phage growth (enzymes involved in DNA or RNA synthesis) are lethal. However, mutants with conditional lethal mutations in genes, mentioned above, can be isolated and studied. Conditional lethal mutations are expressed only under certain conditions (non-permissive conditions) which do not prevent growth of the wild type. Under other conditions the mutated genes function normally and the mutants can be propagated. There are several examples of conditional lethal mutants. Temperature-sensitive mutants (ts mutants) grow normally at low temperature but are unable to do so at high temperature (Horowitz and Leupold, 1953; Campbell, 1961; Edgar and Lielausis, 1964). Suppressor sensitive mutants of phage can propagate in one host bacterium but not in another (Epstein et al, 1963; Campbell, 1961). pH sensitive mutants grow at high pH but do not grow at low pH (Campbell, 1961).

In what follows we will discuss in more detail the nature of ts mutations. Studies of ts mutants of phage T4 indicate that in non-permissive conditions the mutants do synthesize a protein which is altered and non-functional. However, if the mutant phage is grown at low temperature, in most cases the phage-induced enzymes

in the lysate have normal activity at 30°C as well as at 45°C, and the mutant phage is as stable, at 45°C, as the wild type. This observation led Edgar to the suggestion that ts mutation result in altered polypeptide chains and that the process of folding the polypeptide chain into a 3-dimensional structure is temperature sensitive. If the folding takes place at low temperature the altered protein is usually not temperature-sensitive.

In the first attempt, ts mutants of phage T4 were isolated from mutagenised phage populations without any selection (Edgar and Lielausis, 1964). The genetic analysis of 382 of these mutants reveals that they are distributed in 37 genes and that the distribution is strikingly non-random. The uneven distribution is not due to the fact that mutations occur at hot-spots as most occur at different sites. Later, by applying various selective techniques, ts mutants were identified in over 80 genes altogether (Epstein, personal communication). By comparing this finding with the distribution of unselected mutants one can conclude that ts mutants may occur in any gene, but that they occur preferentially in some.

Other examples of uneven distributions of ts mutants were reported. In a population of randomly isolated ts mutants of E. coli, mutants which prevent any protein synthesis at high temperature but allow some residual DNA synthesis were chosen. (Yaniv et al, 1966). Biochemical analysis shows that 6 out of

the 7 chosen mutants were defective in the enzyme valyl-SRNA-synthetase. This finding was tentatively attributed to selection.

Temperature sensitive amino acid auxotrophs of E. coli were isolated without selection. The mutations seem to occur in some amino acid genes but not in others (E. Signer, personal communication).

1.3. Temperature-sensitive bacterial mutants defective in DNA synthesis

A complete explanation of the genetic control of DNA synthesis in bacteria could be obtained only if all the genes coding for the enzymes involved in DNA replication (DNA genes) were identified. As a mutation preventing the synthesis of any of these enzymes is lethal, only conditional lethal mutations can be isolated in DNA genes. Temperature sensitive mutants defective in DNA synthesis were isolated from several species of bacteria, and below we will summarise the data so far obtained with ts mutants of Escherichia coli and Bacillus subtilis.

About 400 ts mutants of E. coli were isolated at random from NG-mutagenised populations (Goldfine and Gross, personal communication). Mutants defective in DNA synthesis were screened by comparing the

amount of protein synthesized at 42°C with the amount of DNA synthesized at 42°C (same method as described in 2. and 3.1.2.). Preliminary analysis shows that about 6% of the mutants are specifically inhibited in DNA synthesis at 42°C.

Mutants of NG-treated populations of E. coli were also isolated at random by Kohiyama (Kohiyama et al, 1966). So far six mutants specifically defective in DNA synthesis at 41°C have been identified. Genetic analysis by conjugation indicates that they are clustered (Hirota, personal communication). However, tests to decide whether the mutants map in one, or in several genes, were not done. By comparing the amount of DNA synthesized at 41°C by different mutants, they were divided in two classes designated as replication and initiation types. Replication type mutants synthesize very little DNA and do not allow multiplication of temperate phages at 41°C. In contrast, initiation type mutants, at 41°C, synthesize an amount of DNA comparable to that required for completion of rounds of replication under way, while multiplication of temperate phages is permitted. The amount of DNA synthesized at 41°C by the initiation type mutant CR34T83 is the same as the amount it synthesizes at 30°C in the presence of chloramphenicol, or when starved of a required amino acid. This

observation leads Kohiyama to suggest that CR34T83 is defective in a protein needed for initiation of new rounds of replication. Crude extracts of all mutants show normal activity of DNA polymerase and of the enzymes needed for the intermediary metabolism of DNA.

Reports about ts mutants isolated from E. coli populations by selective techniques are numerous (Bonhoeffer and Schaller, 1965; Fangman, 1966; Rassmussen and Weywadt, 1968). There is little information about the ts mutants isolated by Bonhoeffer. Two temperature sensitive mutants in DNA genes isolated by Fangman, and one by Rassmussen, had normal DNA polymerase activities. The deoxyribose triphosphate pool after incubation at 41°C were normal which means that the intermediary metabolism enzymes were functioning normally. At sufficiently high temperatures the amount of DNA synthesized was too small to account for completion of rounds of replication, so that the mutants do not appear to be involved in initiation. The mutant of Rassmussen seems to degrade its DNA into low molecular weight components on incubation at 42°C.

Temperature sensitive mutants of B. subtilis, defective in DNA synthesis, have also been isolated from populations by techniques believed to be selective for ts mutants in DNA genes (Mendelson and Gross, 1967). The first mutant to be characterised was 168-ts B134 which is also a tryptophan auxotroph. The amount of DNA

synthesized at 45°C by this mutant is identical to that synthesized at 30°C in the presence of chloramphenicol or when starved in tryptophan, and corresponds to the amount of DNA needed for completion of rounds of replication. By pulse-labelling the cells at 45°C and analysing the incorporation of 3H-thymine by autoradiography it was found that DNA synthesis does not slow down progressively in all cells but ceases abruptly in a proportion of cells which is increasing with time. Both experiments suggest that 168-ts B134 may be unable to initiate new rounds of replication at 45°C.

All these results lead to the conclusion that the mutants so far isolated are not defective in any of the enzymes known to be involved in DNA synthesis. Although there is suggestive evidence that some mutants may be defective in the process of initiation of new rounds of replication, this is far from conclusive. This situation seems paradoxical. However, it is most likely that the number of genes so far identified is too few to draw conclusions from, and that more enzymes are involved in DNA synthesis than are at present known. The work described in this thesis concerns the isolation and characterisation of a large number of ts mutants of B. subtilis defective in DNA genes. We shall try to determine:

1) the number of DNA genes
2) the functions of identified genes
3) their relation to cell division and other activities of the cell.

2. MATERIALS AND METHODS

2.1 Materials

Bacterial strains. The strains used are listed below.

Name	Genotypic designation	Origin
10	thy⁻ ind⁻ PBSX⁻	see remark II
10-tsLn	thy⁻ ind⁻ ts	see remark I
W23	prototrophe, PBSX sensitive,	Dubnau et al, 1967 (Strain BD 1037)
SB19	prototrophe,	Dubnau et al, 1967 (Strain BD21)
SB5	hisA⁻ ura⁻ try⁻	Dubnau et al, 1967 (Strain BD2)
172	leu⁻ metB⁻ ileu⁻	Dubnau et al, 1967 (Strain BD54)
173-tsLn	metB⁻ ileu⁻ ts	By transformation, ts and leu⁺ from 10 were introduced into 172 (Remark II)
174-tsLn	ts	met⁺ and ileu⁺ from SB19 were introduced into 173-tsLn
M22	$\overline{ad_{16}}^{-}$ leu⁻ metB⁻ ileu⁻	By transformation, ileu⁻ and lys⁺ from 172 were introduced into BD46 ad^{-}_{16} leu⁻ metB⁻ lys⁻ (Dubnau et al, 1967)
168-tsB134	thy- ind⁻ tsB	Mendelson and Gross, 1967

Remark I: For temperature-sensitive mutants the following notation is used:

S-*ts*Ln

where S designates the strain which carries the *ts* mutation, L the linkage group in which the mutation is located and n the number of each particular mutant. For example *10-ts* B12 is the mutant number 12 located in group B and carried by strain *10*.

Remark II: Construction of new strains was based on pseudo-linkage. Let the donor be A^- and the recipient B^-C^- (A and B being unlinked). If the transformation is performed with high concentrations of DNA 5-10% of B^+C^- transformants will be A^- as well. The A^-C^- recombinants can easily be identified. When *ts* mutations from *10* were introduced into 172, *leu*$^+$ recombinants were tested for temperature sensitivity. In each case 6 *ts* colonies were picked, purified by streaking for single colonies and finally compared to the original mutant with respect to the *ts* character (reversion frequencies, residual synthesis of DNA), before one recombinant was chosen and kept for the collection.

Remark III: Strain *10* is a $PBSX^-$ derivative of strain 168. PBSX is a phage-like killer particle produced by 168 strains (Seaman *et al*, 1964). When 168 is thymine starved or treated with mytomycin C, or uv irradiated the cells lyse and liberate PBSX. When strain *10* is treated in a similar way lysis, if any, occurs to a much lesser extent. The lysates do not contain any phage-like material (as judged by the electron microscope) and they have no PBSX activity (Karamata, to be published).

Phage PBS1 was used for transduction (Takahashi, 1961). Stocks were obtained in the following way: A thick overnight culture of host cells in SATT medium (see below) was diluted into several tubes of Veal Infusion Broth (VIB) to a scattered light (SL) reading of 5 to 10 and incubated. Different tubes were infected with 2-4 x 10^4 PBS1 particles/ml at different times following the dilution in VIB,

(0, 30, 60 and 90 min.). The cultures were incubated 8-10 hours at 37° and then allowed to lyse over night at room temperature. The bacterial debris was centrifuged for 15 min. at 5000 rpm, the lysates treated with 80 γ/ml DNase for 30 min. at 37°C, and sterilized by filtration. Transducing activities of lysates from different tubes can vary by a factor of 10 to 50. Stocks on ts mutants were made at 34°C.

Mutagen. N-methyl-N'-nitro-N-nitrosoguanidine (NG) from Aldrich Chemical Co. was used. 30 min. before use the crystals were dissolved in distilled water (500 γ/ml) by shaking at room temperature.

Media:

L-media: Difco Tryptone 10 gms, yeast extract 5 gms, NaCl 10 gms, Water 1 l.
For plates, 15 gms Difco Agar, for soft agar 6 gms Difco agar is added. pH is adjusted to 7.

Spizizen's minimal salts x 5: $(NH_4)_2SO_4$ 10 gms, $K_2H\ PO_4$ 70 gms, $KH_2\ PO_4$ 30 gms. Sodium citrate $2H_2O$ 5 gms, $MgSO_4\ 7H_2O$ 1 gms, Water 1 l. After sterilisation by autoclaving, add 0.001 g $MnSO_4\ 4H_2O$.

Spizizen-amino acids-thymine-tryptophan (SATT): x 5 Spizizen minimal salts 25 ml, 20% casamino acids 6.4 ml, 20% glucose 3.2ml, 0.2% thymine 1.3 ml, 0.2% L-tryptophan 1.3 ml., water 100ml.

Spizizen-thymine-tryptophan (STT): Same as SATT without casamino acids. For solid media add 1.5% Difco Agar.

Spiz I: x 5 Spizizen minimal salts 25 ml, 20% glucose 3.2 ml, 0.2% tryptophan 3.2 ml, 0.2% thymine 1.3 ml, 20% Casamino acids 0.125 ml, 5% Yeast Extract 2.5 ml, water 100 ml.

Spiz II: Spizizen I 10 ml, 0.05 M Spermine 0.1 ml, 0.1 M $CaCl_2$ .005 ml, 0.25 M $MgCl_2$ 0.1 ml.

Agar for selecting transductants and transformants (T-S agar): x 5 Spizizen minimal salts 80 ml, 20% Na-glutamate 10 ml, 20% glucose 10 ml, 0.4% Yeast Extract 1 ml, 2% Difco Agar 300 ml. If any supplements are required, 4 ml of 0.2% solutions are added.

Synthetic Medium (SM): x 5 Spizizen minimal salts 25 ml, 20% glucose 3.2 ml, 0.2% thymine 0.31 ml, 0.2% leucine 3.2 ml, 0.2% solution of all amino acids except leucine 1.3ml, water 100 ml.

Slants (Schaeffer agar): Difco nutrient broth 8 gms, SO_4 Mg $7H_2O$ 0.25gms, KCl 1 gm, 0.01M $MnCl_2$ 1 ml, 0.001 M $FeSO_4$ 1 Ml, water 1 l., Difco Agar 17 gm. Adjusted to pH7 with NaOH. After sterilisation add 1 ml of 0.5 M $(NO_3)_2$ Ca.

ViB: Difco Veal Infusion Broth 25 gms, Yeast Extract 5 gms, water 1 l. Adjusted to pH 7.2.

Saline sodium citrate (SSC): NaCl 0.88 gms, Sodium citrate $2H_2O$ 0.44 gms, water 100 ml. Adjusted to pH 7 with NaOH.

^{14}C thymine: For measurements of DNA synthesis, ^{14}C thymine with specific activity 40.2 mc/mM. (The Radiochemical Centre, Amersham, Bucks) was used.

Isotope mixture: The mixture contained 4×10^{-4} mc/ml of ^{14}C leucine and 0.4 mc/ml of ^{3}H thymine. ^{14}C leucine (specific activity 34 mc/mM) and ^{3}H thymine - T(G) (specific activity 19.6 curies/mM) from the Radiochemical Centre, Amersham, Bucks, were used.

PVP solutions consisted of 6 gm PVP (Polyvinyl pyrollidon K15, Fluka AG, Bucks, SG, Switzerland), 8 ml SATT and 0.05 gm Difco agar. The mixture is dissolved by heating in boiling water and kept in a 55°C water bath.

Filters: Oxoid membrane filters were used for harvesting cells and TCA-precipitable materials. Phage stocks were sterilised by filtration through HA filters (Millipore Corp., Bedford, Mass).

2.2. Methods

Growth of Cells. Unless otherwise stated cells were grown in liquid cultures with aeration.

Scattered light measurements. Cell concentration and mass variations were determined with an EEL nephelometer (Evans Electroselenium Ltd., Halstead, Essex). All figures are referred to a calibration such that the standard reads 60 (corresponds to about 2×10^7 CFU/ml in log phase populations). When cultures reach higher densities ($SL > 150$) readings are made on x 10 dilutions.

Double-labelling (Hempstead, 1968). The following method was used to determine rapidly for many mutants the variation of the ratio of the amount of protein synthesized to the amount of DNA.

synthesized. The first day 70 mutants at a time are streaked for single colonies on L plates and incubated overnight at 30°C. The second day, one colony of each mutant is inoculated in 1 ml SATT media and incubated overnight at 27°C. In this and all subsequent incubations the cells were aerated by shaking in a Gallenkamp shaker bath. The third day the overnight cultures are diluted in 1 ml SM medium to SL readings of 5 to 10 (about 20 x dilutions). After 2 hours incubation at 30°C one drop (about 0.05 ml) of isotope mixture (see above) is added to each tube and the incubation then continued at 45°C. 0.5 ml samples are taken at 30 min. and precipitated in 5 ml cold 5% TCA. At 90 min. 5 ml cold 5% TCA are added to the remaining 0.5 ml of culture in each tube. The tubes are stored for 2 hours in the cold and the TCA precipitable material harvested on filters and washed twice with 10 ml 5% TCA. The ^{14}C and ^{3}H counts are determined with a Packard Scintillation counter.

Extraction of DNA. 20 ml samples of cells in late log phase (SL = 200-300) are harvested, resuspended in 4 ml SSC and 100 γ/ml lysozyme is added. After 30 min. incubation at 37°C, 0.5 ml of 10% Dupanol is added and the incubation continued for 5 min. When lysis is complete, 1 ml of 5M Na-perchlorate and 6 mls of $CHCl_3$-amylacetate mixture (24:1) are added. The two phases are

mixed by gentle shaking for 30 min. on a Griffin Microid flask shaker. The two phases are then separated by 10 min. centrifugation at 4000 rpm. The aqueous phase containing the DNA is then withdrawn and the $CHCl_3$-amylacetate extraction repeated. After the second extraction, the DNA in the aqueous layer is precipitated by pouring it into 18 mls absolute alcohol. DNA fibers are finally collected on a spatula and resuspended in 10-fold diluted SSC.

Transformation. Competent recipient bacteria are prepared as follows: Overnight cultures of recipient bacterial strains are diluted in SPIZ I medium to SL = 5. Growth is followed by SL measurements. About 45 min. after the end of the exponential growth-phase, the cells are diluted 10 times in SPIZ II medium, and incubated with strong aeration for another 90 min. For transformation 0.1 ml DNA is added to 0.5 ml cells and the mixture incubated on a shaker-bath 30-60 min. Recombinants are selected by spreading the cells on appropriately supplemented T-S plates. For the selection of ts+ recombinants, L-agar plates are incubated at 45°C without preincubation at 30°C. All other plates are incubated at 30°C or 37°C. When the recipient is a ts mutant, the cells are made competent and the transformation performed at 34°C.

Transduction. Recipients are grown in SATT, pH 7.4, until the late log phase (SL = 250). 0.5 ml of a PBS1 lysate is added to 0.5 ml of cells. After 20 min. incubation without aeration the cells are plated on L agar or appropriately supplemented T-S agar plates. The ts recipients are grown at 34°C, and when ts^+ recombinants are selected the plates are incubated overnight at 45°C without preincubation at 30°C.

DNA synthesis is measured by ^{14}C-thymine incorporation. The cells are grown in SATT (with only 10 γ/ml thymine) to which 8×10^{-5} mc/ml of ^{14}C-thymine is added. 0.5 ml samples are precipitated in 5 ml cold 5% TCA, harvested on filters after 2 hours in the cold, and then washed twice with 10 mls 5% TCA. After drying, the filters are counted in a Nuclear-Chicago gas-flow counter.

Viable counts. The numbers of colony forming units (CFU) are determined by adding appropriate dilutions of cell suspensions to 2.5 ml soft L-agar, molten and cooled to 45°C, and pouring onto L agar plates, which are incubated at 30°C. The dilutions were made in SATT medium.

PBSX test. All B. subtilis strains listed in materials, except 10 and W23, harbour the killer particle PBSX (Seaman et al, 1964).

Its presence in lysates is tested by spotting 0.02 ml samples of serial dilutions of the lysate on L-agar plates, seeded with a culture of strain W23, and incubating at 37°C. W23 lawns are obtained by mixing 0.25 ml W23 cells (SL = 20-50) with 2.5 ml L soft-agar and pouring onto L-agar plates. If the lysate has any activity the spots will give clear plaques, and the final dilution with an inhibitory effect provides an estimate of PBSX concentration.

Microscopy. 0.05-0.2 ml of cells is added to 0.4 ml of a PVP solution. One drop is spread on slides prewarmed at 45°C and covered with a prewarmed cover glass. The slides are examined with a 100 x phase contrast oil-immersion objective on a Zeiss photomicroscope.

3. RESULTS

3.1. Isolation and genetic analysis of ts mutants defective in DNA synthesis

3.1.1. Isolation

The following procedure was designed to avoid selection of any particular type of mutant. When an exponentially growing culture of strain 10, in SATT medium at 37°C, reached an SL of 100, (about 2×10^7 CFU/ml), NG, to a final concentration of 30 γ/ml, was added and the culture was further incubated for 20 min. The cells

were filtered, washed and resuspended in the initial volume of SATT. There were about 50% survivors. One ml of this suspension was diluted in 100 mls of SATT and incubated overnight at 25°C. The overnight culture contained 5×10^8 cells/ml. It was diluted and plated onto L plates (50-100 cells/plate). After 20 hours incubation at 30°C the plates were replicated on pre-warmed L plates and the replica plates incubated for 6 hours at 45°C. A few plates were also replicated on STT agar for determination of the percentage of auxotrophs. All the colonies present on master-plates and absent from replica-plates were streaked for single colonies on L plates and incubated overnight at 30°C. One colony of each mutant was inoculated in 1 ml SATT, grown overnight in a shaker-bath and restreaked on 2 L plates, one of which was incubated at 30°C and the other at 45°C. The mutants which failed to grow at 45°C were finally chosen and kept on slants.

Twelve experiments of this type were performed. The maximum number of mutants isolated in one experiment was 170. The quantitative data concerning the isolation are summarised as follows.

% survivors after treatment with N.G.	% auxotrophs in the overnight cultures	% of ts mutants in the overnight culture	Total number of ts mutants isolated	% auxotrophs among ts mutants
20-60	1.5-2	0.15-0.25	655	19.3

One can see that the mutagenised populations contain about 2% auxotrophs whereas there are about 20% auxotrophs among the temperature sensitive mutants (the mutants are ts and auxotrophs). This shows that each mutant isolated from our NG-mutagenised populations has a high probability of being a multiple mutant.

The experiment was designed to minimise the risk of isolating several mutants from the same clone. As mentioned above the total inoculum of cells, allowed to express overnight, was about 10^7 CFU, and it contained approximately 2×10^4 ts mutants (0.2% of the total). During the incubation each cell gave rise to a clone of 5×10^3 cells. If 200 ts mutants were randomly isolated from the whole population the size of each clone would remain unchanged, and therefore, the sampling would obey Poisson's distribution. From these assumptions one can calculate that in an isolate of 200 mutants there would be only 2 from the same clone. Thus as each of our experiments yielded on the average about 50 mutants, the chance to pick two from the same clone was even smaller.

3.1.2. Screening

To identify mutants defective in DNA synthesis at 45°C (ts DNA mutants) the following criterion was used:

A mutant was considered as ts DNA if, when incubated at 45°C, the ratio of the amount of protein to DNA increased with the time of incubation, and at 90 min showed an obvious difference as compared to the wild type.

The change of ratio is actually measured by a double-labelling technique (Hempstead, 1968; see 2.). The synthesis of protein is measured by ^{14}C-leucine incorporation and that of DNA by ^{3}H-thymine incorporation. The results are most suitable for analysis when plotted in graphs. Each mutant is represented by a point corresponding to the ratio of $^{14}C/\ ^{3}H$ counts at 30 min (abscissa) and at 90 min (co-ordinate). Figure 1 shows a random sample of 54 ts mutants, with wild strain 10 and the ts DNA mutant 168-ts B134 as controls. Points corresponding to a large majority of the mutants are clustered in the neighbourhood of that of the wild strain 10. The few points located well outside the cluster correspond to mutants with abnormally high $^{14}C/^{3}H$ ratios, which we defined as ts DNA mutants.

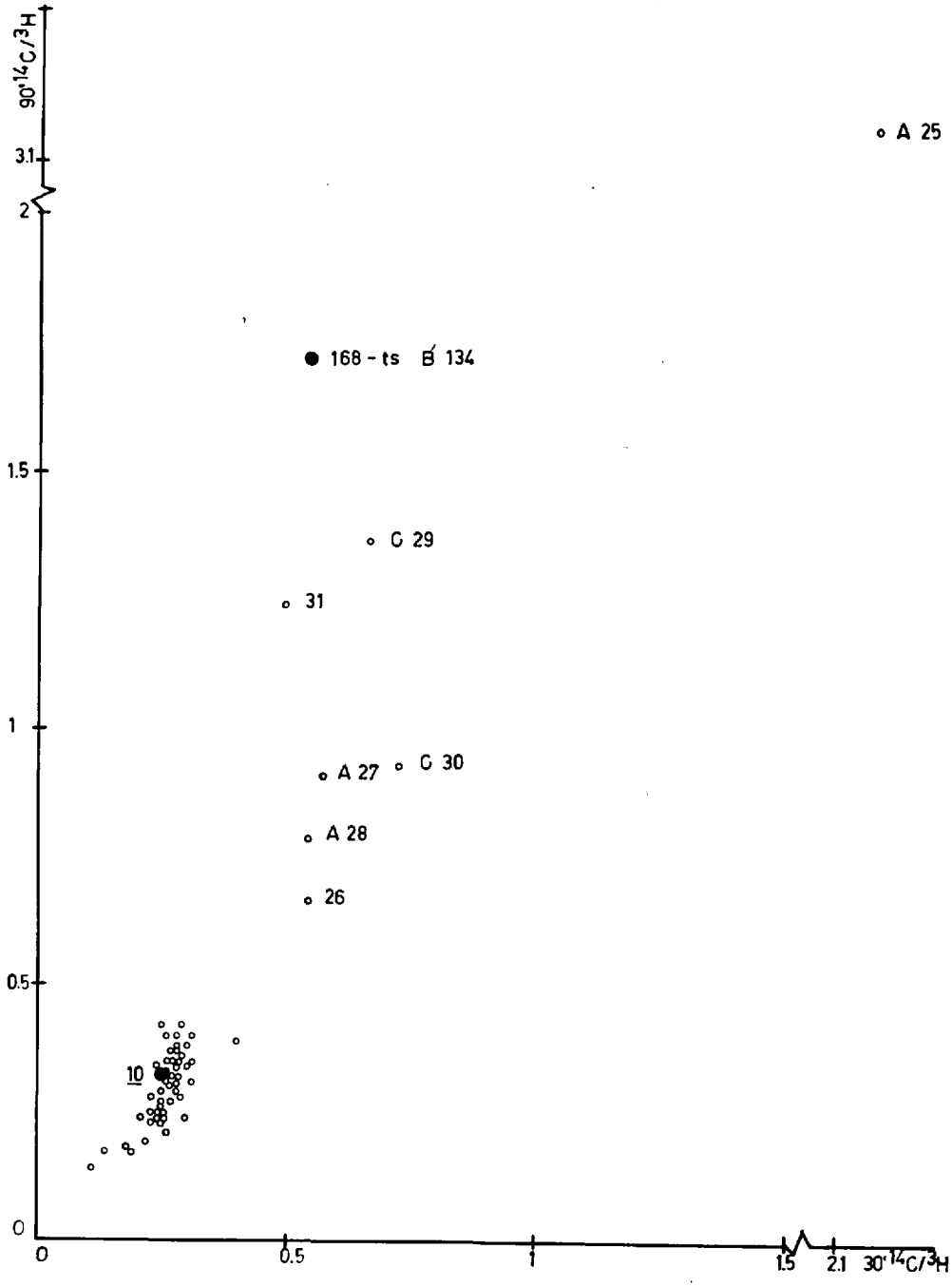

90 14C/3H
3.1
2
1.5
1
0.5
0
A 25
168 - ts B 134
C 29
31
A 27
C 30
A 28
26
10
0
0.5
1
1.5
2.1
30 14C/3H

FIG. 1

Figure 1

Screening for ts DNA mutants in a random sample of 54 ts mutants.

Exponentially growing cells, in SM medium at 30°C, are shifted to 45°C and a mixture of ^{14}C-leucine and ^{3}H-thymine is added. Samples of each mutant are taken after 30 and 90 min. incubation at 45°C, and the TCA precipitable material counted. Each mutant is represented by an open circle corresponding to the ratio of $^{14}C/^{3}H$ counts at 30 min. (abscissa) and at 90 min. (co-ordinate). Possible ts DNA mutants are numbered. Wild strain 10 and the ts DNA mutant 168-ts B134 are represented by solid circles.

All 655 ts mutants, so far isolated, were examined by this test. 15-20% of the mutants were indistinguishable from wild-type up to 90 min, and 10-15% ceased synthesizing protein as well as DNA within 30 min after transfer. Fifty-two mutants appeared to have the ts DNA phenotype. However, when detailed kinetics of mass and DNA increase were determined during three hours after shifting to 45°C (see 2. and 3.2.1) only 38 out of the 52 ts DNA mutants showed an appreciable change in the mass to DNA ratio. Thus, although the limits of the criteria for defining ts DNA mutants are, to some extent, arbitrary, we can conclude that these mutants represent between 6 and 8% of all the ts mutants isolated.

The criterion by which our ts DNA mutants are defined is designed to identify mutants in as many DNA genes as possible. However, there may be genes whose immediate function is in DNA synthesis but which, whenever defective, lead to rapid cessation of all cellular activities. Mutants in these genes could not be identified by our criterion because they would not show an appreciable specific inhibition of DNA synthesis.

3.1.3. Genetic analysis

The linkage between ts DNA mutants was determined by two point crosses. Let us consider two ts mutants: the donor, ts 1, is ileu$^+$ and the recipient, ts 2, is ileu$^-$. Crosses ts 1 ileu$^+$ x ts 2 ileu$^-$ as well as ts$^+$ ileu$^+$ x ts 2 ileu$^-$ are performed. The ts$^+$ and ileu$^+$ recombinants from both crosses are counted. The ratios of the numbers of recombinants, ts$^+$/ileu$^+$ (= R') from the first cross and ts$^+$/ileu$^+$ (= RO) from the second cross, are calculated. The mutations ts 1 and ts 2 are linked if $^{R'}/_{RO} < 1$, they are unlinked if $^{R'}/_{RO} = 1$. The ratio $^{R}/_{RO}$ is called map distance. It expresses the reduction of the recombination frequency between two linked markers. The closer the mutations are the smaller is $^{R}/_{RO}$.

Twenty-nine ts DNA mutants were chosen for genetic analysis. The crosses were performed by transformation. Donor DNA was extracted from mutants in strain 10 (ileu$^+$). Strain SB19 DNA was used as wild-type control (ileu$^+$ ts$^+$). ts mutants in strain 173 were used as recipients (ileu$^-$). The ileu$^+$ recombinants were selected by plating on T-S agar plates appropriately supplemented. Initial crosses were performed on recipients with particularly low frequencies of reversion and

three unlinked mutations were identified in mutants number 13, 19 and 30. Then the remaining 26 mutants were crossed to these three, used as recipients. Only one mutation was not linked to any of the three recipients. All the others were linked either to 13 or to 19 or to 30 but none was linked to two of them. The linkage groups thus established were designated A, B, C and D. The distribution of ts DNA mutants among different groups, and the minimum and maximum map distances between mutants from the same groups are shown below.

Linkage group	Number of mutants	Minimum and maximum map distances
A	12	0.01-0.25
B	9	0.05-0.25
C	7	0.07-0.25
D	1	

A complete fine-structure linkage map of each group was not established. However, all crosses, so far made, between different mutants from the same group yielded significant numbers of ts^+ recombinants. This means that the uneven distribution of mutants among the groups is not due to the existence of hot-spots (sites which would be mutagenised by NG with a high probability).

No technique for making complementation tests in B. subtilis has been devised. Therefore the only way to get an approximate idea of the number of genes in each group is to compare the size of the group with that of genes of known size by means of map distances. Analysis by transformation of mutations in tryptophan synthetase genes of B. subtilis shows that the map distance between the two most distant mutations in one gene is about 0.14-0.2 (Carlton, 1966). Let us assume that the genes involved in DNA synthesis are, on the average, of the same size as the tryptophan synthetase gene, and that the ts mutations in each group are randomly distributed. If the mutants A13, B19 and C30 are located in the middle or on one extremity of the group the maximum map distances given above correspond to half or to the whole size of the group. Thus, comparing the maximum map distances between mutations in groups A, B and C to that given by Carlton for the tryptophan synthetase gene one can see that each group contains one, or at the most, 2 genes.

Location of groups on the B. subtilis chromosome. PBS1 is a large transducing phage which can transduce between 5% and 10% of the B. subtilis chromosome (Thomas and MacHattie, 1967). Such a fragment of DNA is about 100 times bigger than DNA molecules

used for transformation. Therefore transduction with PBS1 provides a tool for determining linkage between relatively distant markers (Dubnau *et al*, 1967). To locate groups A, B, C and D, a stock of phage PBS1 was made on strain M22 ad^-_{16} leu^- met^- $ileu^-$. Then by transduction strain M22 was crossed with recipient mutant strains 174-*ts* A13, 174-*ts* B19, 174-*ts* C30 and 174-*ts* D23 selecting for ts^+ recombinants on L plates. To determine if any of the ts^+ recombinants received any of the four auxotrophic requirements of strain M22 all plates were replicated on appropriately supplemented T-S plates, incubated overnight at 45°C, and the proportion inheriting each different auxotrophic requirement of the ts^+ donor counted. The result is summarised in Table 1.

Table 1

Cross	Total number of ts^+ colonies examined	$ts^+ ad^-_{16}$ colonies	$ts^+ leu^-$ colonies	$ts^+ met^-$ colonies	$ts^+ leu^-$ colonies	linkage of *ts* DNA mutation to auxotrophic markers
M22 x 174-*ts* A13	305	0	0	0	0	none
M22 x 174-*ts* B19	265	0	87	0	0	0.33 to *leu*
M22 x 174-*ts* C30	200	676	0	0	0	0.97 to ad_{16}
M22 x 174-*ts* D23	258	0	0	130	97	0.38 to *met* 0.5 to *ileu*

Linkage of A, B, C and D group mutants to auxotrophic markers. PBS1 phage stocks were grown on the strain M22 ad^-_{16} leu^- met^- $ileu^-$. By transduction M22 was crossed to *ts* mutants 174-*ts* A13, 174-*ts* B19, 174-*ts* C30, 174-*ts* D23 and the ts^+ recombinants scored on L plates. The plates were then replicated on appropriately supplemented T-S plates, incubated overnight and the proportion inheriting each different auxotrophic requirement of the ts^+ donor counted. The linkage is measured as the percentage of auxotrophs among the total number of ts^+ colonies examined.

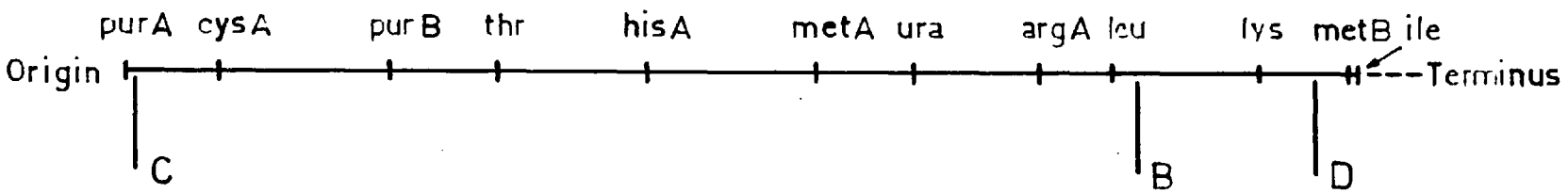

purA
cysA
purB
thr
hisA
metA
ura
argA
leu
lys
metB
ile
Origin
Terminus
C
B
D

FIG. 2

Figure 2

Location of groups B, C and D on the *B. subtilis* genetic map (Dubnau *et al*, 1967)

ts DNA markers were mapped by cotransduction with auxotrophic markers of known location (see Table 1).

From Table 1 and the B. subtilis genetic map (Dubnau et al, 1967) one can see that groups B, C and D are located in different regions of the map (see Fig. 2). As Table 1 shows, group A is not linked to any of the strain M22 auxotrophic markers. Linkage of group A to his A, ura and try was tested in the similar way by using PBS1 phage stocks made on strain SD5, his⁻ A ura⁻ try⁻. This cross revealed no linkage so that the location of group A so far remains unknown.

The observation that groups A, B, C and D are scattered in distant regions of the genetic map is unusual. In other systems all identified genes involved in DNA synthesis are clustered. In phage T4 almost all genes are in one cluster (Epstein et al, 1963) and in E. coli all six identified ts DNA mutants are linked (Hirota, pers. comm.).

3.1.4. Discussion

Although our procedure was designed to isolate at random ts mutants in a large class of genes, a few points which could introduce some kind of selection during isolation as well as during identification of ts DNA mutants should be discussed.

It is likely that NG-induced mutations occur in many points of any gene. Reports of NG-induced mutations in many sites of

the same gene are numerous. Analysis of a large number of NG-induced amino acid auxotrophs of B. subtilis shows that the relative number of auxotrophs requiring a given amino acid is roughly proportional to the number of genes known to be involved in the synthesis of that amino acid. Thus one can safely assume that the probability of NG-induced mutations in any gene is proportional to its size.

We have seen that NG induces multiple mutations (see 3.1.1.). On the other hand there have been reports that NG-induced multiple mutations are closely linked (Hirota, personal communication) probably due to the fact that NG acts preferentially on the growing point (Cerada-Olmedo and Hanawalt, 1968). ts mutations occur on the average with a frequency 10x lower than temperature-stable mutations (Karamata, to be published). Thus it seems that if two essential genes (see below) are closely linked, it would be difficult to isolate a ts mutation in one of them, because there is a good chance that the cell will have simultaneously suffered a lethal mutation in the other gene. There is, however, some evidence against this possibility. C group mutants are closely linked to adenine. If both mutations occur simultaneously the mutant tsC ad$^-$ would be eliminated during the time allowed to the

mutation to express in SATT (no adenine) after NG treatment. In practice, tsC mutants are found to occur with high frequency.

Among other types of mutant which may fail to be isolated are those which already grow slowly at 30°C and are selected against during the expression period. Again, mutants with high frequencies of reversion are not detected by replica plating and those which do not express their phenotype within 90 min. at 45°C do not show up in the double-labelling test. However, mutations in almost any gene may exhibit one or the other of these phenotypes but there is no gene one can think of, which should give predominantly one of the mentioned phenotypes.

Thus, we may conclude that there is no obvious reason to believe that NG induces mutations in some specific genes or that during isolation we have selected specifically against ts mutations in any gene.

The distribution of ts DNA⁻ mutants into the four linkage groups is strikingly non-random (see 3.1.3.). As it is likely that each group corresponds to only one gene, and since a total of 29 ts DNA mutants were genetically analysed our procedure has yielded on the average 7 mutants per gene. Thus the system of isolation (not the genome) seems to be saturated in the sense that it provides us always with ts mutations in a relatively small number of genes.

The idea that D is special and that A, B and C are the only genes involved in DNA synthesis can be easily rejected. First, we know that there are at least 10 enzymes involved in DNA synthesis (see 1. and 3.2.4.). Moreover, a ts mutant in a gene not linked to A, B, C or D has already been isolated by selective techniques (Gross, personal communication). As we have eliminated the possibility that ts mutants in some genes have been selected for, or against, the results suggest that ts mutations occur in different genes with different probabilities. A ts mutation results in an altered polypeptide chain. Usually a correct amino acid is replaced by a different one in such a way that the protein becomes thermo-labile. It is likely that in some proteins many changes produce a thermo-labile product whereas in others almost any substitution may have no effect or, on the contrary, lead to a non-functioning of that protein at any temperature. This interpretation can explain the uneven distribution of unselected ts mutants in phage T4 (Edgar and Lielausis, 1964), as well as the high frequency of occurrence of ts mutants of the valyl-s-RNA synthetase in E. coli (Yaniv et al, 1966).

Our results show that there are at least four genes involved in DNA synthesis. It would be interesting to see if the information they provide can help to get an estimate of the number of all the

genes involved in DNA synthesis. It is impossible to estimate this number from the distribution of ts mutants among groups A, B, C and D because the distribution is not random. Had the distribution been random it would have fitted the Poisson formula for a certain multiplicity (number of mutations:number of all genes) and the number of all genes could readily have been calculated.

However, if all DNA genes represent a sufficiently large set so that the probability of getting a ts mutation in it is the same as the probability of getting a ts mutation in any set of genes of the same size, then the fact that 6-8% of all ts mutants are involved in DNA synthesis means that 6-8% of all essential genes (see below) are involved in DNA synthesis. This agrees well with the observation that 8-10% of all phage T4 genes are DNA genes. As knowledge of the number of essential genes would provide the number of DNA genes the following indirect approach to determine this number was devised. Let us consider the following classes of bacterial genes:

E, the essential genes, defined operationally as genes which when not functioning lead to the death of the cell and which are not involved in the synthesis of metabolits present in the media in which the cells grow.

L, genes involved in synthesis of metabolits present in L-broth.

AA, genes involved in amino acid synthesis (they are part of class L)

U, genes outside class L but which when not functioning would not prevent cell growth on L-agar (drug resistance, sporulation, inducible enzymes .....).

All four classes are big enough so that we can assume that the probability of getting ts mutations per unit of length is the same in each of them. Thus the proportions of ts mutations in each group would give the relative size of the group. Preliminary analysis of the distribution of 69 NG-induced ts mutants in these groups gave the following result:

Class	ts mutant	% of the total examined
E	42	60%
L	27	40%
AA	22(subclass of L)	

Remark: The B. subtilis cells were mutagenised as in 3.1.1., and allowed to express overnight in L-broth.

These figures allow us to estimate the upper and the lower limit of the number of DNA genes. Let us take the generally accepted figure of 3000 genes in the bacterial chromosome (Watson, 1965). Ignoring the existence of class U, one obtains an over-

estimate of the number of genes in class E: 60%, or 1800. This yields a maximum figure of 100 DNA genes (6% of 1800). On the other hand, from known figures for E. coli one can count at least 150 genes involved in amino acid synthesis. As the ratio of ts mutants in class E to ts mutants in class AA is 1.9, it follows that there are at least 300 genes in class E and at least 18 DNA genes (6% of 300). On the basis of these arguments we can conclude that the number of DNA genes is between 20 and 100.

Conclusion: We have isolated ts mutants in at least four genes. Although the study of these mutants is important (see 3.2.) it would be desirable to isolate ts mutants in more DNA genes which we know to exist, and in which the ts mutations arise with a low probability. We have seen that our system of isolation is saturated; it yields only ts mutants which appear with high frequencies. Selection of ts mutants with specific properties may help in the discovery of new DNA genes. Isolation of other types of conditional lethal mutants (cold-sensitives, pH-sensitives) seems more promising. Suppressor-sensitivesmutants, which occur randomly in phage T4 (Epstein, personal communication) are especially interesting.

3.2. Basic physiological properties of ts DNA mutants

To see if ts DNA mutants from the same group have any common properties which may suggest, or eliminate, a possible function of the mutated gene, we have determined a few basic physiological properties, in non-permissive conditions, of almost all the genetically analysed mutants. In what follows we will give the measures of residual mass increase and DNA synthesis, viabilities, PBSX-particle induction and morphological changes at 45°C. All experiments, except PBSX-particle induction, were done with mutants in strain 10. Particle PBSX-induction was determined on mutants in strain 173 which is $PBSX^+$.

3.2.1. Residual DNA synthesis and mass increase

These measurements were made in parallel. A log-phase culture in SATT medium at 30°C (SL = 100-130) was diluted in two SATT-containing tubes to a scattered light reading of 7-10 corresponding to about 2×10^6 CFU/ml and transferred to 45°C. One tube was used for reading the SL. The other, to which ^{14}C-thymine was added (see 2) was used for measuring the residual DNA synthesis. Samples were withdrawn at regular intervals of time and the incorporated ^{14}C-thymine determined as indicated in 2.

The results obtained on 27 ts mutants are represented in Figs. 3-14. On some of the figures the curves corresponding to wild strain 10 and the ts mutant 168-ts B134, both examined at 45°C, are plotted for comparison.

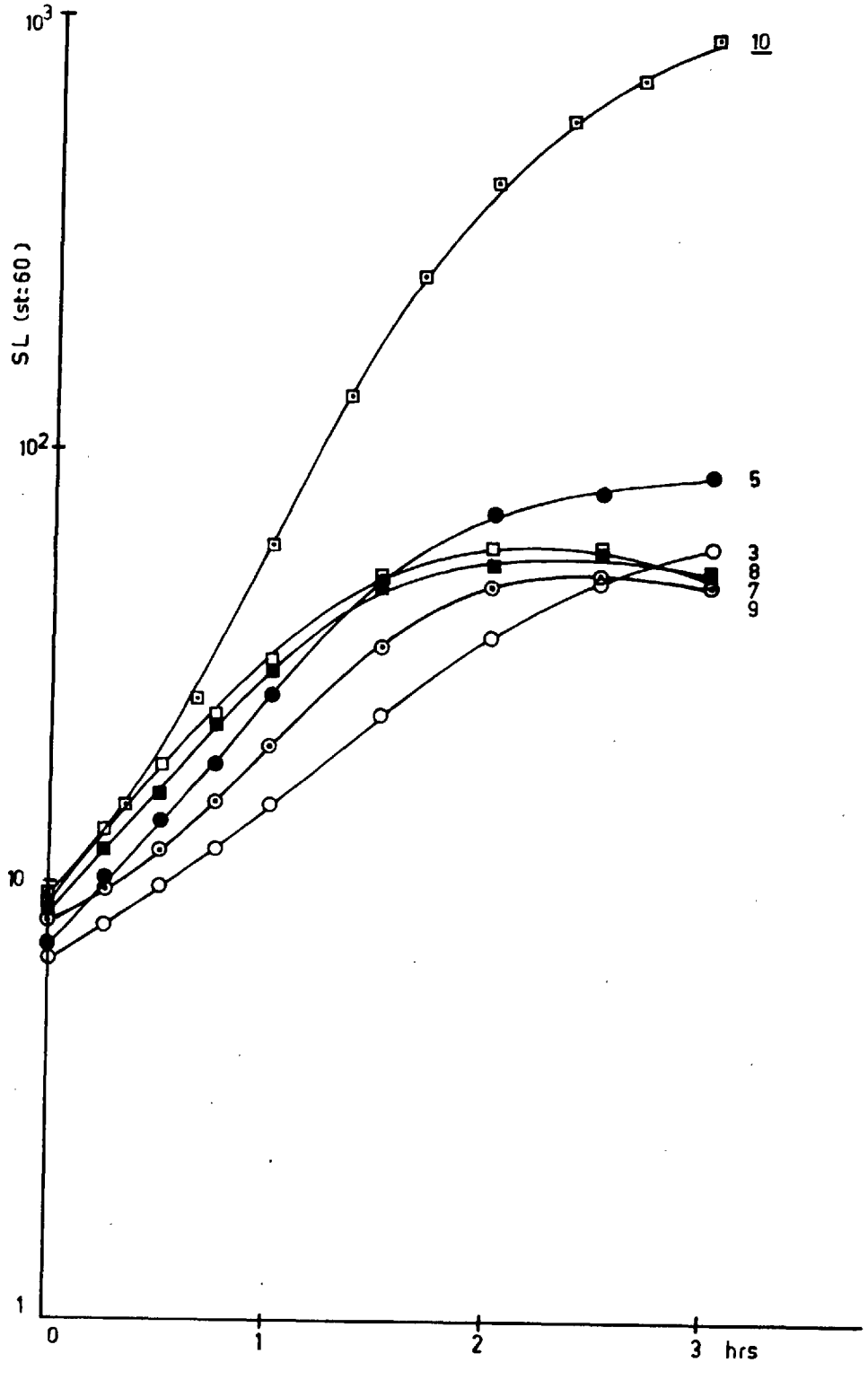


FIG. 3

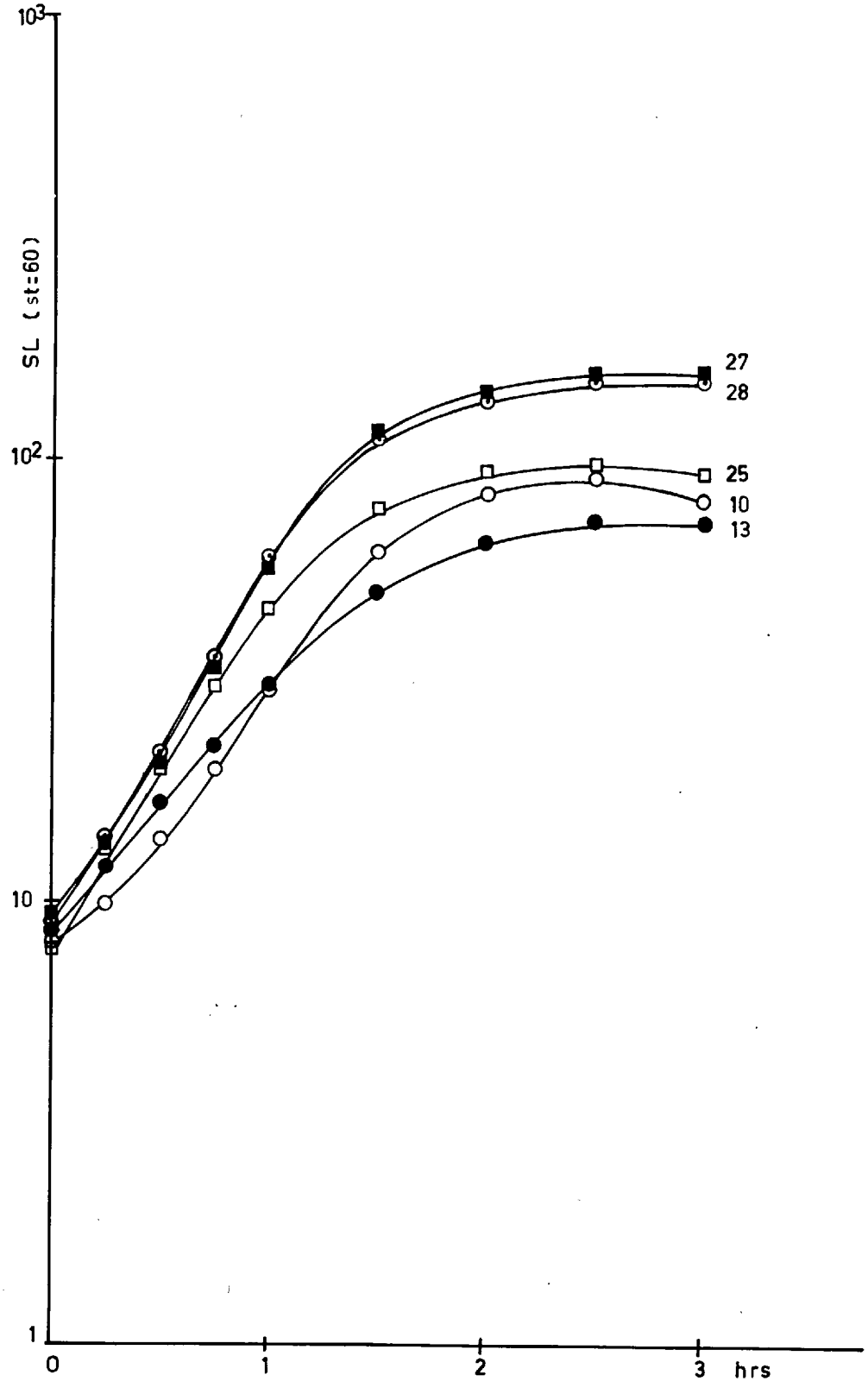


FIG. 4

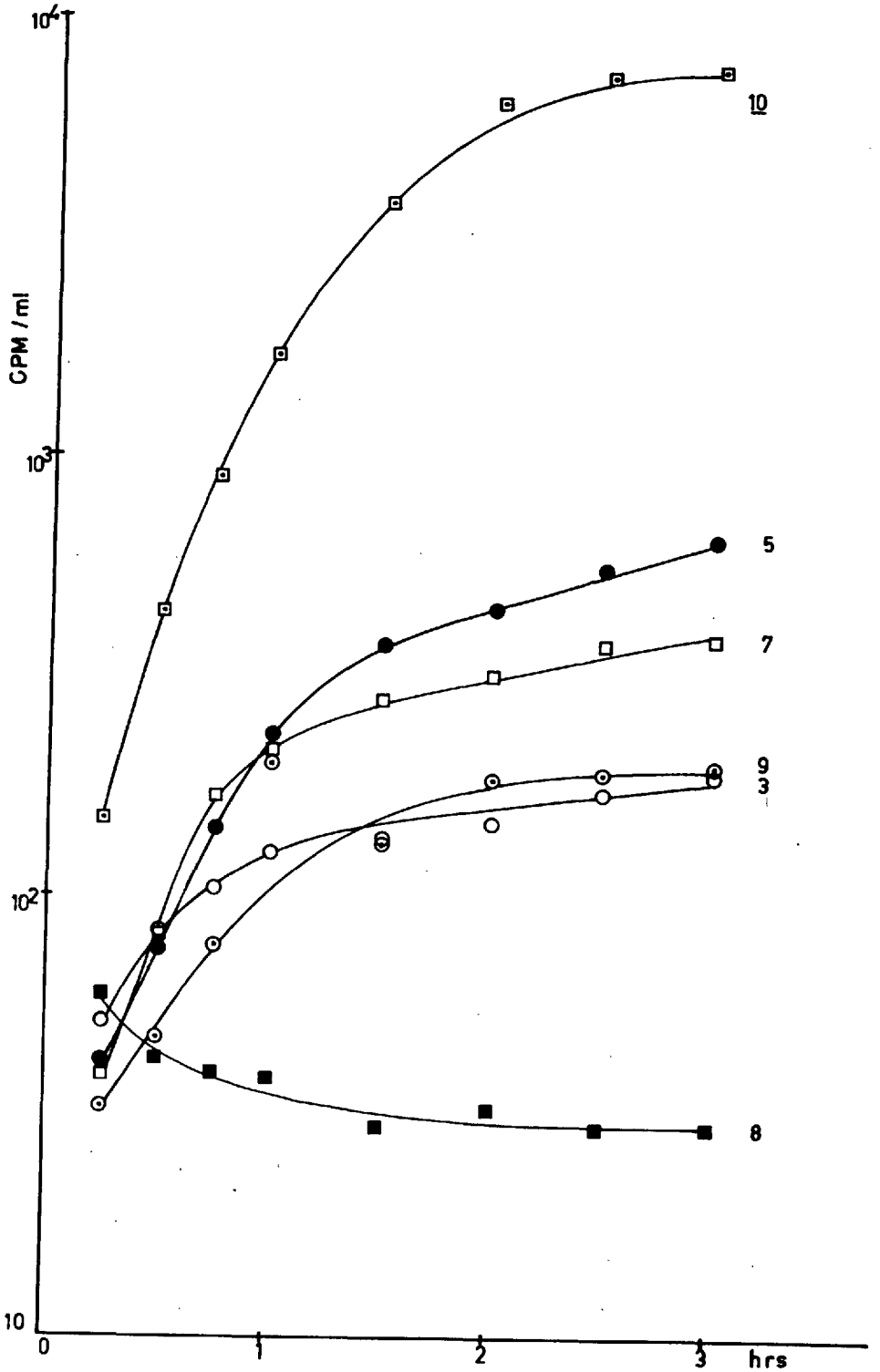


FIG. 5

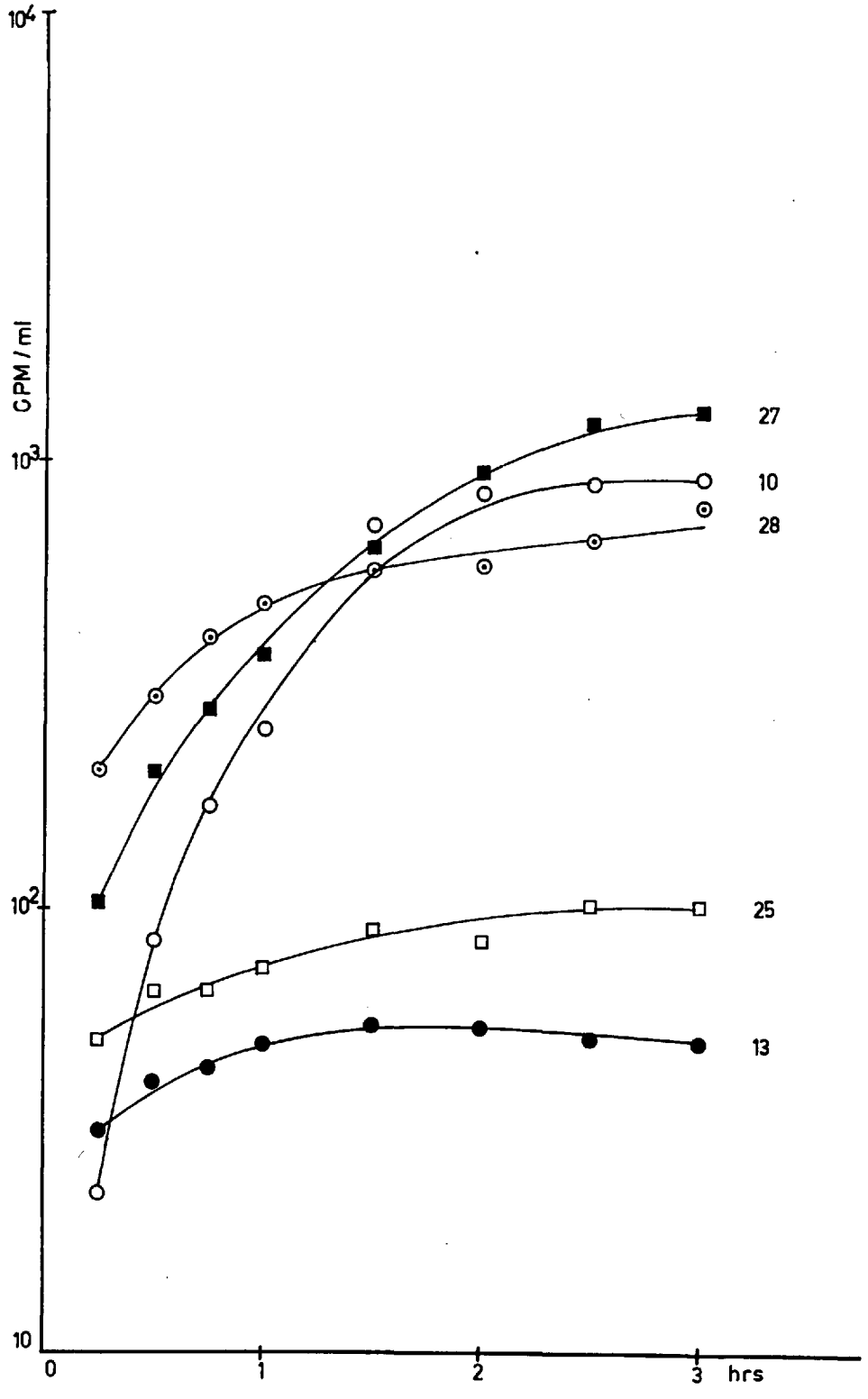


FIG. 6

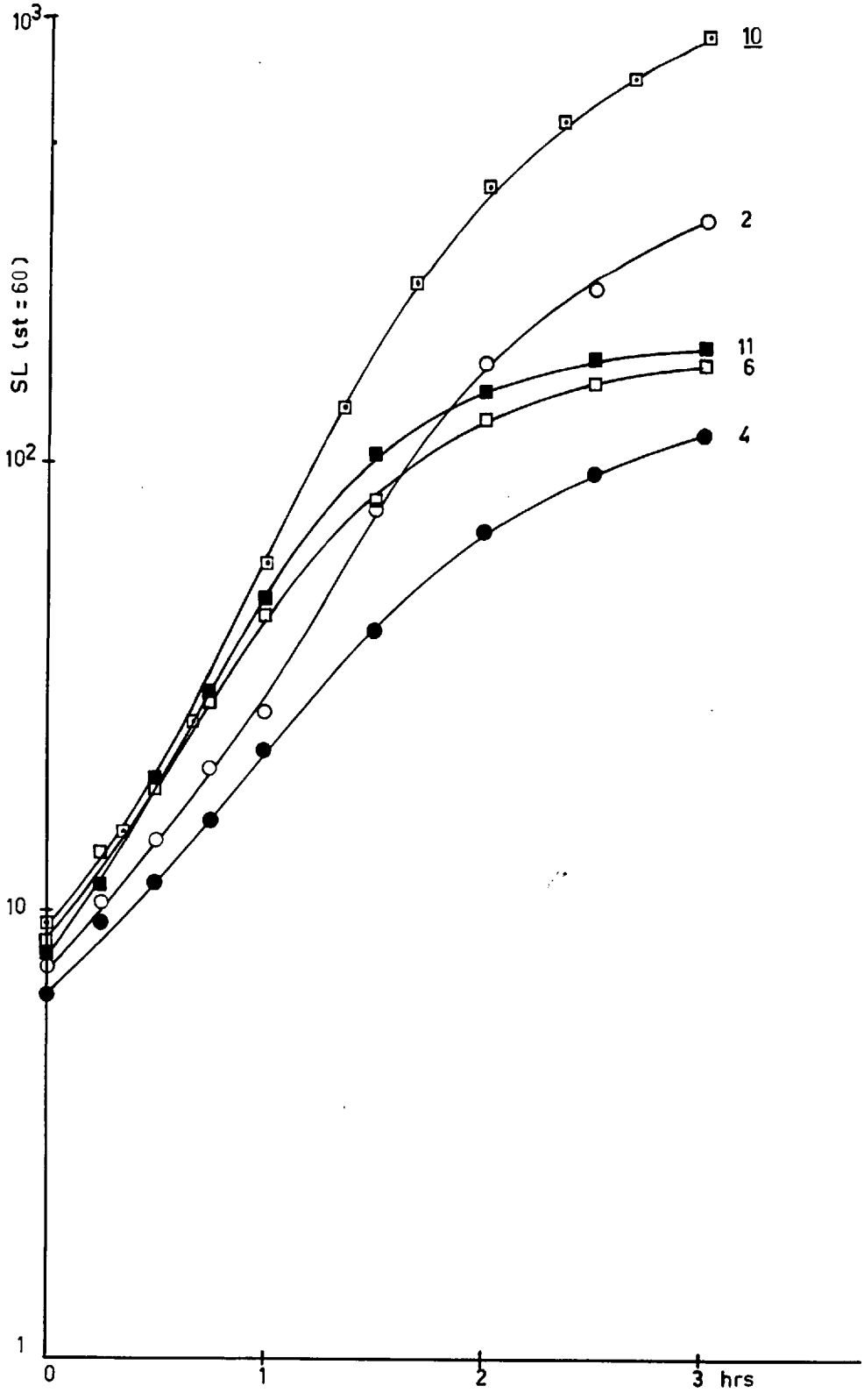
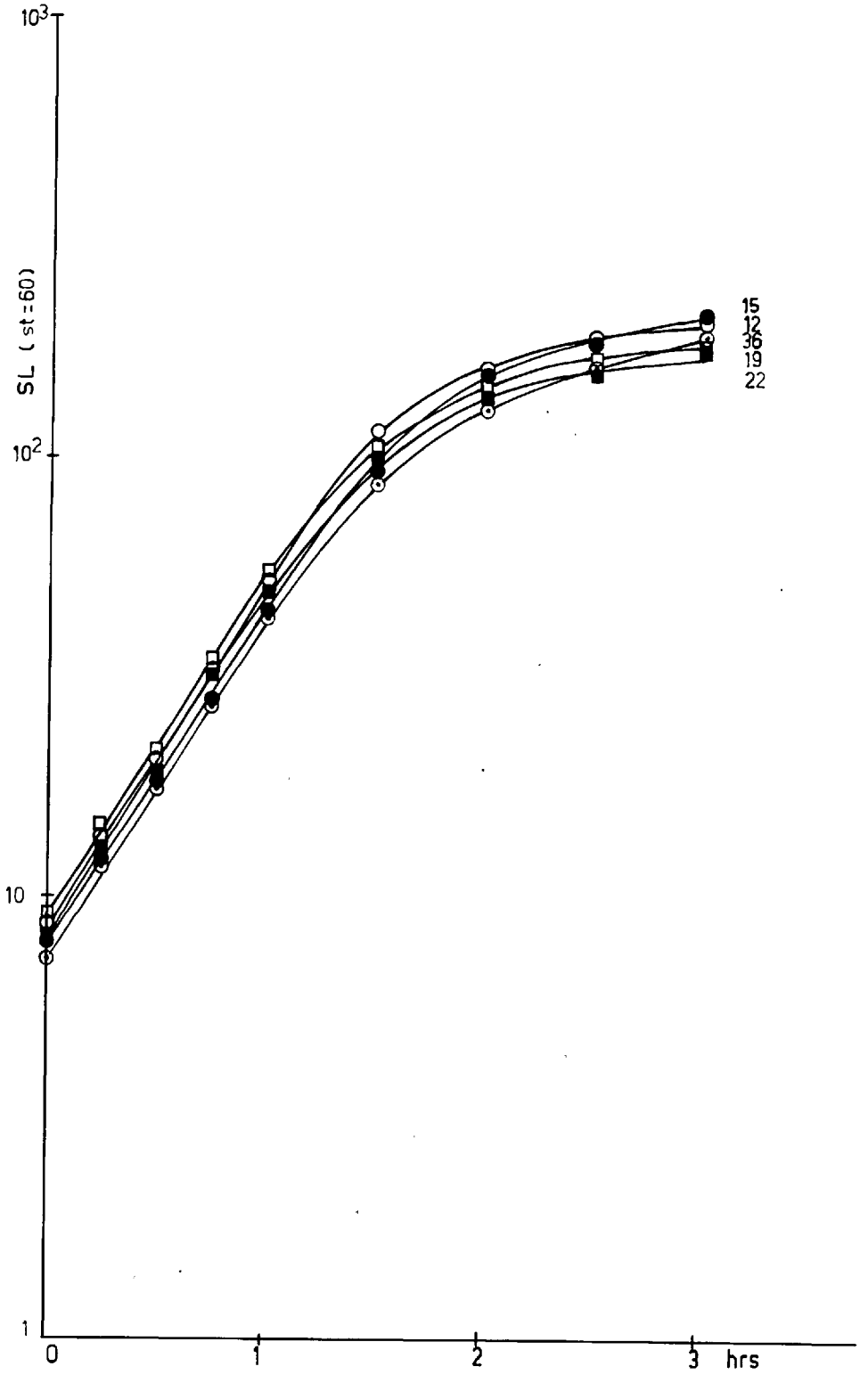


FIG. 7


10^3
SL (st=60)
10^2
10
1
0
1
2
3 hrs
15
12
36
19
22

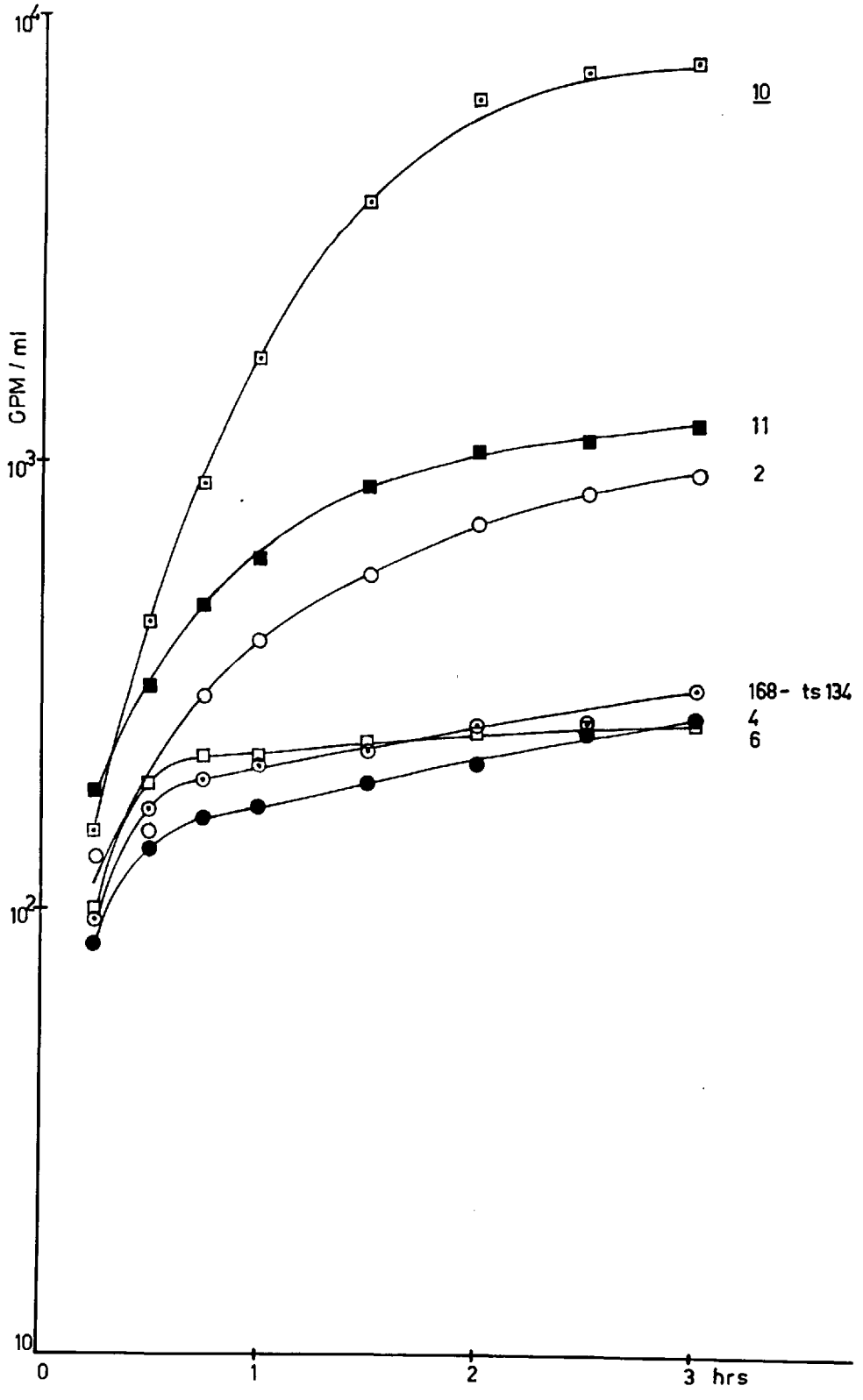


FIG. 9

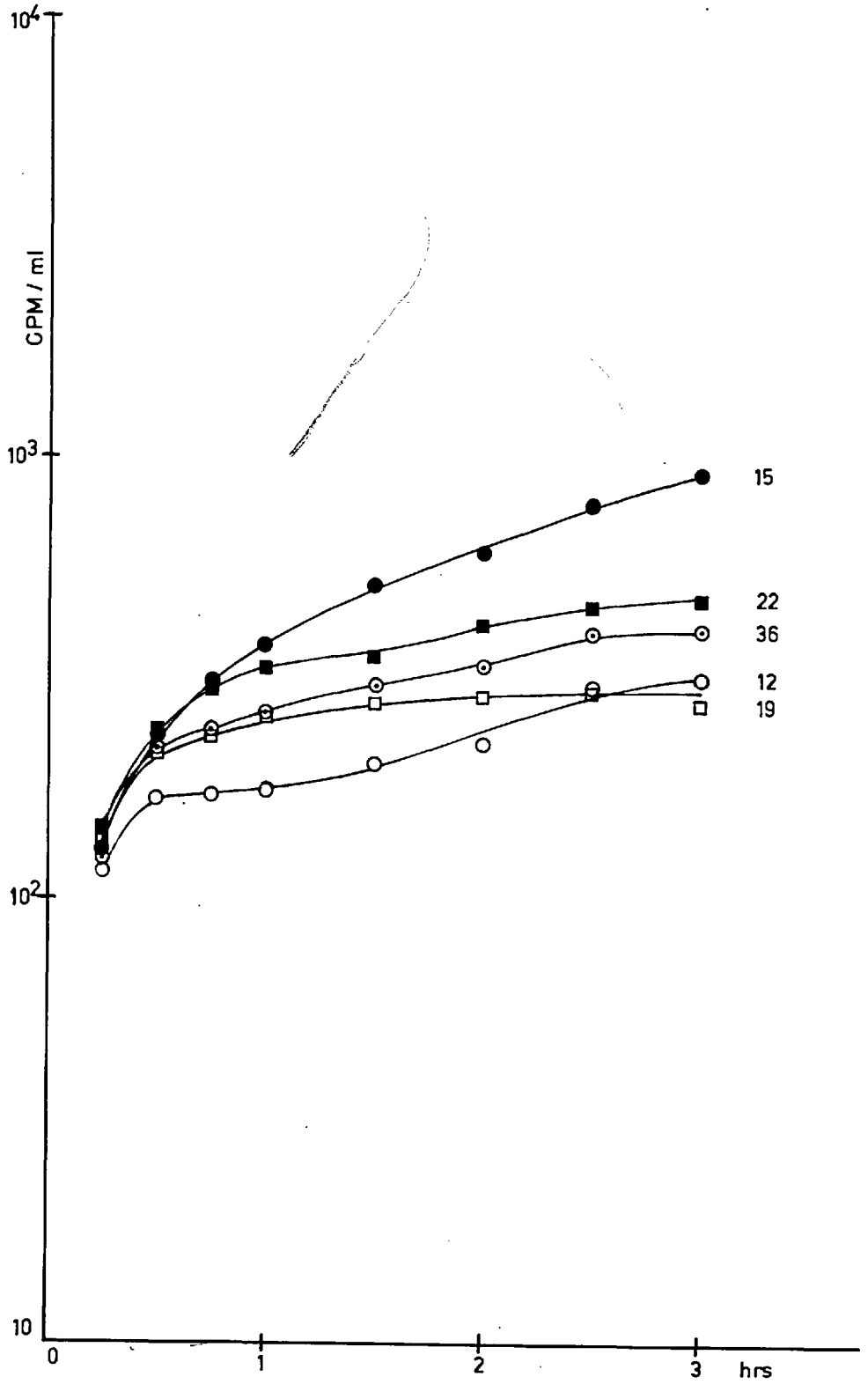


FIG. 10

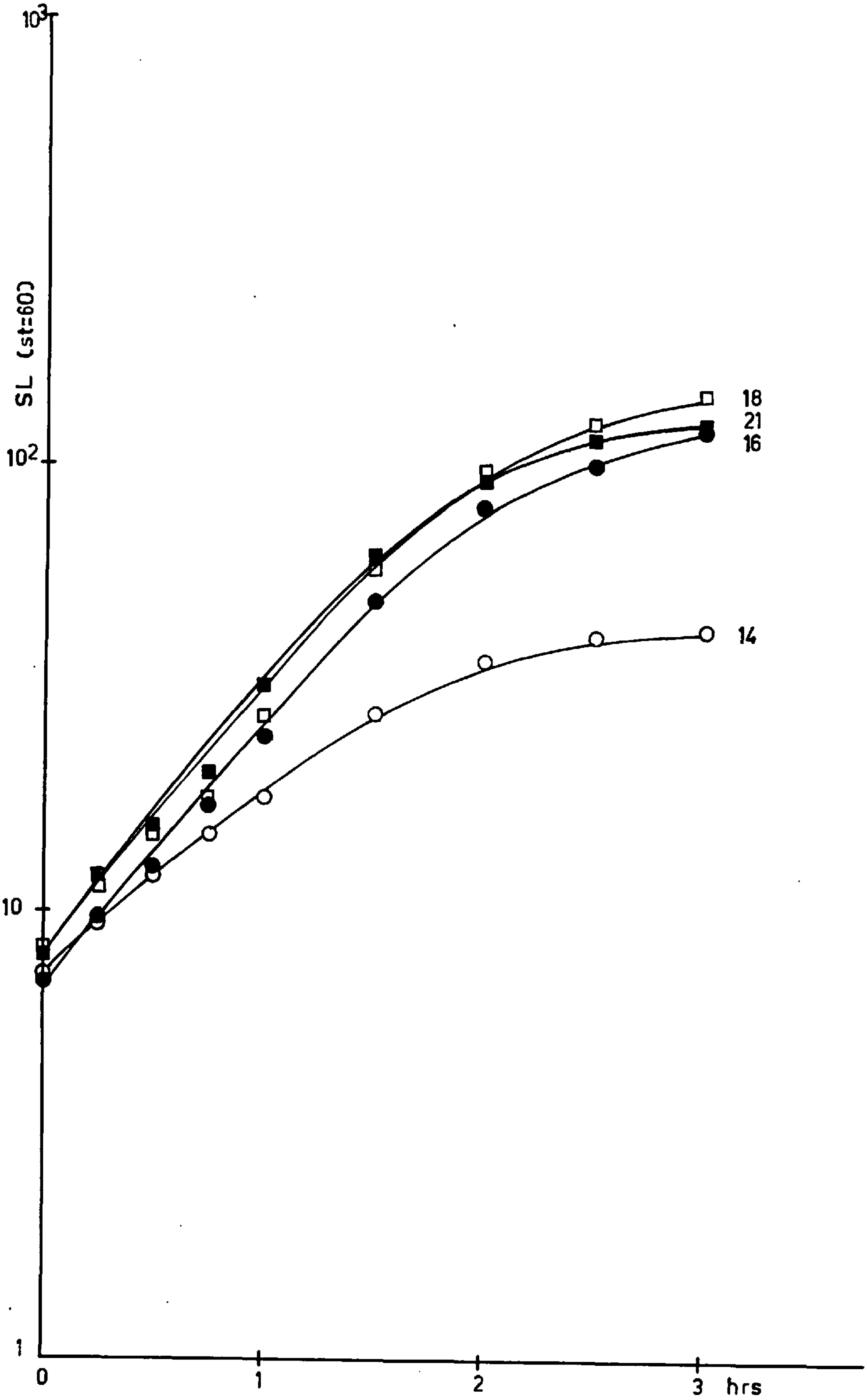


FIG. [illegible]

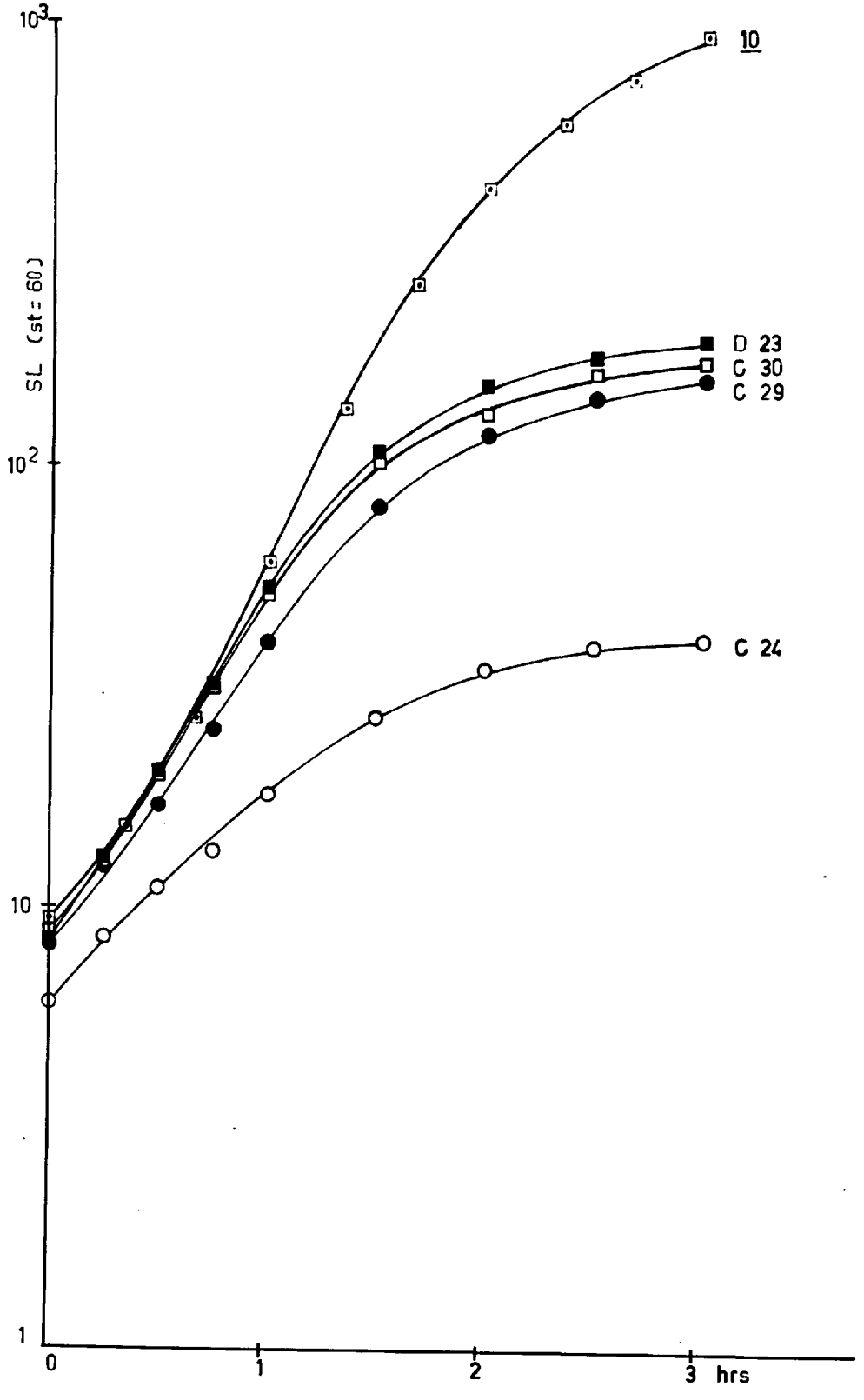


FIG. 12

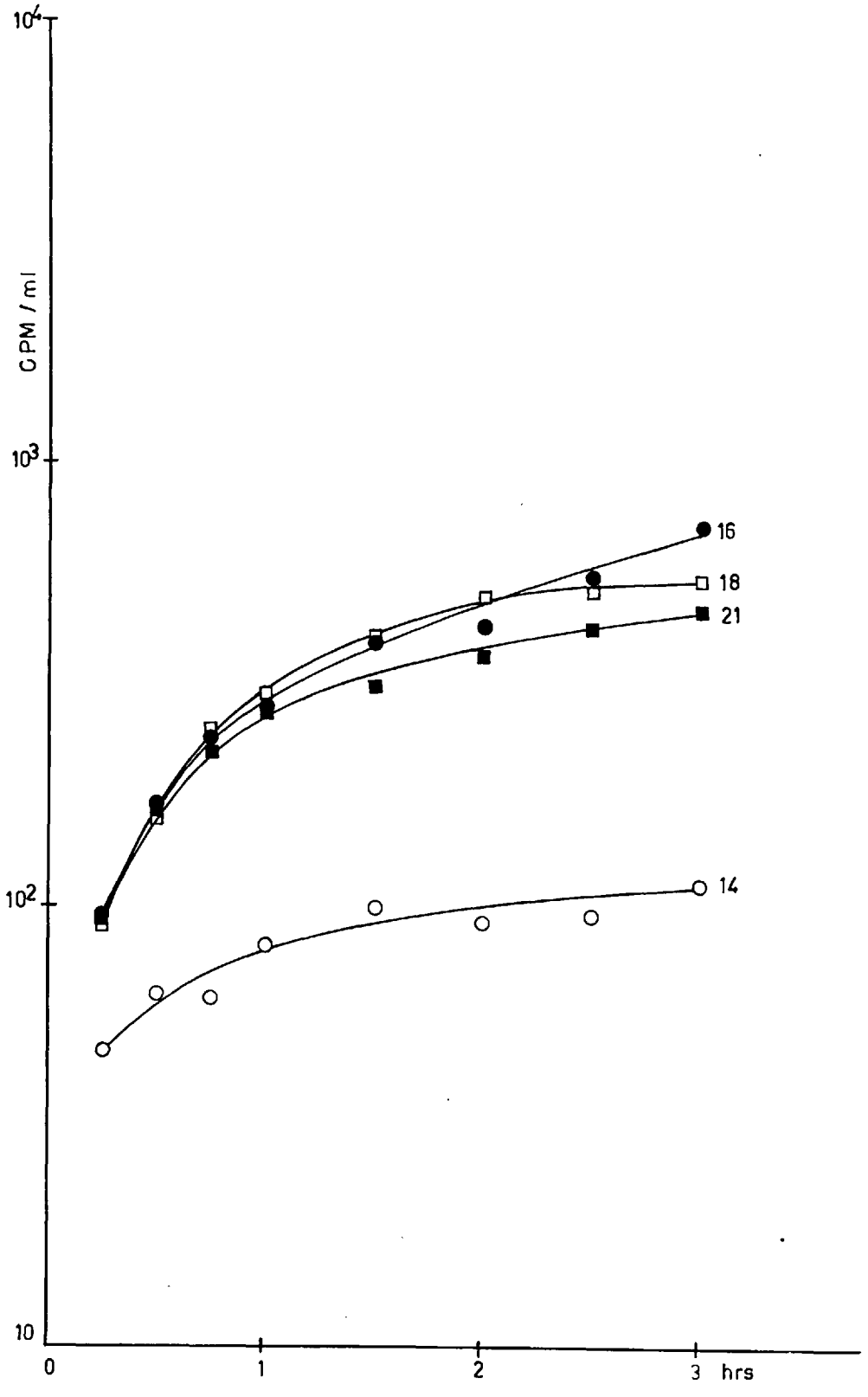
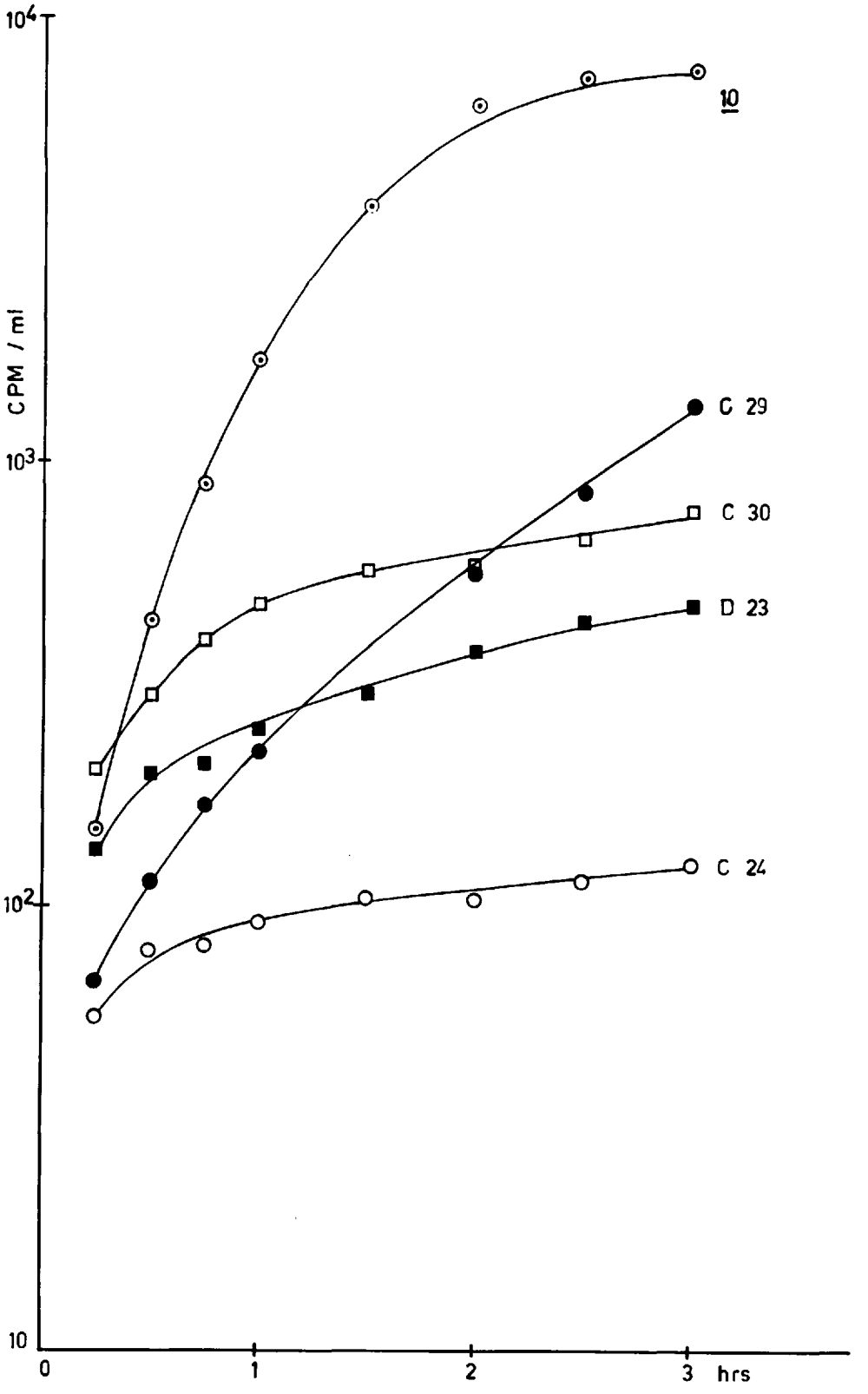


FIG. 33


10^4
10^3
10^2
10
CPM / ml
0
1
2
3
hrs
10
C 29
C 30
D 23
C 24

Figures 3-14: Kinetics of mass increase and DNA synthesis by ts DNA mutants after transfer to 45°C. Log-phase cultures of ts DNA mutants in strain 10 grown at 30°C in SATT medium were diluted in two tubes to a SL reading of 7 to 10, and further grown at 45°C. One tube was used for determination of mass increase as measured by SL readings. The other, to which ^{14}C-thymine was added, was used for measuring the residual DNA synthesis. Samples were withdrawn at regular intervals and the TCA precipitable radioactivity measured. On some figures the curves corresponding to wild strain 10 and the ts DNA mutant 168-ts B134 are plotted for comparison.

Figs. 3 and 4: Mass increase at 45°C by A group ts DNA mutants 3, 5, 7, 8, 9, 10, 13, 25, 27, and 28 in strain 10.

Figs. 5 and 6: ^{14}C-thymine incorporation at 45°C, by the A group ts DNA mutants 3, 5, 7, 8, 9, 10, 13, 25, 27 and 28 in strain 10.

Figs. 7 and 8: [illegible] Mass increase at 45°C by B group ts DNA mutants, 2, 4, 6, 11, 15, 19, 22 and 36 in strain 10.

Figs. 9 and 10: ^{14}C-thymine incorporation at 45°C, by the B group ts DNA mutants 2, 4, 6, 11, 15, 19, 22 and 36.

Figs. 11 and 12: Mass increase at 45°C by C group ts DNA mutants 14, 16, 18, 21, 24, 29, 30, and the D group ts DNA mutant, 23 in strain 10.

Figs. 13 and 14: ^{14}C-thymine incorporation at 45°C by C group ts DNA mutants 14, 16, 18, 21, 24, 29, 30 and the D group ts DNA mutant 23 in strain 10.

We are first going to describe groups A and C, and then groups B and D.

Groups A and C (Figs. 3-6 and 11-14). Ten A group mutants and 7 C group mutants were examined. As compared to wild strain 10 all mutants show a clear reduction in the amount of protein synthesized. The change of rate of protein synthesis appears at about 30 min. for A group mutants and between 45 and 60 min. for C group mutants. The total mass increase in 3 hours is different for each mutant; it varies from 5 to 15 times and is generally proportional to the amount of DNA made. Most A group mutants start slowly lysing after 2 or 3 hours at 45°C. The total amount of DNA synthesized is different for each mutant and is not characteristic of either groups A or C. The change in the rate of DNA synthesis is already apparent at 15 min. with most A group mutants and between 15 and 30 min. with most C group mutants. The total amount of DNA synthesized by some mutants was determined on prelabelled cells (grown at 30°C and 45°C in ^{14}C-thymine containing media). For mutants 10-tsA13 and 10-tsA25 the total increase in 3 hours is about 25%.

Groups B and D (Figs. 7-14). Nine B group mutants were analysed. The curves representing the SL readings are very similar for all mutants. The change in the rate of mass increase, as compared with

wild strain 10, appears between 45 and 60 min. after transfer to 45°C. The total mass increase is about 20 times for almost all mutants. Only in the case of mutant 10-tsB2 is it higher. The pattern of DNA synthesis is almost identical for all mutants, except the mutants 10-tsB2, 10-tsB11 and 10-tsB15. The rate of DNA synthesis is virtually normal until 30 min. Between 30 and 40 min. a sudden change occurs and DNA synthesis continues for at least 3 hours at a considerably reduced rate. Mutants 10-tsB2, 10-tsB11 and 10-tsB15 do not show an abrupt change of rate and synthesise considerably more DNA. Studies with prelabelled cells of mutants 10-tsB4 and 10-tsB19 show that the total increase in DNA synthesized amounts to about 80%. Compared with groupsA and C, group B mutants are much more homogenious with respect to DNA and protein synthesis.

The DNA and protein synthesis curves of mutant 10-tsD23 are identical with those of the standard B group mutants (for example 10-tsB19).

3.2.2. Viability

All ts DNA mutants are conditional lethals. If cultures of mutants are streaked on L-agar plates, incubated for 24 hours at 45°C and then further incubated at 30°C, only a few colonies formed by ts^+ revertants arise. Kinetic studies of the viabilities of 27 ts DNA mutants during 4 hours after the shift to 45°C, were made in the following way: Log-phase cultures in SATT medium at 30°C were diluted in the same medium, so as to get an SL reading of 7 to 10, and then grown at 45°C. At regular time intervals samples were withdrawn and viable counts determined as described in 2. The results are represented in Figures 15-20.

All A group mutants (Figs. 15 and 16), except the mutant 10-ts A3, die in a similar way. After a lag period of 30 mins. the cells die exponentially to a survival of less than 1 in 10^3 in 4 hours. Mutant 10-tsA28 reaches a plateau at a survival of 1 in 10^2. The exception, mutant 10-tsA3 shows little loss of viability in 4 hours. The 30 min. lag may be due to the fact that B. subtilis forms chains when grown in SATT medium, and a decrease in colony-forming units is apparent only when all the cells forming a chain are dead. When the exponential part of the death curve is extrapolated to time zero, one finds that the number of cells in a chain should be about 10.

This is comparable to the length of a chain as determined microscopically. The death pattern is unrelated to the amount of DNA synthesized or to the time at which the DNA synthesis stops. It seems to be a general character of the group.

The majority of B group mutants (Figs. 17 and 18) show a small increase in the number of colony-forming units during the first 45 min. at 45°C. Between 45 min. and 60 min. the cells start dying exponentially and within the next hour or two drop to a survival of 20-50%. From then on, until 4 hours after transfer to 45°C, death continues slowly. Mutants 10-tsB2 and 10-tsB11, which synthesize more DNA, have a slightly different death curve, the number of colony-forming units increases for about 2 hours and then follows a pattern similar to that of the majority of mutants. Mutant 10-tsB12 also shows aberrant behaviour; after the initial rise, the number of colony-forming units drops to the zero value and then remains constant up to 4 hours. However, the death pattern of B group mutants seems to be related to DNA synthesis: when a mutant is synthesizing more DNA its viable counts increase for longer periods of time after transfer.

Death curves of C group mutants (Figs. 19 and 20) split into two clearly different classes. Mutants 10-tsC18, 10-tsC24 and

10-tsC29 fail to die for at least 4 hours; the number of colony-forming units even increases slightly. Mutants 10-tsC14, 10-tsC16, 10-tsC21 and 10-tsC30 have a lag period of 30 to 90 min, after which they die exponentially and at 4 hours drop to a survival of 1 in 10^2 or 1 in 10^8. Here, as in case of A group mutants, the lag period can be explained by the existence of chains of cells. Death is clearly unrelated to DNA synthesis; both 10-tsC14 and 10-tsC24 hardly synthesize any DNA at 45°C, but 10-tsC14 dies whereas 10-tsC24 does not die at all in 4 hours. However, the death curves of the C mutants which die are different from a typical A group mutant death-curve; C group mutants start dying later and die more slowly.

The D group mutant 10-tsD23 (Fig. 20) has a constant viable count for 90 min. and then dies exponentially to drop to a survival of 1 in 10^3 at 4 hours.

We can conclude that, broadly speaking, the death pattern seems to be characteristic for each group, even taking into account the fact that C group mutants split into 2 classes. In groups A and C DNA synthesis and death are not related, but in group B they seem to be related.

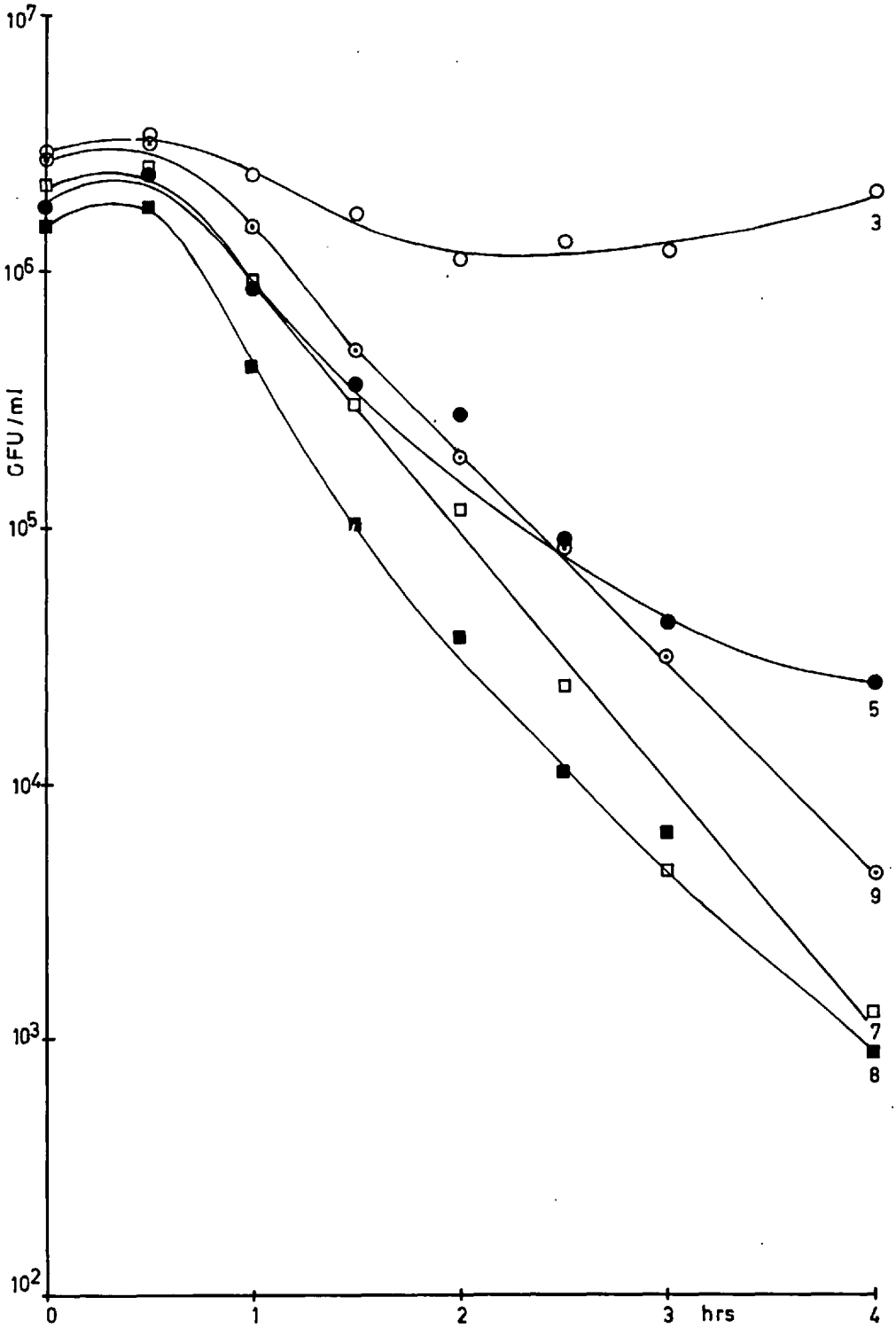


FIG. 16

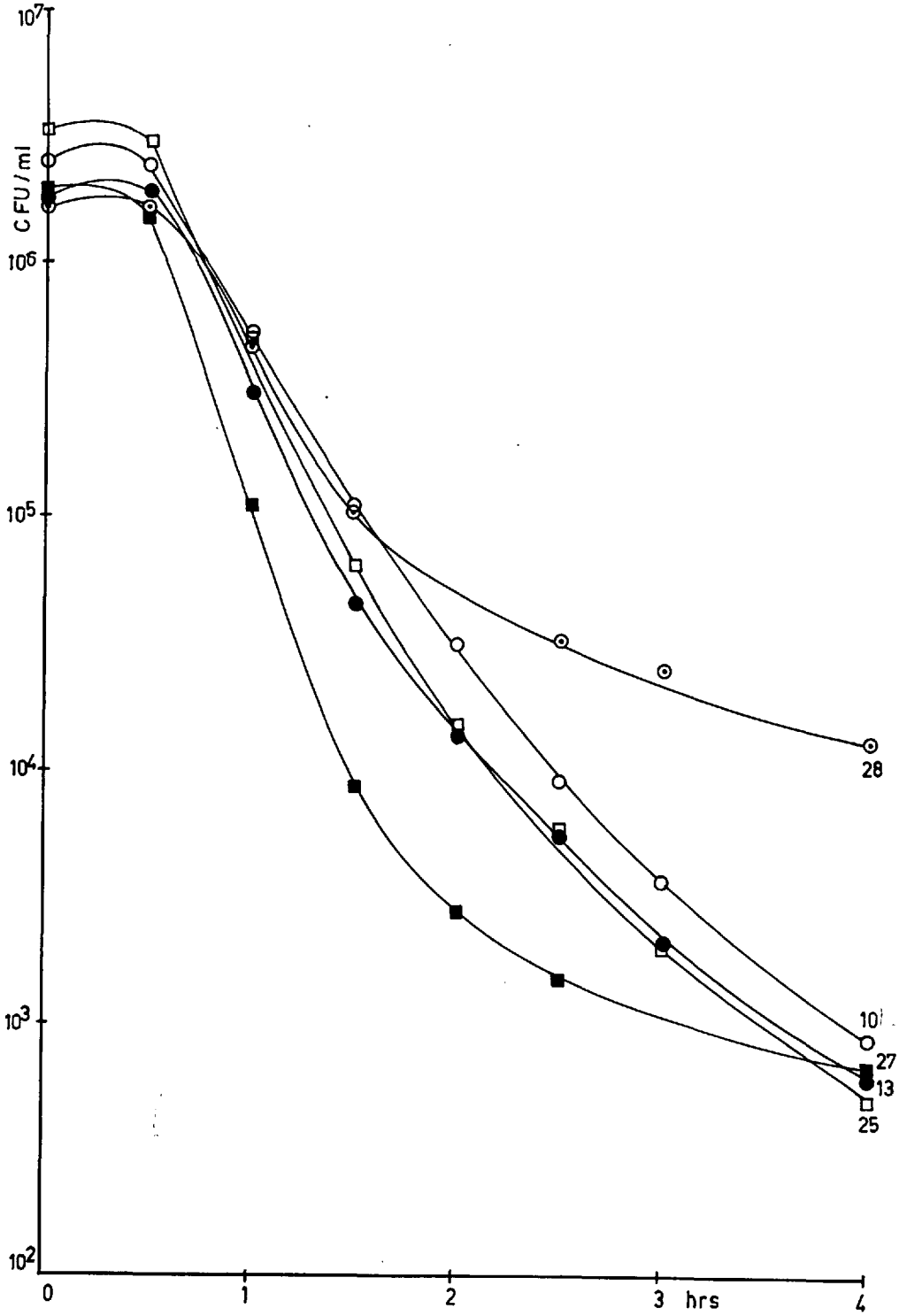


FIG. 10

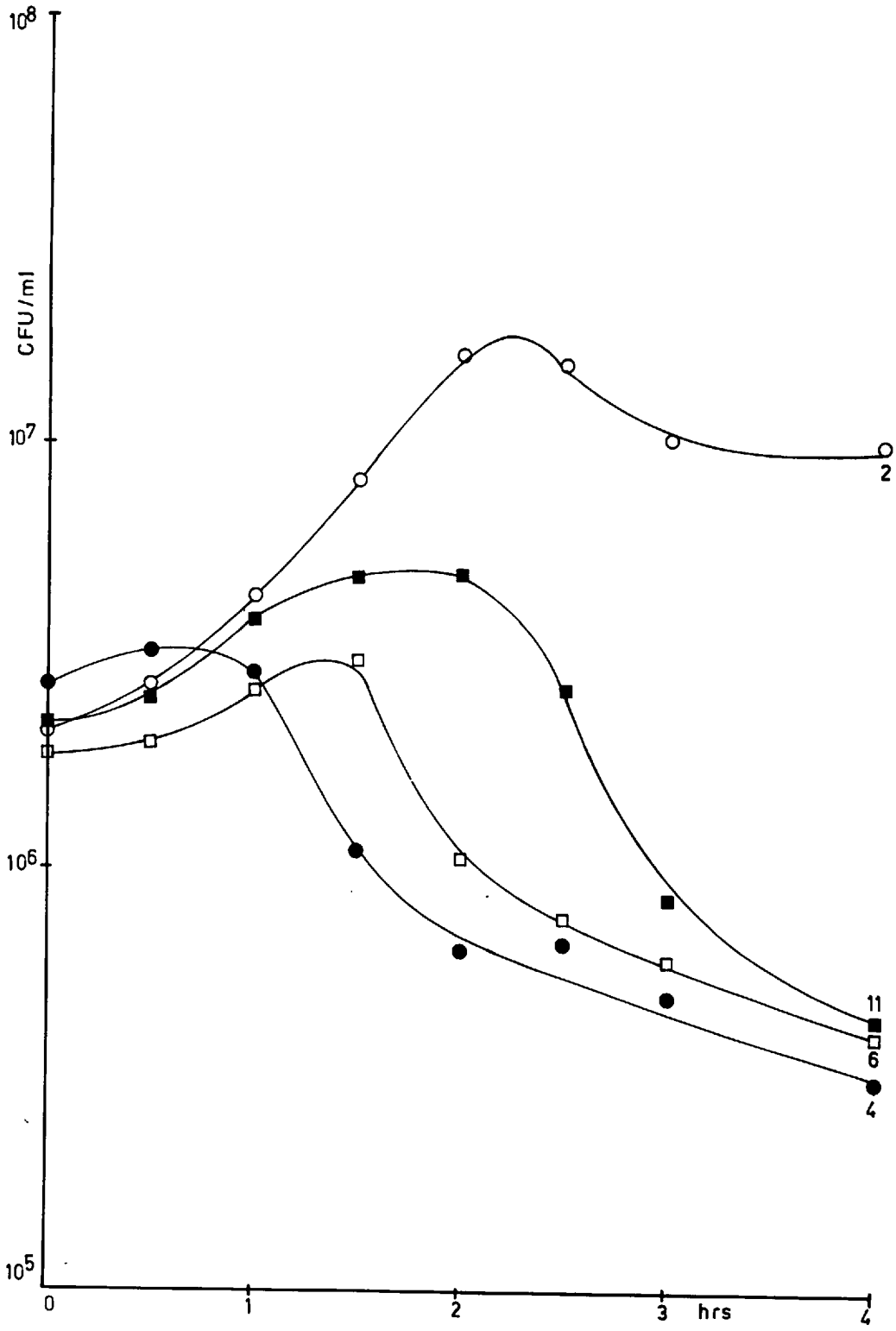


FIG. 15

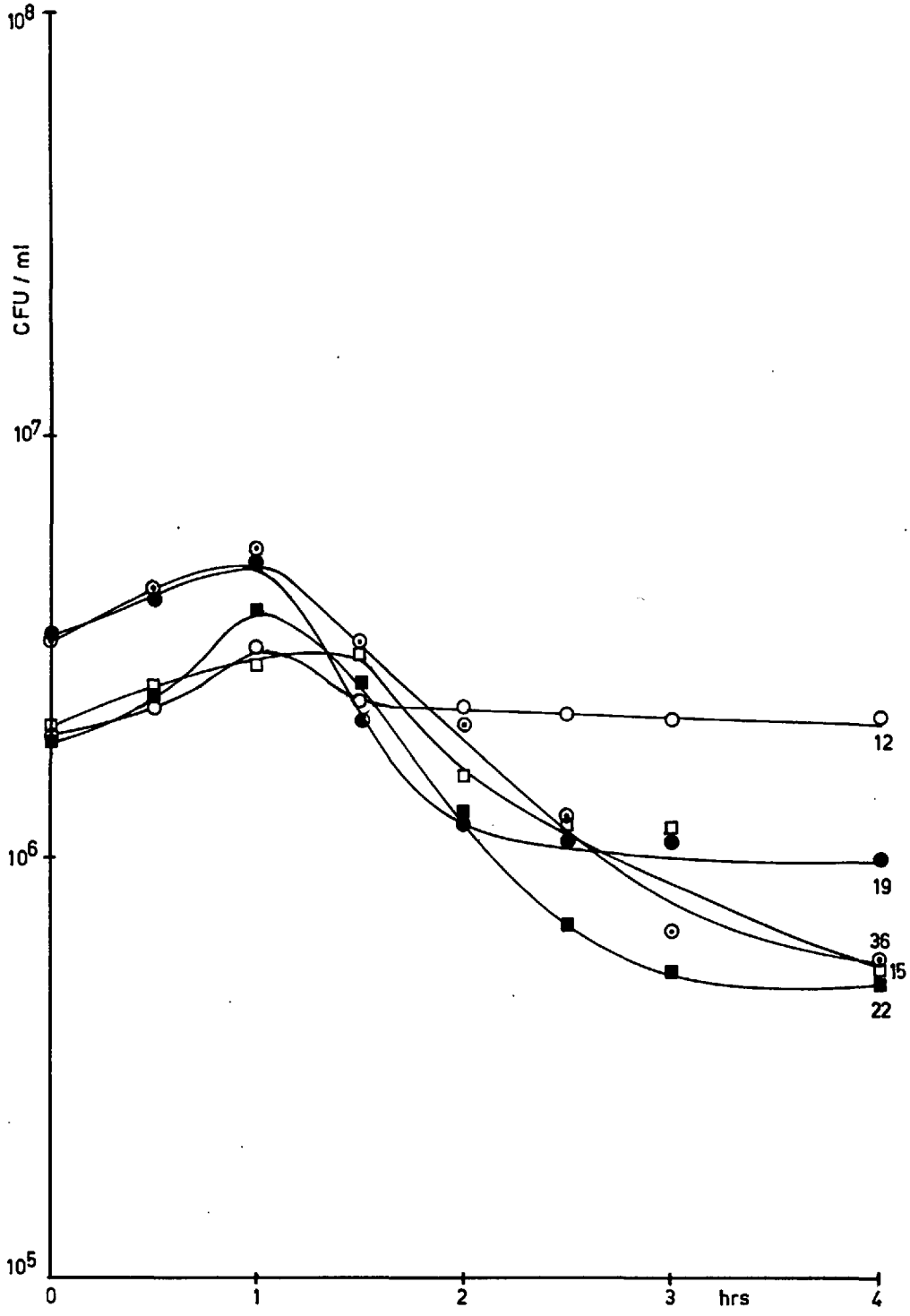


FIG. 20

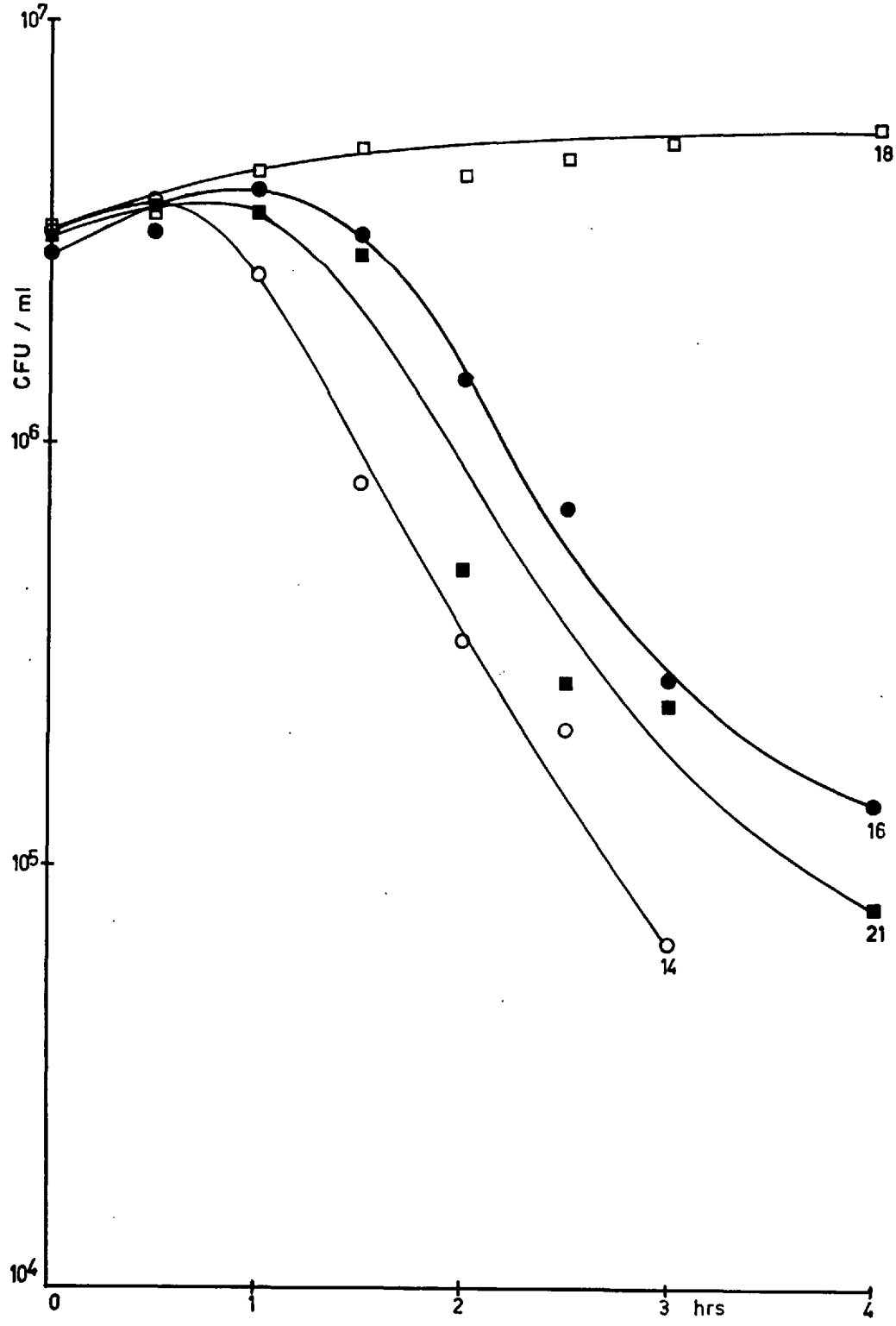


Fig. 12

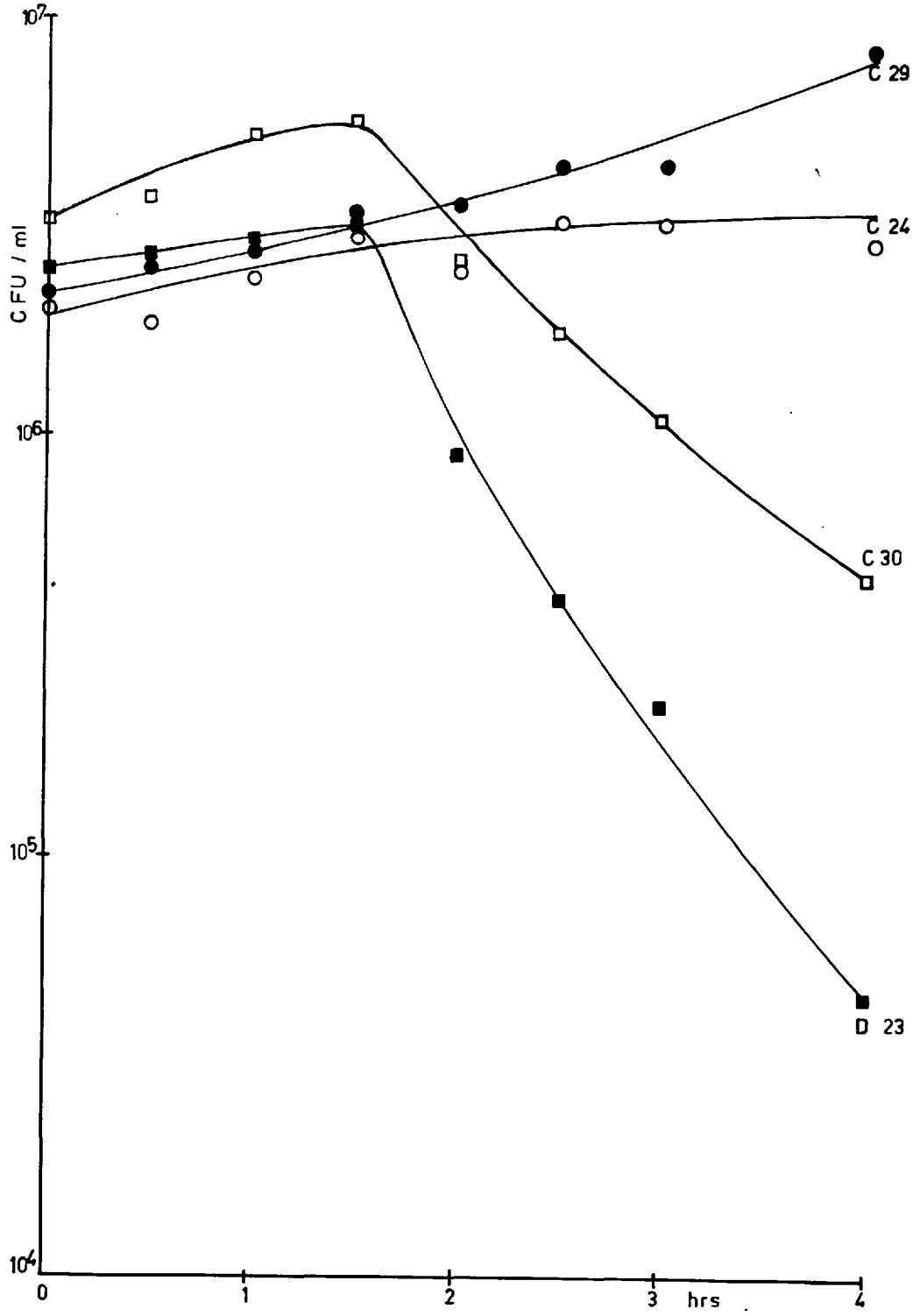


FIG. 22

Figs. 15-20: Viability of ts DNA mutants at 45°C. Log-phase cultures of ts DNA mutants in strain 10, grown at 30°C in SATT medium, were diluted in the same medium to an SL reading of 7 to 10, and grown at 45°C. At regular intervals samples were withdrawn and viable counts determined.

Figs. 15 and 16: Viability at 45° of the A group ts DNA mutants 3, 5, 7, 8, 9, 10, 13, 25, 27 and 28, in strain 10.

Figs. 17 and 18: Viability at 45° of the B group ts DNA mutants 2, 4, 6, 11, 12, 15, 19, 22 and 36, in strain 10.

Figs. 19 and 20: Viability at 45° of the C group ts DNA mutants 14, 16, 18, 21, 24, 29, and 30, and the D group ts DNA mutant 23, in strain 10.

3.2.3. Induction of PBSX

To determine whether mutations result in induction of PBSX particles at 45°C we used the ts mutants in strain 173, which is PBSX⁺. The experiment was performed in the following way: A log-phase culture in SATT medium, at 30°C, is diluted in the same medium to an SL reading of 7-10 and incubated at 45°C for 3 hours. After centrifugation of bacterial debris, and filtration, the lysates are assayed for PBSX activity as indicated in 2. One mutant from each group was examined, namely mutants 173-tsA13, 173-tsB19, 173-tsC30 and 173-tsD23. Strain 172 was used as ts⁺ control.

Lysates of all 4 mutants showed PBSX particle activity even when diluted 30 times, whereas the undiluted lysate of 172 had no activity. It is well known that inhibition of DNA synthesis, for example by thymine starvation of thy⁻ strains or by mytomycin C or uv treatment, induces PBSX particles in B. subtilis PBSX⁺ strains (Seaman et al, 1964; Ionesco et al, 1964). Our results show that whichever of the four identified DNA genes is defective, the cessation of DNA synthesis leads to PBSX particle production. In other words, irrespective of the way in which DNA synthesis is prevented, one of the consequences is always PBSX induction. However, protein synthesis is obviously required for PBSX production, as shown by Mendelson and Gross (1967) for the ts DNA mutant 168-tsB134: Indeed if chloramphenicol is added at the

time of shift to 45°C there is neither lysis nor PBSX activity. PBSX particles contain bacterial DNA and their production does not require synthesis of either bacterial or phage DNA (Seaman et al, 1964). Therefore one cannot say whether any cell enzyme is involved in phage DNA synthesis, or whether any of the four identified genes codes for an enzyme common to host and PBSX particle DNA synthesis.

4.2.4. Morphology

The morphological changes of ts DNA mutants, occuring at 45°C, were studied by phase-contrast microscopy. By the method used (see 2.2.) one can see cells and cell nuclei. Thus one can follow in time the changes of size and shape of the cell and the nucleus, or see if the cell lacks a nucleus. Log-phase cultures in SATT medium at 30°C were diluted in the same medium at 45°C and further incubated. Samples were withdrawn every 20 to 30 min., mixed with PVP and examined as described in 2.2. In what follows we will first describe the observations on A and C group mutants and then the observations on B and D group mutants.

Groups A and C. Only the mutants 10-tsA13 and 10-tsC30 were examined. Upon transfer to 45°C the cells start to elongate and after about 2 hours some cells are at least 3 times longer than

normal. Before the maximum length is reached, the cells begin to swell at one end and become pear-shaped. After two hours some cells of 10-tsA13 start lysing. The nucleus usually remains at one extremity of the cell and becomes more diffuse with time. However, it is difficult to decide whether the diffuse appearance is due to dispersion of DNA (dislocation of the nucleus) or to a modification of the cytoplasm such that the contrast is no longer sufficient to observe the nucleus properly. At some points towards one extremity of the cell one can often see the cell wall narrowing as if the cell was going to divide. No anucleate cells were seen. The mutant 10-tsC30 differs slightly from 10-tsA13: the cells increase in width and in length at the same time and so look much thicker.

Groups B and D. We are first going to describe in detail the observations on the mutant 10-tsB19. After transfer to 45°C the cells grow normally and divide during the first 40-60 min. Later they continue to elongate normally, but every 20 min. a septum is formed which separates a normal cell from an anucleate cell of normal length. This process continues for at least 80-100 min. and, as strain 10 cells remain joined in SATT medium, one can see chains of four to eight anucleate cells connected to one normal cell. Every cell seems to produce anucleate cells; when anucleate

cells start appearing in chains of normal cells one get chains in which normal and anucleate cells alternate. As anucleate cells are always of the same length they obviously do not divide. Mutants 10-tsB4 and 10-tsB12 behave in the same way whereas the mutant 10-tsB2 seems to start producing anucleate cells only after 3hhours or more at 45°C. This is understandable because it synthesizes DNA at 45°C for at least 2 hours.

Mutant 10-tsD23 is very similar to 10-tsB19. However, the anucleate cells appear in a less regular way and the cells look slightly thicker than at 30°C.

This comparison of the effects on morphology of arresting DNA synthesis by B or D mutations suggests that the cells divide and segregate nuclei until there is only one nucleus per cell, and then continue dividing and producing anucleate cells (see 3.2.5.).

3.2.5. Discussion

It would be interesting to know what are the functions of the ts DNA mutants isolated so far. Genetic analysis shows that the ts DNA mutants are distributed into four small linkage groups and, based on the estimate of the size of the groups, it is suggested that each group is composed of one or at the most of two genes. In 3.2.1-4 we have seen that all mutants belonging to any single group have some

common physiological properties. It seems that, even if each group is composed of several genes, all the genes belonging to one group are involved in a single pathway. We are therefore going to try to identify the function of each group and in what follows we will use the terms gene and group as interchangeable. The first task would be to classify genes A, B, C and D into the three classes defined in the introduction, namely, to see if they are involved in the intermediary metabolism, replication or regulation of DNA synthesis.

As far as intermediary metabolism is concerned, our results provide only two hints. First, the morphology and the viability of A group mutants at 45°C are similar to those of a thymine-requiring strain starved of thymine. Thus A group mutants exhibit the phenotype of mutants deficient in some precursor. Second, group C is very closely linked to a purine marker, and if all genes involved in deoxypurine triphosphate synthesis were clustered C could be one of them. Far more information is provided by the preliminary experiments done by Dr. G. Bazill (personal communication). In these experiments the sizes of the pools of all four deoxynucleoside triphosphates is measured. The cells are incubated for one hour at 45°C in inorganic ^{32}P-containing media and then lysed. The lysate is fractionated by thin-layer chromatography and the ^{32}P content

of the four deoxynucleoside triphosphate spots measured. If intermediary metabolism is normal, these pools should be comparable to those obtained with the wild strain. In this way one mutant of each of the groups A, B, C and D was analysed. The result shows that there is no significant qualitative or quantitative difference between any of the mutants and the wild strain 10. If one assumes that the deoxynucleoside triphosphates are the ultimate precursors of DNA molecules, then this evidence strongly suggests that none of the genes A, B, C or D codes for enzymes involved in intermediary metabolism.

The only enzymes so far known to be involved in DNA replication are the DNA polymerase (Kornberg, 1962) and the DNA ligase (Gellert, 1967). Genes coding for the DNA polymerase and the DNA ligase have been identified in phage T4 (de Waard et al, 1965; Richardson et al, 1968). Conditional lethal mutations in these genes block phage DNA synthesis (Epstein et al, 1963). The DNA polymerase activity of all our ts DNA mutants was examined in in vitro systems by Hempstead (1968). Crude extracts were prepared from cells grown at 30°C as well as from cells incubated at 45°C for different intervals of time. The extracts were assayed for DNA polymerase activity in in vitro systems at 45°C. Extracts of all genetically analysed ts DNA mutants were found to have normal activity. These experiments seem to indicate that none of

the genes A, B, C or D are coding for the DNA polymerase. No assays for ligase activity have been done, so that we cannot exclude the possibility that one of the identified DNA genes is coding for it. So far there is no positive evidence that any of the genes A, B, C or D is involved in DNA replication at the growing point.

The physiological and genetic analysis provide indications that B group mutants may be involved in regulation and be of the initiator type. The evidence for this is as follows:

a) The mutant 168-tsB134, which is believed to be involved in initiation (see Introduction), maps in group B.

b) Our experiments show that the amount of DNA synthesized at 45°C by B group mutants represents a total increase of about 80%, or much over 80% in few exceptional cases. No B group mutant so far isolated shows an increase of less than 80%. If B is coding for an initiator protein one would expect that the ts B mutants would complete at 45°C all the rounds of replication underway, and thus synthesize at least the amount of DNA needed for completion. If the mutations were leaky the mutants could, of course, synthesize more DNA. Our cells were grown in SATT medium in which, according to Oishi et al (1964) the majority of chromosomes should have three growing points. One can calculate that if all rounds of replication under way can terminate, but no new ones can be initiated, the total increase of DNA synthesized should be about 80% which is exactly the amount synthesized by most B group mutants at 45°C.

To test the suggestion that B genes are involved in initiation the following preliminary experiments were carried out:

a) The first experiment is designed to distinguish between two classes of genes designated I and R. Products of I genes are assumed to be needed for initiation of new rounds of replication but not for completion of those under way. Products of R genes are assumed to be involved in replication or intermediary metabolism so that some R mutations could result in rapid cessation of DNA synthesis regardless of the positions of the growing points on the chromosome. We have seen that ts mutations result in thermo-labile proteins and we can assume that the stability of most thermo-labile proteins will decrease with increasing temperature. Therefore ts mutations in R genes could result in a decrease in the amount of DNA made, whose rate of cut-off would increase with increasing temperature. On the contrary, independently of the rate at which the gene products are disintegrated, ts mutants in I genes should show at least an 80% increase, corresponding to completion of rounds of replication under way, as long as the temperature is below the maximum temperature at which the wild-type can grow. As B. subtilis grows normally at 48°C, and as all our ts mutants already express the mutant phenotype at 45°C, we compared the amounts of DNA synthesized at 45°C and at 47°C by mutants from groups A, B, C and D. The result shows that some A and C group mutants synthesize much less DNA at 47°C than at 45°C;

the total increase is sometimes reduced to 20%. The amount of DNA_n^N made by most B group mutants, as well as by the D group mutant, is 80% at both temperatures. The higher amount synthesized by a few exceptional B group mutants tends to be reduced to an 80% increase at 47°C. Judging by this criterion B as well as D genes may be involved in initiation.

b) A second way of distinguishing between ts mutants in I and R genes is to prevent DNA synthesis for some time after transfer to 45° C. During the period of inhibition R genes products should decay and the amount of DNA synthesized after resumption of synthesis should be considerably reduced as compared to the amount normally made at 45°C. I gene ts mutants, which are potentially capable of completing already initiated rounds of replication, should continue to be able to do so after resumption of DNA synthesis, and the total amount of DNA synthesized should be the same as if there had been no inhibition period. As ts mutants in strain 10 are thy⁻ we prevented DNA synthesis by withdrawing thymine during the first 30 min. after transfer to 45°C; we then restored DNA synthesis by adding thymine and determined the amount of DNA synthesized during the following 2 hours. The experiment was performed with mutants from all four groups. It shows that ts mutants of genes A and C make considerably less DNA after thymine starvation. On the

contrary, the amount of DNA made by B and D group mutants is the same whether the mutant was or was not starved of thymine. This kind of experiment is more difficult to interpret than a) because thymine starvation initiates new points of replication (Pritchard and Lark, 1964). Therefore the number of growing points of B and D group mutants should increase during the starvation period and so should the total amount of DNA synthesized after the starvation. However, initiation by thymine starvation has not been demonstrated for cells which already have 3 points of replication (which is the case of B. subtilis grown in SATT medium). This result strengthens the idea that genes B and D are involved in initiation.

c) In the introduction we have seen that DNA replication in B. subtilis starts at a fixed origin and proceeds unidirectionally. This allows a more direct approach to distinguishing between I and R genes by determining the ratio of the number of copies of any gene near the initiation point (origin) to the number of copies of any gene near the terminus of the chromosome. We will designate that ratio $^{O}/_{T}$. Exponentially growing cultures of ts mutants, in SATT medium at 30°C, have three growing points and the ratio $^{O}/_{T}$ is 4:1. In R gene ts mutants, DNA synthesis stops at 45°C independently of the position of the growing points, so that the $^{O}/_{T}$ ratio should remain 4:1

as in exponentially growing cells. On the contrary ts mutants involving I genes should complete, at 45°C, the rounds of replication under way, so that the ratio O/T should become 1:1. The O/T ratios were determined in the following way: We choose ad as a marker close to the origin and ileu as a terminal marker (see Fig. 2). DNA was extracted from cultures of ts mutants in strain 10 (ad^+ileu^+) grown at 30°C, as well as from cultures grown at 45°C for one hour. The ratio of ad to ileu markers was determined by using the DNA to transform an ad^- $ileu^-$ recipient, selecting for ad^+ and $ileu^+$ transformants. Results show that ts mutants of genes A and C have a temperature-independent O/T equal to 2.5-3.5:1. For mutants in genes B and D the ratio O/T changes from 2.5-3.5:1 at 30°C to 1:1 at 45°C. Although not very accurate these measurements provide additional evidence that B and D genes are involved in initiation.

All the experiments to date indicate that B and D group mutants complete at 45°C all rounds of replication already initiated, but that they cannot initiate new rounds. The possibility that two proteins may be involved in the initiation of E. coli DNA replication has been already suggested by Lark and Lark (1964) (see Introduction). The two proteins can be distinguished by the different sensitivity to chloramphenicol of their mechanisms of synthesis. However, we have not tested the sensitivity to chloramphenicol of the mechanisms of

synthesis of B and D genes products and cannot compare our results with those of Lark. As far as groups A and C are concerned the analysis of their physiological properties, and the experiments recounted above, eliminates the possibility that they are concerned with initiation.

On the basis of the results discussed above we will conclude by briefly examining the relations which exist between cell division and DNA synthesis, and which we mentioned in 3.2.4. In 3.2.4. we saw that B and D group ts mutants start producing anucleate cells at 45°C as soon as DNA synthesis ceases, and that all the existing nuclei segregate. Under the same conditions, A and C group ts mutant cells elongate but no cell division occurs, nor do anucleate cells appear. On the other hand the preliminary results, discussed above, show that B and D group mutants complete at 45°C all initiated rounds of replication, whereas the replication of A and C mutant chromosomes stops at any point. Thus it seems that cell division and DNA synthesis are related in the sense that cells can, and do, divide if all the genomes are completed, but do not divide so long as a round of replication is under way. This may be interpreted on the assumption that as long as a round of replication is under way the formation of the corresponding septum is repressed and that septum formation can begin only when replication is completed. It follows that when all rounds of replication are

completed, as is the case with B and D group mutants at 45°C, cell division is no longer under repression and anucleate cells are produced.

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