TEMPERATURE-SENSITIVE DNA SYNTHESIS MUTANTS OF *BACILLUS SUBTILIS*—APPENDIX: THEORY OF DENSITY TRANSFER FOR SYMMETRIC CHROMOSOME REPLICATION[1]

KALPANA WHITE AND NOBORU SUEOKA[2]

Departments of Biochemical Sciences and Biology, Princeton University
Princeton, New Jersey 08540

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ABSTRACT

A simple method for characterizing temperature-sensitive DNA synthesis mutants is described. The method uses density transfer and transformation techniques and is based on expected theoretical behavior of the chromosome population. A direct proof of inhibition of initiation of DNA replication is provided. The mutant *dna*-1, showing quick inhibition of initiation, is further characterized and mapped. An independent method for mapping genetic markers close to the origin, based on their transfer behavior after inhibition of initiation, is presented.

A variety of temperature-sensitive mutants which synthesize limited amounts of DNA at high temperature are known in *Escherichia coli* and in *Bacillus subtilis*. The interest in residual synthesis has been primarily focused on the amount of DNA synthesis and kinetics of shut-off under various physiological conditions (KOHIYAMA *et al.* 1963; BONHOEFFER and SCHALLER 1965; KOHIYAMA *et al.* 1966; MENDELSON and GROSS 1967; FANGMAN and NOVICK 1968; GROSS, KARAMATA and HEMPSTEAD 1968).

In general, some of the causes that could lead to gradual shut-off are 1) inhibition of initiation of new rounds of chromosome replication, 2) gradual inhibition of initiation, 3) leaky inhibition of replication, 4) anomalous replication which could result from abnormal initiation or replication or precursor synthesis.

We have isolated ts-DNA mutants in *B. subtilis*; several of these synthesize varied amounts of DNA at high temperature. To distinguish between causes which result in gradual shut-off, we have examined the DNA made at the restrictive temperature for the distribution of various genetic markers in a density shift experiment. As normal replication in *B. subtilis* has been shown to start at a fixed origin and proceed in a definite temporal sequence (YOSHIKAWA and SUEOKA

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[2] Present address: Department of Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, Colorado 80302.

1963a,b; O'SULLIVAN and SUEOKA 1967; OISHI, YOSHIKAWA and SUEOKA 1964), it is possible to predict the pattern of behavior of genetic markers at known positions on the chromosome in each case. The methodology for such analysis is established.

Both in *E. coli* and *B. subtilis*, mutants which fail to initiate DNA replication at restrictive temperature have been reported (KOHIYAMA *et al.* 1963; MENDELSON and GROSS 1967; FANGMAN and NOVICK 1968; GROSS, KARAMATA and HEMPSTEAD 1968; HIROTA, MORDOH and JACOB 1970; ABE and TOMIZAWA 1971). The evidence, although convincing, has been indirect. Moreover, the criteria often have not been sensitive enough to distinguish between immediate inhibition and leaky inhibition of initiation. Here we provide a direct proof for inability in a mutant to initiate at non-permissive temperature. Further, we have investigated the kinetics of transfer of various genetic markers after inhibition of initiation and have shown it to be consistent with the theoretical expectation. In the APPENDIX, theoretical treatment of DNA and marker transfer kinetics is presented for density transfer during steady-state replication, and also for a special case where initiation is inhibited at the time of density transfer.

MATERIALS AND METHODS

1. *Bacterial strains:* The bacterial strains used were:

Strain designation	*Genotype*	*Source*
B. subtilis 168*TT*	*try2,thy*	F. ROTHMAN
168 *Try*	*try*	J. SPIZIZEN
Mu8u5u6	*leu8,met5,ade6*	This laboratory
Mu8u5u16	*leu8,met5,ade16*	This laboratory
BC 200	*thy,leu8,met5*	J. COPELAND
But 23.1	*thy,his But*23.1	This laboratory

168 *Try* is the source of all strains in this table.

168*TT* is the parent strain for all the tsDNA mutants isolated.

2. *Media and buffers:*

C medium: The minimal salt-glucose medium of SPIZIZEN (1958).

C+ medium: The C medium supplemented with 500 μg/ml casamino acids (Difco), 50 μg/ml tryptophan, 20 μg/ml uracil and 10 μg/ml thymine.

BrU-C+ medium: The C medium supplemented with 500 μg/ml casamino acids, 50 μg/ml tryptophan, 20 μg/ml uracil and specified amounts of bromouracil and thymine.

Penassay medium: Antibiotic medium 3 for penicillin assay (Difco), 12 g per liter.

TBA plates: Tryptose blood agar base (Difco), 33 g per liter.

Potato extract plates: The C medium supplemented with 0.5% potato extract (Difco), 40 μg/ml $CaCl_2$, 4 μg/ml $MnCl_2$, 50 μg/ml of each auxotrophic requirement and 4% agar.

Transformation medium: BOTT and WILSON (1967) medium for competent cells.

3. *Mutagenesis and enrichment for ts-dna mutants:* Our approach for isolating ts-dna mutants was the same as that of MENDELSON and GROSS (1967). Exponentially growing cells were mutagenized with N-methyl-1-N'-nitro-N-nitrosoguanidine and mutants were enriched by allowing the incorporation of bromouracil at non-permissive temperature. 168TT was the parent strain; 30°C and 45°C were the permissive and non-permissive temperatures, respectively.

4. *Radioactive assay:* ^{3}H-methyl-1-thymine (5 mc/0.0362 mg) and 2-^{14}C-thymine (0.25 mc/0.565 mg) and ^{3}H-leucine were obtained from New England Nuclear Corp. Radioactive samples were assayed for acid precipitable counts. Samples were precipitated by adding 4 to 20

times as much 10% ice-cold TCA and 100 μg of carrier DNA (salmon sperm DNA). Normal total volume was 2 to 2.5 ml. The precipitate was collected on a glass fiber filter (Whatman GF/A) and washed with 15 ml of 10% ice-cold TCA, dried and counted in a liquid scintillation counter.

5. *Screening for ts-dna mutants:* The criteria for ts-dna mutants were essentially similar to those of HEMPSTEAD (1968). Exponentially growing mutant cells along with a parallel culture of the parent strain 168TT were shifted to high temperature. At the shift, ^{3}H-leucine and ^{14}C-thymine were added. Incorporation was terminated at the end of 90 min by adding 10% TCA and acid precipitable radioactivity was counted. The culture showing a low ratio of $^{14}C/^{3}H$ as compared to the parent strain were considered to have a lowered DNA metabolism.

6. *Density transfer experiments:*

Uniform labelling with ^{14}C thymine: An overnight culture was diluted fifty-fold into C+ medium containing ^{14}C-thymine (0.2 μc/ml to 0.5 μc/ml) and allowed to grow for several generations.

Labelling with bromouracil and ^{3}H thymine: Cells were collected over 55 mm Millipore filter (GSWP05500) by suction, washed with 50 ml C salts supplemented with glucose, and resuspended in a BrU-C+ medium (bromouracil to thymine, 10 to 1, 2 μc/ml ^{3}H-thymine).

Preparation of lysates and cesium chloride centrifugation: Cultures were quick-chilled on ice, centrifuged and resuspended in 1.5 ml NaCl (0.15 M) + EDTA (0.1 M) pH 8.2. Cells were lysed with lysozyme (0.5 mg/ml, 30 min, 37°C) and sodium lauryl sulfate (0.5%, 15 min, 37°C). The lysates were centrifuged in a CsCl density gradient (final density 1.70 g/cm^{3}) in Tris (0.01 M) + EDTA (0.001 M) buffer (pH 8.4) for 72 hr at 25°C, 35,000 rpm in SW 50.1 rotor. Three drop fractions were collected and each fraction was diluted with 1 ml SSC (0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0). 0.5 ml aliquots were removed for radioactive counting.

Transformation: Transforming activity was determined by adding 0.1 ml aliquots to 1 ml competent cells and plating on selective media after 40 min incubation. Competent cells were prepared by the method of BOTT and WILSON (1967).

7. *Preparation of crude lysates for marker frequency analysis:* Cultures were quickly chilled on KCN (0.02 M)-ice. Cells were centrifuged and resuspended in 1.5 ml NaCl (0.15 M) + EDTA (0.1 M) pH 8.2, lysed with lysozyme (0.5 mg/ml, 30 min, 37°C) and then digested with pronase (0.1 mg/ml, overnight at 4°C).

8. *Preparation of spore DNA:* 168 *Try* spores were prepared on potato extract medium plates and harvested and purified by treatment with lysozyme and sodium lauryl sulfate (MANDEL and ROWLEY 1963). Lyophilized spores were ground to a fine powder with sea sand (under liquid nitrogen), suspended in NaCl-EDTA and incubated with lysozyme (1 mg/ml, 1 hr at 37°C) and then with sodium lauryl sulfate (1%, 30 min, 37°C). Sodium perchlorate was added to a final concentration of 1.25 M. After deproteinization with an equal volume of isoamyl alcohol-chloroform (24:1) DNA was precipitated with ethanol and resuspended in 1 × SSC.

9. *Temperatures used:* For all initial experiments 30°C was the permissive temperature. For experiments with *dna*-1, 34°C was used as the generation time was shorter at 34°C compared to 30°C. 45°C was used as the non-permissive temperature.

10. *Mapping dna-1:* Mutant *dna*-1 was mapped using density transfer techniques (O'SULLIVAN and SUEOKA 1967).

RESULTS AND DISCUSSION

Analyses of ts-dna mutants: From two independently mutagenized batches, 35 temperature-sensitive mutants showed lowered DNA metabolism but normal protein synthesis. As previously observed by other workers (BONHOEFFER and SCHALLER 1965; KOHIYAMA *et al.* 1966; FANGMAN and NOVICK 1968) the mutants fall into two main classes, immediate shut-off and gradual shut-off. The mutants showing residual synthesis were analyzed for the nature of synthesis at restrictive temperature.

In normal replication, an exponentially growing culture, when shifted to a heavy (DNA precursor) medium, will show an equal transfer of all genetic markers, provided n (number of replication positions on the chromosome) remains unaltered (see APPENDIX, also SUEOKA and YOSHIKAWA 1965). Therefore, it was of interest to investigate the mutant behavior after a simultaneous shift to heavy medium and high temperature. It is expected that leaky replication mutants, which initiate normally but keep replicating for a short time at restrictive temperature, will show limited synthesis but retain equal percent transfer of all markers. For most other types of mutants this balance will be destroyed. Mutants unable to initiate at high temperature will show a gradient of transfer, the genetic marker nearest to the origin showing the least transfer and the one farthest showing the most. The amount of transfer of the early marker will depend on its distance from the origin and effectiveness of inhibition of initiation

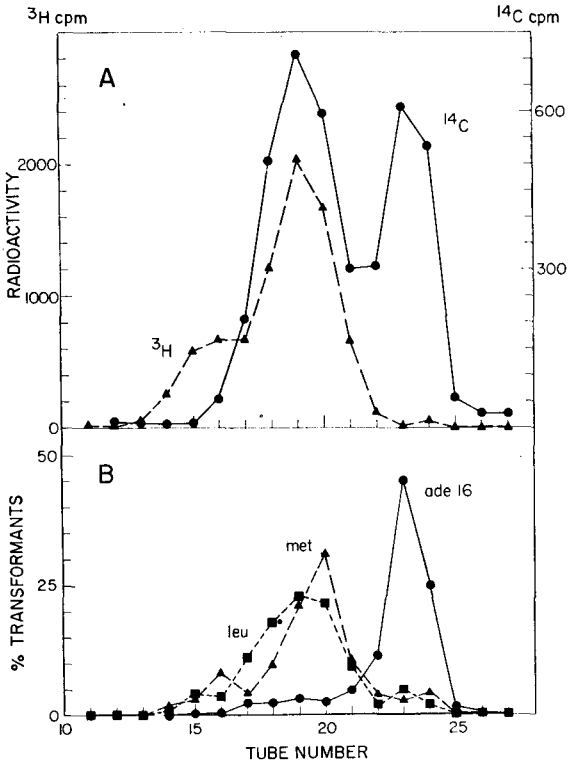

FIGURE 1.—Replication in mutant *dna*-1 at 45°C. The mutant culture was uniformly labelled with ^{14}C-thymine at 30°C. The growth was monitored by Klett-Summerson colorimeter (66 Filter). In logarithmic growth between Klett units 30 to 40 cells were quick-filtered, washed and shifted to 45°C in a BrU-C+ medium (25 μg/ml bromouracil and 2.5 μg/ml thymine) containing ^{3}H-thymine (2 μc/ml). At the end of 80 min at 45°C crude lysates were prepared and DNA subjected to CsCl density gradient centrifugation. Fractions were analyzed for TCA precipitable radioactivity and transforming activity. A. Radioactivity profile; B. Transforming activity profile.

(see APPENDIX). Mutants with anomalous synthesis will show patterns of transfer that somehow differ from those expected from sequential replication.

To analyze the nature of synthesis under restrictive conditions, exponentially growing mutant cells, uniformly labelled with ^{14}C-thymine at 30°C were harvested by suction, washed and resuspended at 45°C in prewarmed BrU-C$^+$ medium. At the end of the indicated time, cultures were chilled, centrifuged and lysates prepared. Lysates were centrifuged in a CsCl solution to separate the newly-made hybrid DNA (D_H) and parental, light DNA (D_P). The fractions from the CsCl gradient were assayed for radioactivity and transforming activity. The experimental details are given in the legend to Figure 1. Figure 1A,B shows the radioactive count and transformation profiles for mutant *dna-1* 80 minutes after the density and temperature shift. Genetic map positions of the markers used in this paper are shown in Figure 2. *Ade*16 is most proximal to the origin, *ade*6 follows at position 0.07, *leu*8 is at position 0.6, and *met*5 is near the terminus (Figure 2). The extent of replication was obtained from the amount of transfer of parental label to the hybrid (percent replication = ^{14}C cpm in hybrid/total ^{14}C cpm × 100). The extent of synthesis of a specific marker was estimated by the percent transformants in the heavy region (T_D), the hybrid region (T_H) and in the parental, light region (T_P).

To ascertain whether normal exponential transfer in the parent strain 168TT did show the expected equal percentage transfer for all markers under the experimental conditions used, control transfer with 168TT was first performed. This is very critical, as BU is known to reduce the rate of DNA synthesis in both *E. coli* and *B. subtilis*. Further, YOSHIKAWA and HASS (1968) have suggested that the reduction in the rate of DNA synthesis probably explains the observation that BU induces premature initiation of replication (ABE and TOMIZAWA 1967; WOLF, NEWMAN and GLASER 1968). For this analysis, it was important that no abnormalities were introduced by BU, at least during the first cycle of replication. Table 1, row 1, gives results of an 80 min transfer for an exponential culture of 168TT shifted from 30°C to 45°C. About 80% of the total DNA is in the hybrid peak, and about equal percent transfer of all markers to the hybrid is observed.

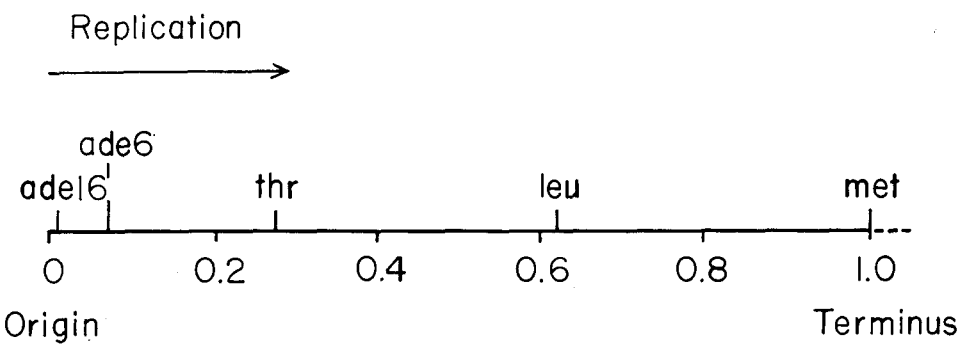

FIGURE 2.—Synchro-transfer map of *B. subtilis* chromosome from the data of O'SULLIVAN and SUEOKA (1967).

TABLE 1

Marker distribution of DNA synthesized in mutants at restrictive temperature

Strain	Time*	Percent R†	*ade*16 Total‡	*ade*16 Percent T_H§	*leu*8 Total	*leu*8 Percent T_H	*met*5 Total	*met*5 Percent T_H
168TT	80	80	7669	76.9	2514	77.4	876	71.4
dna-1	65	61	8356	11.7	6845	80.6	1616	76.9
dna-3	50	46	1504	42.2	537	74.1	714	82.0
dna-5	50	48	1914	27.5	1045	68.8	720	80.1
dna-9	50	17	2334	13.5	1669	51.0	1008	46.9
dna-10	50	29	2501	10.9	1558	49.1	1501	81.4
dna-15	50	48	709	25.5	225	76.4	260	88.8
dna-17	50	34	13420	28.7	8766	50.5	4154	66.7
dna-18	60	62	6145	37.8	1634	87.3	1072	89.9
dna-21	50	19	4661	44.6	1771	26.1	1215	23.1

* Time in minutes at 45°C.
† ^{14}C cpm in hybrid/total ^{14}C cpm × 100.
‡ Total number of transformants.
§ Transformants in hybrid region/total transformants × 100.

The slight edge that *ade*16 transfer has over *leu*8 and *met*5 in its transfer to the heavy peak does not interfere with the conclusions in this section as the analysis is restricted to the first round of replication for any gene.

The use of BU may also be objected to because BU was used to enrich the mutants for their isolation. So we may be looking at the behavior somehow induced by BU. We would like to point out, however, that the test for both the ts character and ts-DNA character did not involve use of BU and so the ts-DNA phenotype is independent of BU.

Table 1 summarizes similar analyses of DNA synthesized (in the indicated time after temperature shift in the presence of bromouracil) for nine mutants along with the parent strain 168TT. The percentage replication gives the extent of synthesis in the transfer experiment and should not be confused with total residual synthesis of the mutant. The analysis is restricted to the first replication of any gene (hybrid peak) and the heavy shoulder or peak in the DNA profile is disregarded. The nature of heavy DNA (D_D) will be considered later. As expected, the 168TT control shows an equal percentage transfer of all markers irrespective of map position. None of the mutants so far tested shows this aspect of normal replication.

Inhibition of initiation: Genetic marker *ade*16 has been estimated to be located within 2% or 3% from the origin of replication (O'SULLIVAN and SUEOKA 1967). If inhibition of initiation occurs immediately after the shift, only those forks which are not yet past *ade*16 will be potentially capable of transferring *ade*16 to the hybrid density position. The amount of *ade*16 transfer will depend on the pre-shift state of the chromosome (see APPENDIX). The expected value of *ade*16 transfer for a chromosome with only one replication position ($n = 1$) is 3% to 4%, for two replication positions ($n = 2$) 5% to 7%, and 7% to 10% for three replication positions ($n = 3$). A delay in inhibition of initiation will be reflected

in a value which is higher than the expected value. All the mutants showing substantial replication at 45°C show a much lower fractional transfer to hybrid for *ade*16 than for either *leu*8 or *met*5 (Table 1). The lowest *ade*16 transfer is observed for mutants *dna*-1 (12%), *dna*-9 (14%), *dna*-10 (11%). Mutants *dna*-5, *dna*-15 and *dna*-17 show about 25% *ade*16 transfer, and mutants *dna*-17 and *dna*-18 show an even higher (35% to 45%) *ade*16 transfer, *dna*-5, *dna*-15, and *dna*-17 could be leaky initiator mutants or alternatively, the gradual shut off may be a secondary effect. This indicates that in some mutants the inhibition of initiation is quite immediate (*dna*-1, *dna*-9 and *dna*-10), whereas most other mutants continue to initiate for some time. Even where a mutant (*dna*-9) shows limited replication (17%), the percentage of *ade*16 under the hybrid peak is much lower than the percentage of *leu*8 or *met*5 under the hybrid peak. This indicates that the efficiency of initiating new rounds of replication is reduced before complete inhibition of synthesis. The residual replication seems to proceed normally in a sequential manner, giving preferential transfer of late markers. The only exception to this is mutant *dna*-21, which shows a higher percent transfer of *ade*16 (44.6%) compared to *leu*8 (26.1%) and *met*5 (23.1%). Further studies on this mutant are in progress.

Nature of heavy shoulder or peak: The presence of a heavy density shoulder or peak along with the hybrid and light peaks in the radioactive profile (Figure 1A) is a result of some segments of the chromosome being replicated more than once while others have not replicated at all. This could result without new initiation at 45°C from continued sequential replication of chromosomes having more than one replication position prior to the temperature shift. In this condition a preferential transfer of later markers to the heavy DNA is expected (see APPENDIX). Alternatively, it could result from anomalous replication. In *E. coli*, active repair or repeated synthesis of a segment of the chromosome has been suggested by HIROTA, RYTER and JACOB (1968). In *B. subtilis* these three alternatives are distinguishable, as active repair would result in non-preferential transfer of all genetic markers, and repeated synthesis of a segment would result in enrichment in the heavy region of markers in that region only. Figure 1B shows a heavy shoulder with no *ade*16 transformants under it, whereas *leu*8 and *met*5 both show corresponding peaks. The preferential transfer of later markers indicates multifork replication prior to temperature shift. Independent evidence for the multifork replication of *dna*-1 prior to the temperature shift is provided later. This preferential transfer of later markers is observed even better in the case of mutant *dna*-18 (Figure 3) where there is enough synthesis to result in a well-defined heavy peak. In all cases tested, normal sequential replication is observed.

PROPERTIES OF MUTANT *dna*-1

Since mutant *dna*-1 showed almost immediate inhibition of initiation, it was further investigated.

Viability at non-permissive temperature: Exponentially growing liquid culture of *dna*-1 was shifted to 45°C. Aliquots were withdrawn and plated for colony-forming units after appropriate dilutions. The results are shown in Figure 4.

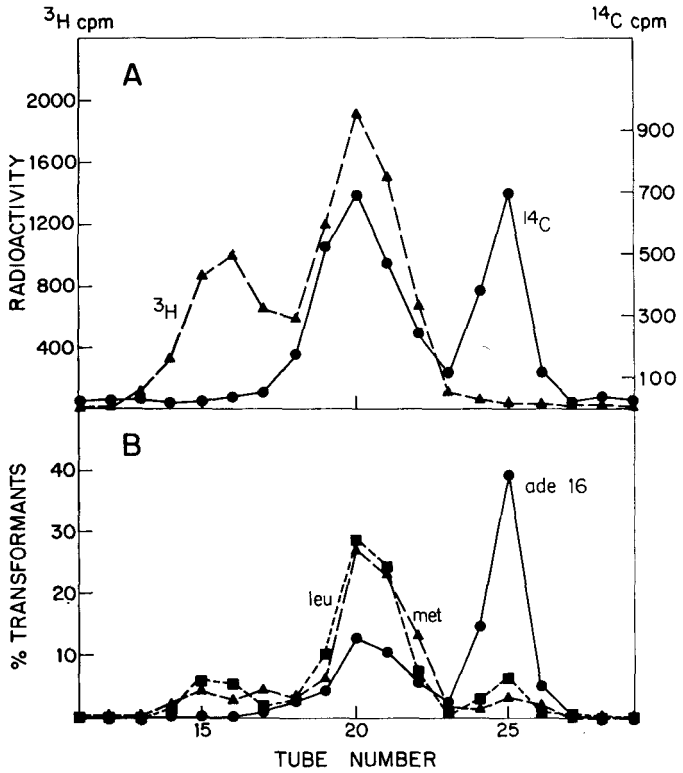

FIGURE 3.—Replication in mutant *dna*-18 at 45°C. The procedure is identical to the one described in Figure 1, except that the lysates were made after 60 min at 45°C.

Cells continued to divide for an hour, and were fully viable for at least three hours.

Multifork replication during growth at 34°C: An estimate for *n*, number of replication positions per chromosome, was obtained by marker frequency analysis (SUEOKA and YOSHIKAWA 1965). Crude cell lysates prepared from exponentially growing *dna*-1 were used as a source of DNA. The *ade*16/*met*5 ratio was normalized by using DNA from 168 *Try* spores. The results are given in Table 2. For cultures growing at 34°C with a generation time of 42 minutes, an approximate value of 2 for *n* was obtained.

TABLE 2

n for exponentially growing dna-1*

Source of DNA	Number of transformants *ade*16	*met*5	*ade*16/*met*5	Normalized *ade*16/*met*5
Spore DNA	1000	1077	0.92	1
dna-1	760	218	3.48	3.78

* Culture was grown at 34°C with a generation time of 42 min.

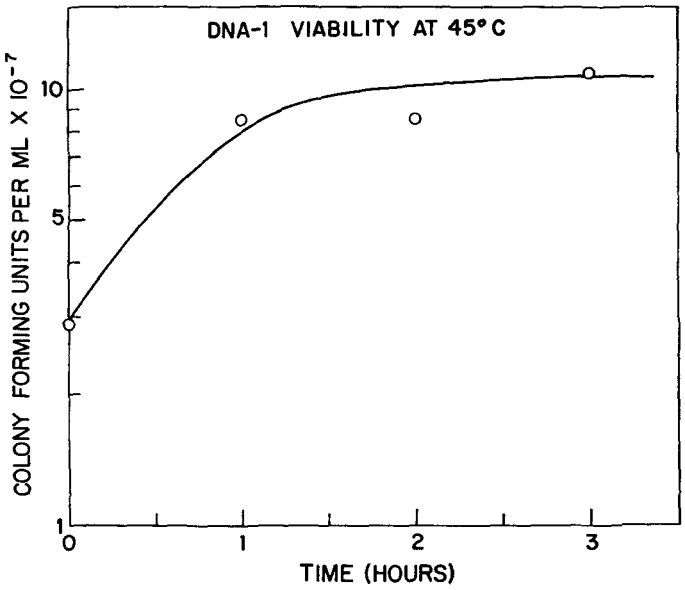

FIGURE 4.—Cell viability at 45°C for mutant *dna*-1. The log phase cell culture growing at 30°C was shifted to 45°C, and at 1 hr intervals appropriate dilutions were plated and plates incubated at 30°C.

Kinetics of finishing up: If cultures are shifted to a heavy DNA precursor medium (bromouracil) at the time of initiation inhibition, the kinetics of distribution (in heavy, hybrid and light DNA) of any genetic marker at a known position on the chromosome is predictable (see APPENDIX). The theoretical behavior is shown in Figure 14 for different values of n (number of replication positions prior to the inhibition). It states that the transfer of a marker at position X on the chromosome scale 0 to 1, will be unaffected in the time range $0 < \tau < X$, and at $\tau = X$ further transfer of that marker to hybrid DNA will stop. Here τ is the time after the density transfer (simultaneous with the inhibition of initiation) in a unit of the time required for the replication fork to travel from origin to terminus.

The experiment was similar to the one described in Figure 1, except that samples were withdrawn at times 15 minutes, 30 minutes, 60 minutes and 120 minutes after the temperature shift up (Figure 5A). Table 3 shows the distribution of transformants for *ade*16, *ade*6, *leu*8 and *met*5 in hybrid (D_H) and heavy (D_D) DNA. The data are plotted in Figure 5B to be comparable to the theoretical curve in the APPENDIX (Figure 14). In the 15 minute sample, the percentage of *ade*16 under the hybrid peak is 7.0, whereas *ade*6, *leu*8 and *met*5 have all replicated to the same extent (*ade*6, 27.3%; *leu*8, 29.3%; *met*5, 26.3%). The 30 minute sample shows that the rate slows down and reaches a plateau between 40% and 50%. Comparison with the theoretical curve shows good qualitative correlation at the early time even though the experimental situation is complicated by the presence of more than one replication position and use of the

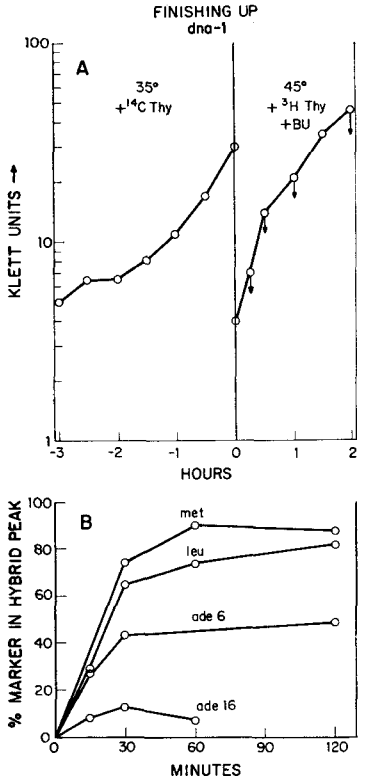

FIGURE 5.—The kinetics of finishing up in mutant *dna*-1. The experimental procedure is similar to that described in Figure 1 except that the cultures were grown at 35°C and were washed and resuspended in five times the amount of medium BrU-C+. Samples were withdrawn at the indicated times. A. Protocol of the experiment; B. Kinetics of transfer of genetic markers to the hybrid DNA.

TABLE 3

Kinetics of marker transfer after inhibition of initiation in dna-1

Time*	Percent R†	*ade*16 Total‡	*ade*16 Percent T_H§	*ade*16 Percent T_D‖	*ade*6 Total	*ade*6 Percent T_H	*ade*6 Percent T_D	*leu*8 Total	*leu*8 Percent T_H	*leu*8 Percent T_D	*met*5 Total	*met*5 Percent T_H	*met*5 Percent T_D
15	28	710	7	0	866	27.3	0	753	29.3	0	72	26.3	0
30	41	685	13.5	0	1051	44.3	0	288	65.2	0	415	73.4	0
60	43	670	7.9	0	...	...	.	186	70.4	3.7	301	90.0	0
120	49	...	...	.	1273	48.2	1.1	461	80.4	1.9	347	83.5	4.6

* Time in minutes at 45°C.
† ^{14}C cpm in hybrid/total ^{14}C cpm × 100.
‡ Total number of transformants.
§ Transformants in hybrid region/total transformants × 100.
‖ Transformants in heavy region/total transformants × 100.

TABLE 4

ade16/met5 ratio after blockage of initiation in dna-1

Time*	Number of transformants *ade16*	*met5*	*ade16/met5*
0 min	760	218	3.48
90 min	2829	1530	1.84
150 min	3562	2436	1.46

* Time at 45°C at which cell lysates were prepared.

density analogue bromouracil. The kinetics of transfer of *ade*16 show an almost immediate inhibition of initiation. The percentage of *ade*6 under the hybrid peak is considerably higher than would be expected from the known position of this genetic marker. (This will be discussed later in detail.) The kinetics of the transfer of *leu*8 and *met*5 indicate that not all chromosomes complete the replication. (Even in the last sample, there is a residual 10% to 15% *leu*8 and *met*5 in the light parental peak.) This could very possibly be an effect of bromouracil. In *B. subtilis*, bromouracil is known to reduce the rate of DNA synthesis (HAUNG *et al.* 1968) and cells also lose viability (BISHOP 1970). Alternatively, it is possible that not all the replicating forks reach the terminus. YOSHIKAWA, O'SULLIVAN and SUEOKA (1964) have demonstrated that chromosomes are not in completed form in the stationary phase in parental strain 168TT. To distinguish between the two possibilities, marker frequency analysis was done for DNA from cells which were allowed to complete chromosomes at 45°C in the presence of thymine. The *ade*16/*met*5 ratio of DNA extracted from cells prior to shift gave an approximate value of 2 for the number of replication positions. With increasing time at 45°C, the *ade*16/*met*5 ratio went down from 3.5 to 1.5, showing a tendency to complete the chromosome (Table 4). The final value of 1.5 when compared to the spore DNA ratio of 0.92 shows that not all chromosomes are completed even in the presence of thymine.

The place and kinetics of reinitiation: OISHI, YOSHIKAWA and SUEOKA (1964) have established a fixed origin for replication during outgrowth of *B. subtilis* spores. *E. coli dna*-B mutant RT46 has been shown to reinitiate at the same place in consecutive shift-up and shift-down experiments (HIROTA, MURDOH and JACOB 1970; WORCEL 1970). Therefore it was of interest to investigate whether the mutant always reinitiates at the same place, and whether the place of reinitiation is the same as the normal origin of replication. Exponentially growing cells were uniformly labelled with ^{14}C-thymine and shifted to 45°C. After 2 hr at non-permissive temperature the cells were washed and resuspended at 35°C in a BrU-C$^+$ medium containing ^{3}H-thymine. Samples were withdrawn at the indicated times and analyzed.

Table 5 summarizes the results. It is clear that *ade*16 is the first marker to be replicated (68.3% and 21.9% under hybrid and heavy peaks, respectively, in the 60 minute sample). There is no substantial transfer of any of the other markers tested till 25 minutes (6.5% for *ade*6, 5.4% for *leu*8 and 7.2% for *met*). Between

TABLE 5

Reinitiation in dna-1

Time*	*ade*16 Total†	Percent T_H‡	Percent T_D§	*ade*6 Total	Percent T_H	Percent T_D	*leu*8 Total	Percent T_H	Percent T_D	*met*5 Total	Percent T_H	Percent T_D
15 min	7545	16.9	0	252	4.7	0	2595	3.3	0	1500	3.3	0
25 min	14369	32.9	0	458	6.5	0	5646	5.4	0	3646	7.2	0
40 min	6528	67.1	2.3	1576	12.9	0	1837	10.5	0	936	10.8	0
60 min	10634	68.3	21.9	3722	57.9	7.2	2644	16.0	0	1509	21.2	0

* Time after shift back to permissive temperature.
† Total number of transformants.
‡ Transformants in hybrid region/total transformants × 100.
§ Transformants in heavy region/total transformants × 100.

40 and 60 minutes, there is a fair amount of *ade*6 transferred (57.9% for 60 minute sample), indicating that by that time replication forks have reached the *ade*6 location and that the replication forks proceed synchronously. It also points at the slow advance of the replication forks and the slow recovery of the cells.

In the 60 min sample (Figure 6) a heavy shoulder is observed. It indicates that a second round of replication is initiated. Further evidence for this is provided by

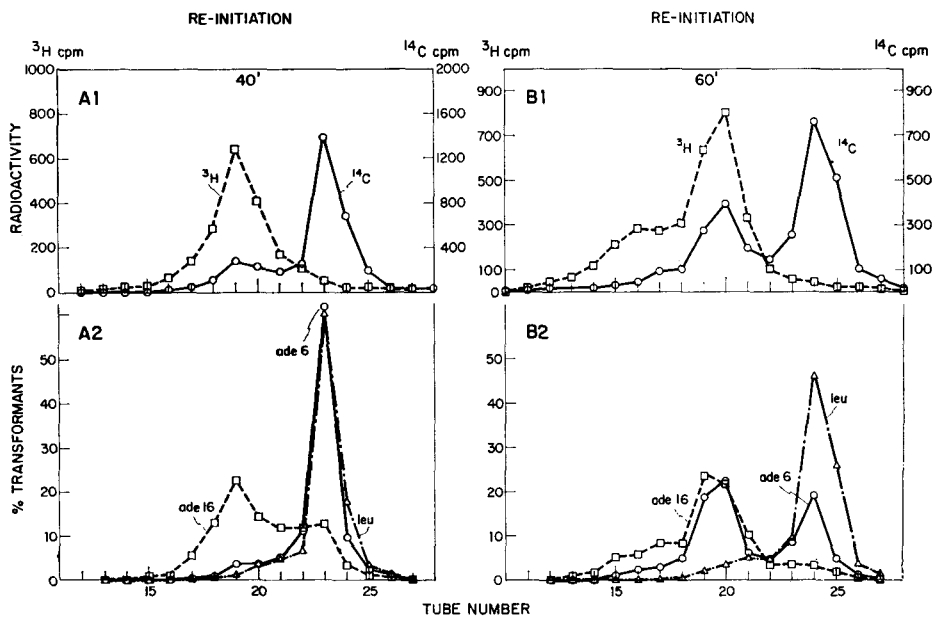

FIGURE 6.—Reinitiation in mutant *dna*-1. *dna*-1 was uniformly labelled with ^{14}C thymine at 35°C. At Klett 30, cultures were shifted to 45°C. After 2 hr at 45°C cells were harvested by suction on a Millipore filter, washed and resuspended at 35°C in BrU-C+ medium (25 μg/ml bromouracil and 2.5 μg thymine and ^{3}H-thymine 3 μc/ml). Samples were withdrawn at 40 and 60 min, cell lysates made and DNA analyzed by CsCl centrifugation. A1, B1. Radioactivity profiles; A2, B2. Transforming activity profiles.

the presence of *ade*16 transformants in the heavy shoulder (21.9% in 60 minute sample). In view of the normal protein synthesis at 45°C and the low rate of replication during the recovery period, the quick second reinitiation is not surprising.

Table 5 shows some transfer to hybrid of *leu*8 and *met*5 even in the earliest time sample (percent under the hybrid peak is 3.3% for *leu*8 and 3.3% for *met*5). About equal transfer of both markers is observed at all times (Table 5). It is very unlikely that the first wave of replication has reached as far as *leu*8 in the period tested. The equal transfer of both markers is characteristic of random synthesis (see APPENDIX). Therefore, the results shown in Table 5 are interpreted as a synchronous wave of replication starting at the origin of all chromosomes superimposing on the continued synthesis at the forks of unfinished chromosomes. The equal percentage transfer of *leu*8 and *met*5 suggests a random population of unfinished chromosomes.

Inhibition of protein synthesis by the addition of chloroamphenicol (150 μg/ml) 10 minutes prior to the shift to permissive temperature suppressed reinitiation by at least 95%.

Mapping of dna-1: Mutant *dna*-1 was mapped by the density transfer technique of O'SULLIVAN and SUEOKA (1967). The parental 168TT strain was found to be non-transformable so the mutation *dna*-1 was put in another genetic background by transformation. The recipient BC 200 (168, *thy*, *leu*8, *met*5) was transformed by the DNA from the *dna*-1 strain and plated for *leu*$^+$ transformants; these were checked for temperature-sensitive growth at 30°C and 45°C. Of the 640 colonies tested, 16 were found to be temperature-sensitive. The high percent of ts colonies among the *leu*$^+$ transformants suggests linkage between *dna*-1 and *leu*8. Of these 16 ts colonies, 2 (3 BC 200-*dna*-1 and 4 BC 200-*dna*-1) were tested for density transfer at high temperature (Table 6, rows 2 and 3). It is clear that these two ts mutants behave in a similar manner to *dna*-1 in a density transfer at restrictive temperature, with very low *ade*16 transfer. So presumably both carry the original *dna*-1 mutation. These two mutants were transformable and could be transformed for the ts character. This indicates that *dna*-1 is a single gene mutation.

The tabulations of the results of the transfer experiment and the position of the *dna*-1 marker on the genetic map are shown in Table 7. KARAMATA and GROSS (1971) report two linkage groups, dnaB and dnaI, one on either side of *leu*8, the order being *dna*B, *leu*8, *dna*I. *dna*-1 at present cannot be assigned to either link-

TABLE 6

First round of replication in the BU transfer of dna-1 *at 45°C*

Strain	*ade*16 Percent T_H	*leu*8 Percent T_H
dna-1	13%	65%
3 BC200 *dna-1*	12%	61%
4 BC200 *dna-1*	6%	58%

TABLE 7

Mapping dna-1 *by density transfer experiment**

Marker	PercentT_H†	X‡
*ade*16	93	0
*leu*8	48	0.62
dna-1	46	0.64

* *ade*16 and *leu*8 were used as reference markers. X for *dna-1* was calculated according to O'SULLIVAN and SUEOKA 1967.
† Only the first round of replication was taken into consideration.
‡ Position of the marker on the chromosome.

age as the resolution of the density transfer method is reduced for distal markers. A member of the *dna*B group (168ts-134) was characterized by MENDELSON and GROSS (1967). They suggest that at high temperature initiation is severely but not entirely inhibited in this mutant. In this respect *dna*-1 could be a member of the dnaB group, though the initiation is entirely inhibited. However, at high temperature 168ts-134 loses viability dramatically after an initial increase in colony-forming units; on the other hand, *dna*-1 shows an initial increase and is fully viable for at least three hours. No information is available on the characteristics of *dna*1.

TRANSFER OF *ade*6 AFTER INHIBITION OF INITIATION IN AN EXPONENTIALLY GROWING CULTURE

The estimated map position of the *ade*6 marker (0.07) is based on the average of the results obtained by marker frequency analysis (OISHI, YOSHIKAWA and SUEOKA 1964). The marker frequency estimate involved assuming the position 1.0 for the *met*5 marker. If 0.07 is the marker map position of *ade*6, then the expected maximal fraction of *ade*6 in hybrid on inhibition of initiation in an exponentially growing culture is 0.10 for $n = 1$, 0.18 for $n = 2$, and 0.25 for $n = 3$ (Figure 14). The experimental value found was between 0.40 and 0.50 (Figure 5B). The high experimental value could result from a delay in inhibition of initiation. However, this seems unlikely in view of the law fraction of *ade*16 found in hybrid (< 0.10). The possibility that the observed *ade*16 transfer is lower, because of its closeness to the origin, cannot be ruled out. A second possibility is that n, the number of replication positions prior to the temperature shift, is unusually high ($n > 4$). This seems highly unlikely, as the value obtained for the same mutant under similar conditions (growing with generation time 42 minutes) by marker frequency analysis is 2 (Table 2). It is also possible that *ade*6 may be translocated to a different position in this strain.

The fourth possibility is that *ade*6 is farther than 0.07 on the chromosome. The fractional value 0.45 for *ade*6 in hybrid would locate its chromosomal position at 0.18 for $n = 2$ and at 0.12 for $n = 3$. This is an independent estimate of the position of *ade*6 on the chromosome.

The recent evidence for bidirectional replication in *B. subtilis* (WAKE 1972) and in *E. coli* (BIRD *et al.* 1972; MASTERS and BRODA 1971; PRESCOTT and

KUEMPEL 1972) hardly affect the picture presented in this paper, either experimentally or theoretically. A minor modification in the relationship between the replicated amount of DNA and the marker position will be made later.

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APPENDIX

GENERAL THEORY OF DENSITY TRANSFER FOR SYMMETRIC CHROMOSOME REPLICATION

In this appendix, theoretical answers will be given for DNA and marker distributions in different density peaks when density transfer experiments are performed with or without concomitant inhibition of chromosome initiation. The entire treatment is based on the steady-state, non-synchronous replication of chromosomes with symmetric initiation. The steady-state replication is obtained when the rate of replication at each replication point and the initiation interval are constant. The basic equations to be used are:

a) generalized equation of marker frequency (SUEOKA and YOSHIKAWA 1965):

$$g_n(X) = 2^{n(1-X)} \tag{1}$$

where X represents the chromosomal position (0 for the origin and 1 for the terminus) of a marker, n is the average number of *replication positions* per chromosome, and $g_n(X)$ is the frequency of marker X. It is also noted that when $0 < n < 1$, the cell has a resting period (G-period) of DNA replication, and when $n > 1$, the cell has, on an average, more than one replication position (multifork replication).

b) generalized equation of age distribution (SUEOKA 1971):

$$f_n(x) = n(1n2)\, 2^{n(1-x)} \tag{2}$$

where x is a chromosomal position and $f_n(x)$ is the frequency of *replication points* at x. Here the distinction between *replication point* and *replication position* is important and is explained in Figure 7. Both equations are applicable to symmetric (dichotomous) replication of the chromosome (YOSHIKAWA, O'SULLIVAN and SUEOKA 1964; OISHI, YOSHIKAWA and SUEOKA 1964; BIRD and LARK 1968; CARO and BERG 1969; QUINN and SUEOKA 1970; FRITCH and WORCEL 1971) during the steady-state growth of exponential cell cultures, and are normalized to the number of chromosomes at the time of transfer in the population (for a detailed discussion, see SUEOKA and YOSHIKAWA 1965; SUEOKA 1971).

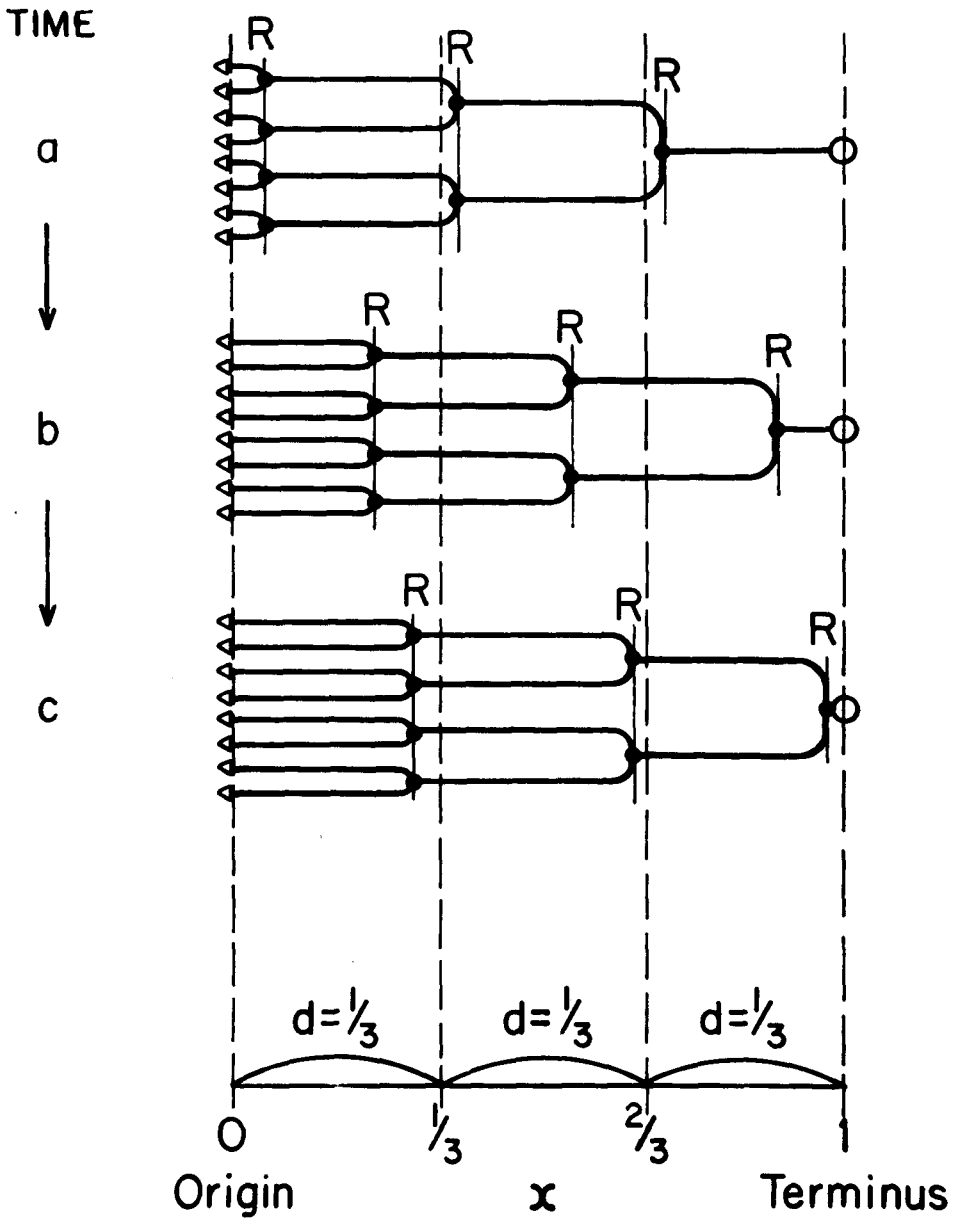


FIGURE 7.—A dichotomous replication of the chromosome. A schematic representation of dichotomous replication with three replication positions per chromosome ($n = 3$) is shown as an example. Each segment has a length of $d = 1/n$, thus $\frac{1}{3}$ in the diagram. This distance, d, corresponds to the initiation interval. Note that each chromosome has three replication positions (R) and seven replication points (●). In general, n is not limited to integers, nor to $n > 1$, and the number of replication points per chromosome is $2^n - 1$ (see SUEOKA and YOSHIKAWA 1965; SUEOKA 1971). R, replication position; ●, replication point; ○, terminus; △, origin.

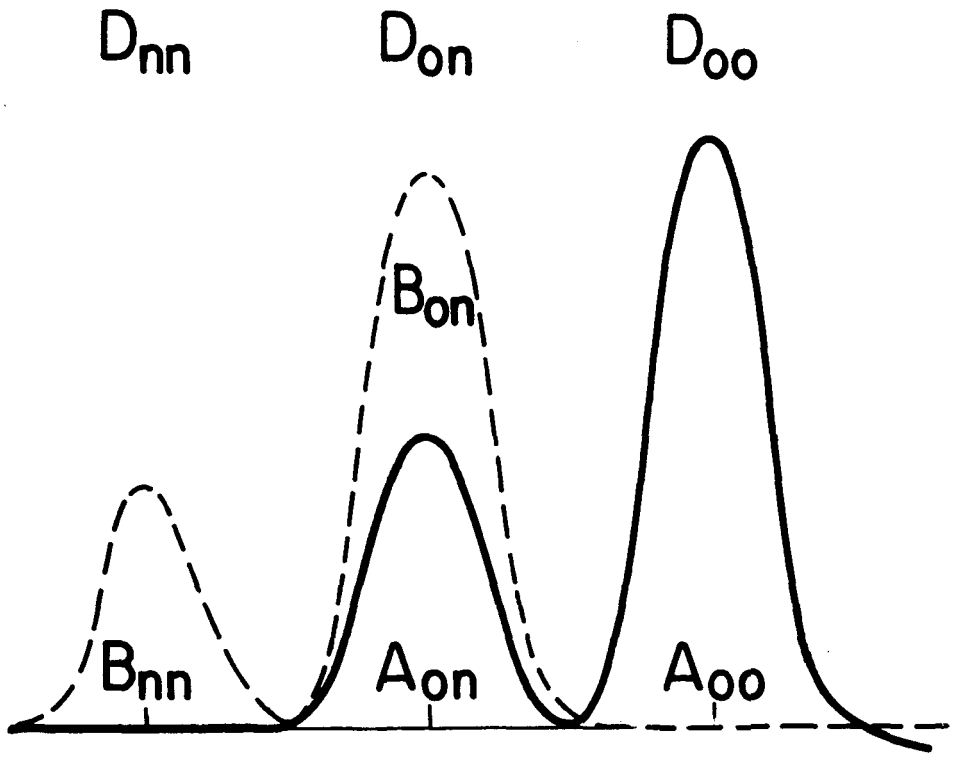


FIGURE 8.—Radioactive profiles of DNA after density transfer. Before density transfer, the chromosome is uniformly labeled with a radioisotope, A (e.g., ^{14}C) and after the transfer with a different radioisotope, B (e.g., ^{3}H). From such a profile, the relative amounts of DNA in the three peaks (D_{oo}, D_{on} and D_{nn}) can be calculated as follows. Since the specific activity of the hybrid molecule is half that of the parental molecule, the following formula can be used in calculating the experimental value:

$$\text{Relative } D_{oo} \text{ (experimental)} = \frac{A_{oo}}{A_{oo} + 2A_{on}}$$

$$\text{Relative } D_{on} \text{ (experimental)} = \frac{2A_{on}}{A_{oo} + 2A_{on}}$$

Here $D_{oo} + D_{on} = 1$.

A_{oo} and A_{on} are fractional radioactive counts of the radioisotope used for uniform labeling of pretransfer DNA (e.g., ^{14}C) in parental and hybrid peaks, respectively. Here $A_{oo} + A_{on} = 1$. The amount of DNA in the doubly-labelled peak (D_{nn}) can be estimated by labelling the newly-synthesized strand with another radioisotope, B. The relative experimental values of D_{on} and D_{nn} are then calculated as:

$$\text{Relative } D_{on} = \frac{2B_{on}}{2B_{on} + B_{nn}}$$

$$\text{Relative } D_{nn} = \frac{B_{nn}}{2B_{on} + B_{nn}}$$

Here $D_{on} + D_{nn} = 1$.

B_{on} and B_{nn} are fractional radioactive counts of the radioisotope used to label the newly-synthesized DNA strands after the transfer in hybrid and doubly-labelled peaks, respectively. When all three peaks exist, the fractional amounts of the three can be calculated as

I. Density Transfer Without Initiation Blockage

A. DNA distribution

The classical density transfer experiment of Meselson and Stahl (1958) consisted of the transfer of exponentially growing cells from a heavy medium (^{15}N) to a light medium (^{14}N) without initiation interval and cell generation time being altered. Under a similar steady-state condition, the kinetics of DNA transfer from parental (*oo*) to hybrid (*on*) to doubly density-labelled (*nn*) DNA is presented here in general form. The amounts of parental, hybrid and doubly density-labelled DNA are designated as D_P, D_H and D_D, and their fractional amounts as D_{oo}, D_{on} and D_{nn}, respectively. Thus, $D_P + D_H + D_D = D_T$ and $D_{oo} + D_{on} + D_{nn} = 1$. D_T represents the total amount of DNA. Figure 8 is a schematic representation of a typical DNA distribution in a density transfer experiment.

The expected amounts of DNA transfer to the hybrid and doubly-labelled peaks can be calculated as a function of time τ. Here τ is the time after the density transfer measured in the unit of time required for a replication point to travel from the origin to the terminus (Sueoka 1971). Note that the rate of transfer is a function of τ and of n. The n in this treatment for steady-state transfer remains unaltered before and after the transfer.

a) $0 \leqslant \tau \leqslant \dfrac{1}{n}$

When the pretransfer population is defined as $f_n(x)$ (equation 2), x can be transformed to τ by $x = 1 - \tau$ (Figure 9A), thus,

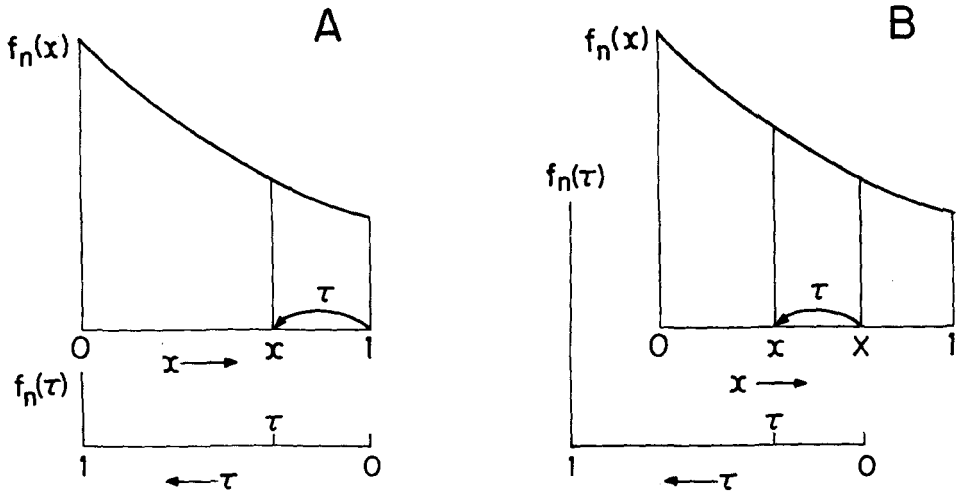


Figure 9.—Transformation of the x-scale to the τ-scale. (A) $x = 1 - \tau$. This transformation allows us to calculate the frequency of replication points which would exist after time τ at the terminus of the chromosome (equation 3). (B) $x = X - \tau$. This transformation allows us to calculate the frequency of replication points which would exist after time τ at chromosome position X (equation 13).

$$D_{oo} = \frac{(1 - A_{on})\, B_{on}}{A_{on} + B_{on}}$$

$$D_{on} = \frac{2\, A_{on} \cdot B_{on}}{A_{on} + B_{on}}$$

$$D_{nn} = \frac{A_{on}\,(1 - B_{on})}{A_{on} + B_{on}}$$

where $D_{oo} + D_{on} + D_{nn} = 1$.

$$f_n(\tau) = n(ln2)2^{n\tau *} \tag{3}$$

Since each replication fork at time τ doubles the DNA at the fork, converting parental DNA (D_P) to hybrid DNA (D_H),

$$\frac{dD_H}{d\tau} = 2\int_{\tau}^{1+\tau} f_n(\tau)d\tau = 2\cdot 2^{n\tau}\,(2^n - 1)$$

When $\tau = 0$, $D_H = 0$

Therefore,

$$D_H = \frac{2(2^n - 1)}{2(ln2)}\,(2^{n\tau} - 1) \tag{4}$$

Similarly, since each replication fork at time τ converts parental DNA to hybrid DNA,

$$\frac{dD_p}{d\tau} = -\int_{\tau}^{1+\tau} f_n(\tau)d\tau = 2^{n\tau}(1 - 2^n)$$

When $\tau = \frac{1}{n}$, $D_p = 0$.

Therefore,

$$D_p = \frac{(1 - 2^n)}{n(ln2)}\,(2^{n\tau} - 2) \tag{5}$$

Since the increase of total RNA is proportional to the total number of replication forks,

$$\frac{dD_T}{d\tau} = \int_{\tau}^{1+\tau} f_n(\tau)d\tau = 2^{n\tau}(2^n - 1)$$

When $\tau = 0$; $D_T = \frac{2^n - 1}{n(ln2)}$.

Therefore,

$$D_T = \frac{2^{n\tau}}{n(ln2)}\,(2^n - 1) \tag{6}$$

Note that there is no D_D in this range of τ. The fractional amounts of parental and hybrid DNA are:

$$D_{oo} = \frac{D_p}{D_T} = 2^{1-n\tau} - 1 \tag{7}$$

$$D_{on} = \frac{D_H}{D_T} = 2 - 2^{1-n\tau} \tag{8}$$

b) $\tau \geqslant \frac{1}{n}$

Note that no D_P is found in this range and D_H stays constant as the value of D_H (equation 4) at $\tau = \frac{1}{n}$, namely

$$D_H = D_H(\tfrac{1}{n}) = \frac{2(2^n - 1)}{n(ln2)} \tag{9}$$

Since D_T of equation 6 is applicable for $\tau \geqslant 0$.

$$D_D = D_T - D_H(\tfrac{1}{n}) = \frac{(2^n - 1)(2^{n\tau} - 2)}{n(ln2)} \tag{10}$$

Therefore, fractional amounts of DNA in the *on* and *nn* peaks are:

$$D_{on} = \frac{D_H(\frac{1}{n})}{D_T} = 2^{1-n\tau} \tag{11}$$

$$D_{nn} = \frac{D_D}{D_T} = 1 - 2^{1-n\tau} \tag{12}$$

The graphic representation of these equations is shown in Figure 10.

* Editor's note: Equation (3) and many following equations in this paper include a term in which 2 is raised to the power $n\tau$ or to the power $1\text{-}n\tau$. The Greek typeface available to our printer cannot be set exactly at the superscript level, so the letter τ appears slightly lower than the rest of the exponent.

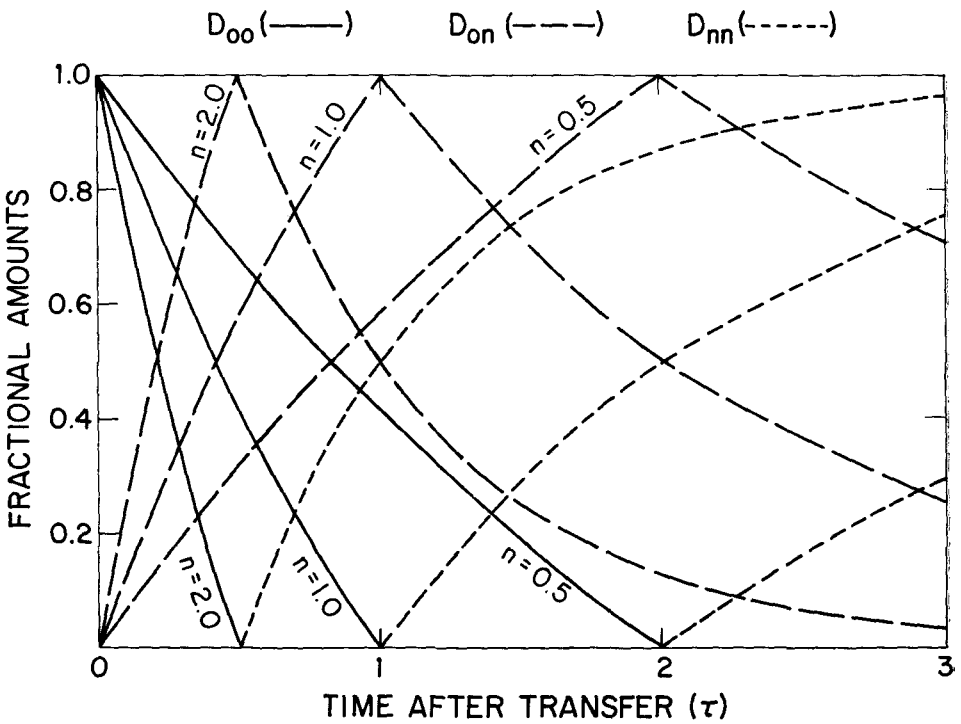


FIGURE 10.—Transfer of DNA by steady-state transfer experiments (no initiation blockage) (equations 7, 8, 11 and 12). The fractional amounts of DNA in parental (D_{oo}), hybrid (D_{on}) and doubly density-labelled (D_{nn}) are shown as the function of n and time τ. The classical MESELSON and STAHL experiment (1958) is seen as the curve for n = 1. The transfer of genetic markers has similar kinetics (equations 17, 18, 21 and 22).

B. MARKER DISTRIBUTION

The amount of marker X in parental, hybrid and doubly density-labelled DNA is designated as X_P, X_H and X_D and its fractional amounts as X_{oo}, X_{on} and X_{nn}, respectively.

$$\text{Thus} \quad X_P + X_H + X_D = X_T$$
$$X_{oo} + X_{on} + X_{nn} = 1$$

where X_T is the total amount of marker X. In the text (see Tables 1, 3 and 5), the percent transformants in the parental, hybrid and doubly density-labelled peaks are designated as T_P, T_H and T_D, respectively. These then are the experimental values (percent) corresponding to X_{oo}, X_{on} and X_{nn} (fractional), respectively.

a) $0 \leqslant \tau \leqslant \frac{1}{n}$

There is no X_D in this period. At τ, the rate of losing X_{oo} is proportional to the frequency of the replication point at $X-\tau$ (see Figure 9B).

Putting $x = X-\tau$ for equation 2,

$$f_n(\tau,X) = n(ln2)2^{n(1-X+\tau)} \tag{13}$$

Thus,

$$\frac{dX_P}{d\tau} = -f_n(\tau,X) = -n(ln2)2^{n(1-X+\tau)}$$
$$X_P = 2^{n(1-X)}(2 - 2^{n\tau}) \tag{14}$$

Since, in general, the increase of a marker during the time interval τ is by a factor of $2^{n\tau}$,

$$X_T = 2^{n(1-X)}2^{n\tau} \tag{15}$$

Therefore,

$$X_H = X_T - X_P = 2 \cdot 2^{n(1-X)}(2^{n\tau}-1) \tag{16}$$

Thus,

$$X_{oo} = \frac{X_P}{X_T} = 2^{1-n\tau} - 1 \tag{17}$$

$$X_{on} = \frac{X_H}{X_T} = 2 - 2^{1-n\tau} \tag{18}$$

b) $\tau \geqslant \frac{1}{n}$

In this range, there is no X_P.

$$X_H = 2g_n(X) = 2 \cdot 2^{n(1-X)} \tag{19}$$

Note that this remains constant. Therefore,

$$X_D = X_T - X_H = 2^{n(1-X)}(2^{n\tau} - 2) \tag{20}$$

Thus,

$$X_{on} = \frac{X_H}{X_T} = 2^{1-n\tau} \tag{21}$$

$$X_{nn} = \frac{X_D}{X_T} = 1 - 2^{1-n\tau} \tag{22}$$

These relationships are graphically identical to the DNA distribution shown in Figure 10 and equations 11 and 12.

C. SPECIFIC ACTIVITY OF MARKERS

The results obtained above show that the transfer rates of both the DNA and the markers are the same (Figure 10) and independent of the marker position. It is concluded, then, that the specific activity of markers is the same in all three peaks. In other words, the fractional rate of transfer of all markers is the same as that of DNA in the case of random steady-state transfer.

II. Density Transfer with Initiation Blockage

When a density transfer is carried out at the time of inhibition of initiation, DNA will eventually distribute in parental, hybrid and doubly density-labelled peaks in a definite proportion which depends on the pretransfer conditions of the chromosome population. The latter can best be defined by n. In the ideal state, the inhibition of initiation should be immediate and absolute (not leaky), and the replication point should proceed until it reaches the terminus. A change in replication rate at each replication point after the inhibition of initiation should not affect the theory as long as the new rate is the same in all replication points and n does not change. The solution for this ideal state gives the theoretical basis with which experimental data can be compared and interpreted.

A. DNA DISTRIBUTION AFTER COMPLETION

a) $0 \leqslant n \leqslant 1$

In this range of n, there is no D_D produced. The amount of hybrid DNA (D_H) at the completion of chromosome replication can be calculated as

$$D_H = 2\int_0^1 (1-x)f_n(x)dx = 2\{2^n - \frac{2^n - 1}{n(ln2)}\} \tag{23}$$

Since the total amount of DNA (D_T) is 2^n,

$$D_{on} = \frac{D_H}{D_T} = 2\{1 - \frac{1 - 2^{-n}}{n(ln2)}\} \tag{24}$$

Therefore,

$$D_{oo} = 1 - D_{on} = \frac{2(1-2^{-n})}{n(ln2)} - 1 \tag{25}$$

Note that for $n = 0$, D_{on} and D_{oo} are 0 and 1, respectively.

b) $n \geqslant 1$.

A presence of doubly-labelled DNA (D_D) suggests $n > 1$. The DNA between the origin and first replication position remains parental and the total amount of DNA after completion of DNA replication is 2^n.

Therefore,

$$D_P = \int_0^{1/n} 2x f_n(x)dx = \frac{2^n(1 - ln2)}{n(ln2)} \tag{26}$$

Therefore,

$$D_{oo} = \frac{1 - ln2}{n(ln2)} \tag{27}$$

The total DNA at $\tau = 0$, minus the remaining D_P after completion (equation 26), yields a double amount of hybrid DNA, so

$$D_{on} = 2[\int_0^1 g_n(X)dX - D_P]/2^n = \frac{2}{n}\{1 - \frac{1}{2^n(ln2)}\} \tag{28}$$

Consequently,

$$D_{nn} = 1 - D_{oo} - D_{on} = 1 - \frac{2^n(1 + ln2) - 2}{n2^n(ln2)} \tag{29}$$

It is noted that for $n = 1$, equations 24 and 25 are exactly the same as equations 28 and 27, respectively. These relations are graphically shown in Figure 11.

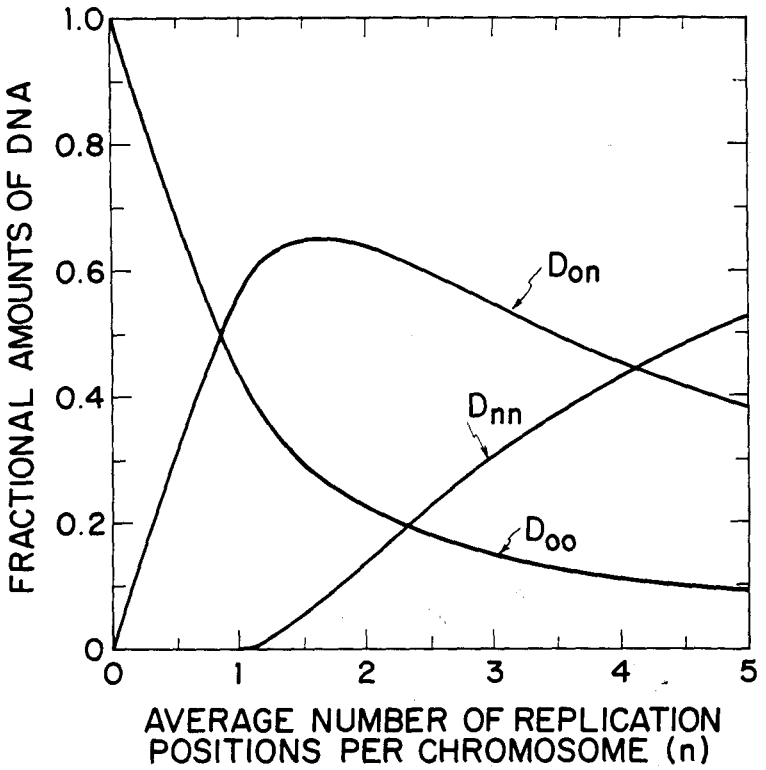


FIGURE 11.—Fractional amounts of parental, hybrid and doubly density-labelled DNA's as the function of n after the completion of chromosome replication in density transfer experiments with concomitant initiation blockage. For $0 \leqslant n \leqslant 1$, equations 24 and 25 are used, and for $n \geqslant 1$, equations 27, 28 and 29.

B. KINETICS OF DNA TRANSFER

The expected amounts of DNA transferred to the hybrid and to doubly labelled peaks can be calculated as a function of time τ. Here τ is the time after the density transfer and the block of initiation and its unit is the time required for a replication point to travel without interruption from the origin to the terminus as before. Note that the rate of transfer is a function of τ and of n prior to the initiation blockage and density transfer.

a) $0 \leqslant n \leqslant 1$

Let us define $\tau = 1-x$ as in equation 3 (Figure 9A). Since each remaining replication point at τ doubles the DNA and changes parental DNA to hybrid DNA, the absolute amount of DNA in hybrid (D_H) at τ can be obtained as follows:

$$\frac{dD_H}{d\tau} = \int_{\tau}^{1} 2f_n(\tau)d\tau = 2(2^n - 2^n\tau)$$

When $\tau = 0$, $D_H = 0$.
Therefore,

$$D_H = 2\{2^n\tau - \frac{2^n\tau - 1}{n(ln2)}\} \tag{30}$$

The absolute amount of DNA in the parental peak (D_P) is obtained as follows:

$$\frac{dD_P}{d\tau} = -\int_{\tau}^{1} f_n(\tau)d\tau = 2^n\tau - 2^n$$

When $$\tau = 0, D_P = \int_0^1 g_n(X)dX = \frac{2^n - 1}{n(ln2)}$$

Therefore,

$$D_P = \frac{2^n\tau + 2^n - 2}{n(ln2)} - 2^n\tau \tag{31}$$

Since there will be no DNA in the doubly-labelled peak, the fractional amount of DNA in the hybrid peak (D_{on}) is:

$$D_{on} = \frac{D_H}{D_P + D_H} = \frac{2^{n+1}\tau n(ln2) - 2^{n\tau+1} + 2}{2^n\tau n(ln2) - 2^n\tau + 2^n} \tag{32}$$

and $D_{oo} = 1\text{-}D_{on}$

b) $n \geqslant 1$

Now the total DNA (D_T) is:

$$\frac{dD_T}{d\tau} = \int_{\tau}^{1} f_n(\tau)d\tau = 2^n - 2^n\tau$$

Since D_T at $\tau = 0$ is $\int_0^1 g_n(X)dX$

$$D_T = 2^n\tau - \frac{2^n\tau - 2^n}{n(ln2)} \tag{33}$$

Note that for $\tau = 0$ and $n = 0$, $D_T = 1$.

For the range $0 \leqslant \tau \leqslant \frac{1}{n}$, no doubly-labelled DNA (D_D) should be found.

$$\frac{dD_E}{d\tau} = -\int_{\tau}^{1} f_n(\tau)d\tau = 2^n\tau - 2^n$$

Since D_P at $\tau = 0$ is $\int_0^1 g(X)dX$

$$D_P = \frac{2^n\tau + 2^n - 2}{n(ln2)} - 2^n\tau \tag{34}$$

D_H and D_{on} can be calculated by equations 30 and 32, respectively. And,

$$D_{oo} = 1 - D_{on}$$

For the range of $\frac{1}{n} \leqslant \tau \leqslant 1$, all three peaks will be present.

When $\tau = \frac{1}{n}$,

$$D_P\left(\frac{1}{n}\right) = \frac{2^n(1 - ln2)}{n(ln2)} \tag{35}$$

The above can be calculated from equation 34, and remains constant.

When $\tau = \frac{1}{n}$,

$$D_H\left(\frac{1}{n}\right) = \frac{2\{2^n(ln2) - 1\}}{n(ln2)} \tag{36}$$

This can be calculated from equation 30, and remains constant.

The amount of doubly-labelled DNA (D_D) can be calculated as,

$$D_D = D_T - D_T\left(\frac{1}{n}\right) = 2^n(\tau - \frac{1}{n}) + \frac{2 - 2^n\tau}{n(ln2)} \tag{37}$$

where $D_T\left(\frac{1}{n}\right)$ is the value of D_T at $\tau = \frac{1}{n}$.

Consequently,

$$D_{oo} = \frac{D_P\left(\frac{1}{n}\right)}{D_T} = \frac{2^n(1 - ln2)}{2^n\tau n(ln2) - 2^n\tau + 2^n} \tag{38}$$

$$D_{on} = \frac{D_H\left(\frac{1}{n}\right)}{D_T} = \frac{2\{2^n(ln2) - 1\}}{2^n\tau n(ln2) - 2^n\tau + 2^n} \tag{39}$$

$$D_{nn} = \frac{D_D}{D_T} = \frac{2^n\tau n(ln2) - 2^n(ln2) - 2^n\tau + 2}{2^n\tau n(ln2) - 2^n\tau + 2^n} \tag{40}$$

The graphic representation of the kinetics is shown in Figure 12.

C. MARKER DISTRIBUTION AFTER COMPLETION

The fractional amounts of marker X in the parental, hybrid and doubly density-labelled DNA are designated as X_{oo}, X_{on} and X_{nn}, respectively.

In the range of $0 \leqslant X \leqslant \frac{1}{n}$, the following relations can be obtained: each replication point between X (chromosomal position of marker X) and $\frac{1}{n}$ has already given two X markers which remain as parental, and the total number of X markers after completion of replication is 2^n, so

$$X_{oo} = \int_X^{1/n} f_n(x)dx/2^n = 2^{1-nX} - 1 \tag{41}$$

Each replication point between 0 and X gives two X markers in hybrid DNA, so
Note that there would be no X_{nn} in this region.

$$X_{on} = 2\int_0^X f_n(x)dx/2^n = 2 - 2^{1-nX} \tag{42}$$

In the range of $\frac{1}{n} \leqslant X \leqslant 1$, the following relations can be obtained: each X-marker in this region will give 2 X-markers in the hybrid DNA, so

$$X_{on} = 2g_n(X)/2^n = 2^{1-nX} \tag{43}$$

Consequently,

$$X_{nn} = 1 - 2^{1-nX} \tag{44}$$

Note that there would be no X_{oo} in this region.

A graphic representation of these relations is given in Figure 7.

D. KINETICS OF MARKER TRANSFER

The transfer kinetics of marker X into hybrid and doubly-labelled peaks can be analyzed as follows.

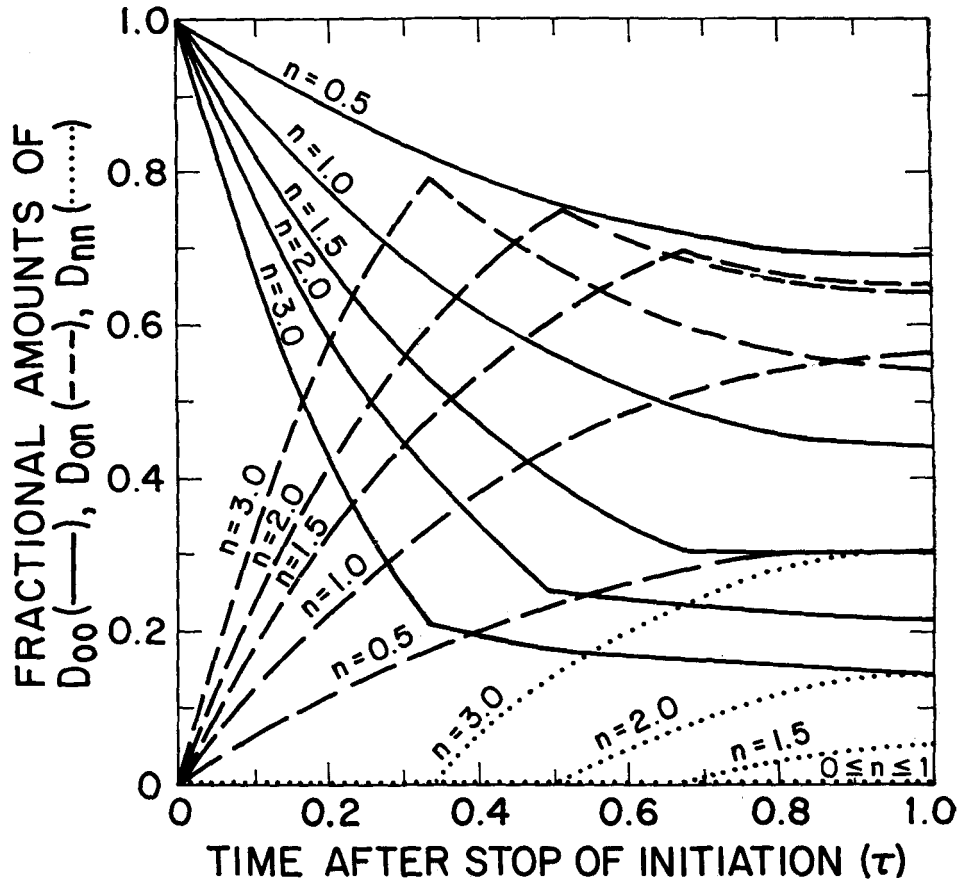


FIGURE 12.—The kinetics of density transfer of DNA with concomitant initiation blockage (equations 32, 38, 39 and 40).

The rate of loss of X_{oo} due to the transfer can be obtained by noting that the rate is proportional to the frequency of the replication point which passes marker X at time τ. Here τ is the time after the stop of initiation, and its unit is the time required for each replication point to travel from the origin to the terminus.

a) $0 \leqslant \tau \leqslant X$ and $0 \leqslant X \leqslant \frac{1}{n}$

Using equation 13, the amount of the X marker in the parental peak, X_P, can be obtained as follows:

$$\frac{dX_P}{d\tau} = -f_n(\tau,X) = -n(ln2)2^{n(1-X+\tau)}$$

$$X_P = 2^{n(1-X)}(2 - 2^{n\tau}) \tag{45}$$

At time τ the total frequency of marker X in the population, X_τ, is

$$X_\tau = g_n(X)2^{n\tau} = 2^{n(1-X+\tau)} \tag{46}$$

Therefore, the fractional amount of X in the parental peak (X_{oo}) is

$$X_{oo} = X_P/2^{n(1-X+\tau)} = 2^{1-n\tau} - 1 \tag{47}$$

Similarly, the absolute frequency of the X marker in the hybrid peak, X_H, is obtained as follows:

$$\frac{dX_H}{d\tau} = 2f_n(\tau,X) = 2n(ln2)2^{n(1-X+\tau)}$$

$$X_H = 2 \cdot 2^{n(1-X)}(2^{n\tau} - 1) \tag{48}$$

The fractional amount of X in the hybrid peak (X_{on}) is

$$X_{on} = X_H / 2^{n(1-X+\tau)} = 2 - 2^{1-n\tau} \tag{49}$$

b) $\frac{1}{n} \leqslant \tau \leqslant X$, and $\frac{1}{n} \leqslant X \leqslant 1$

$$X_H = 2g_n(X) = 2 \cdot 2^{n(1-X)} \tag{50}$$

Note that this is constant, so

$$X_{on} = 2 \cdot 2^{n(1-X)} / 2^{n(1-X+\tau)} = 2^{1-n\tau} \tag{51}$$

Since in this range $X_{on} + X_{nn} = 1$,

$$X_{nn} = 1 - 2^{1-n\tau} \tag{52}$$

These relationships are shown in Figure 14. It is important to note that (a) the change of X_{oo}, X_{on} and X_{nn} occurs only in the range of $0 \leqslant \tau \leqslant X$, where in the range of $\tau \geqslant X$, these values stay constant (Figure 5B is an experimental example); (b) Figures 13 and 14 take exactly the same shape. In other words, X and τ are interchangeable.

The rate of transfer of a marker is not dependent on its location on the chromosome until τ reaches the equivalence of the distance between the origin and the locus. After that time, no transfer of the marker is seen. The situation for $n = 1$ is shown in Figure 15A; that for $n = 2$ is shown in Figure 15B.

In conclusion, the following are salient points which should be useful for the interpretation of experimental results:

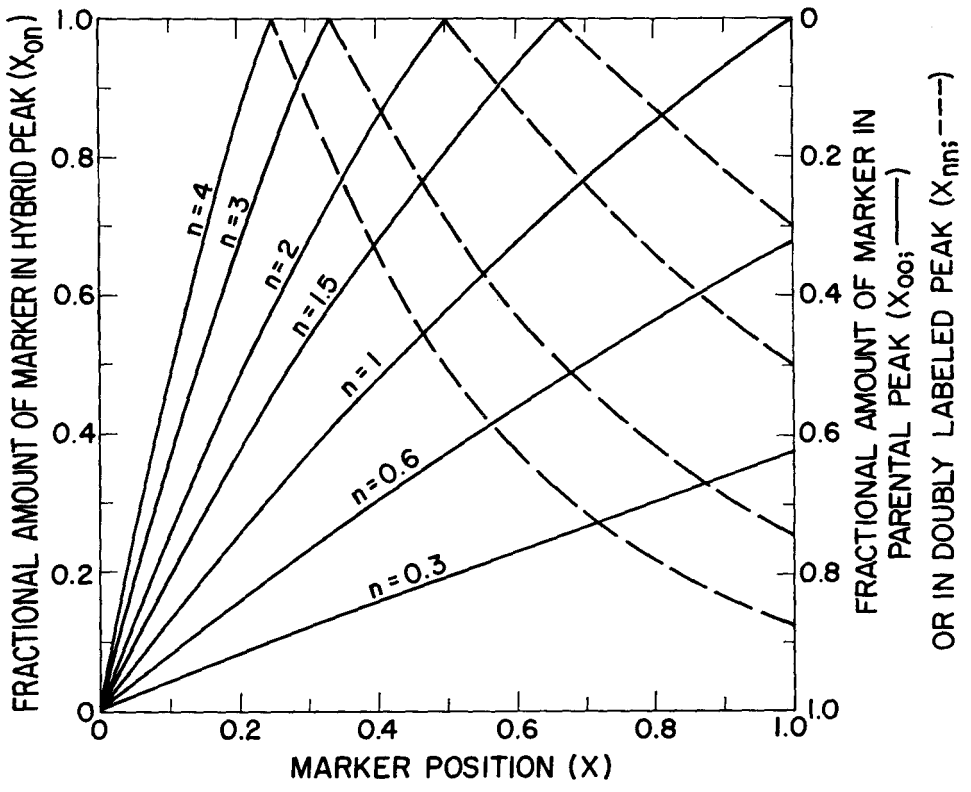


FIGURE 13.—Fractional amounts of markers in parental (*oo*), hybrid (*on*) and doubly density-labelled (*nn*) peaks after the completion of chromosome replication with concomitant initiation blockage (equations 41 and 42).

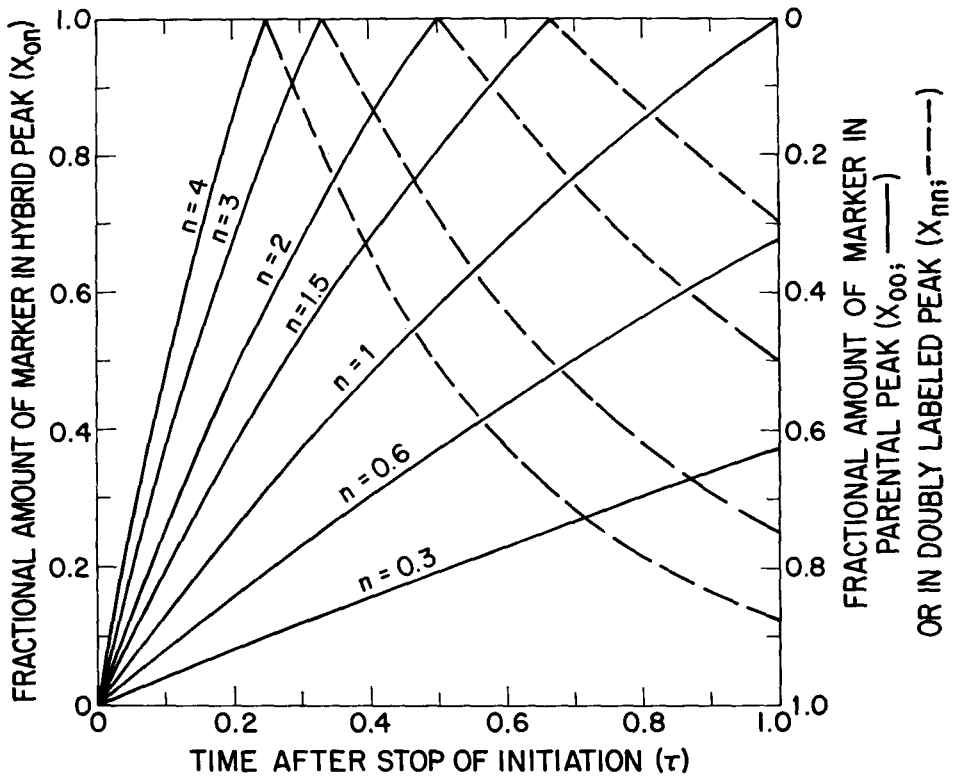


FIGURE 14.—The kinetics of density transfer of markers with concomitant initiation blockage (equations 47, 49, 51 and 52).

i) *Without blocking of initiation:* All genetic markers are transferred to hybrid and doubly-labelled peaks at the same rate, and the specific (transforming) activity of a marker remains the same in all three peaks.

ii) *With the concomitant blocking of initiation:*

a) For $0 \leqslant n \leqslant 1$, no D_{nn} nor X_{nn} is found.

b) In $0 \leqslant \tau \leqslant \frac{1}{n}$, no D_{nn} nor X_{nn} is found.

c) All markers show the same rate of transfer until the time which corresponds to the distance between 0 and their position on the chromosome is reached, after which point no more transfer occurs (Figures 14, 15A).

d) When $n > 1$, the markers which are located beyond the $\frac{1}{n}$ position on the chromosome will eventually have activity in the doubly-labelled peak. The transfer of these markers also stops when time reaches the equivalence of the distance between 0 and their position on the chromosome (Figures 8, 15B).

e) The fractional amounts of markers at completion give estimates of the chromosomal positions of the markers, if an estimate of n prior to the transfer is available. If, on the other hand, the position of a marker is known, an estimate of n can be obtained.

f) After completion of replication, an estimate of pretransfer n can also be calculated from the DNA amount in hybrid (equations 24 and 28) and/or that in doubly-labelled (equation 29) (Figure 11).

iii) It is important to remember that the theoretical expectations are bound by the following assumptions:

a) Steady-state replication.
b) Non-synchronous replication.
c) Symmetric initiation.
d) Immediate and complete blockage of initiation.
e) A constant rate of post-transfer replication of each replication point.
f) Completion of all chromosomes.

The design of experiments and the interpretation of experimental results can be, and has been, aided by theory. At the same time, knowing the assumptions, discrepancy from the theoretical expectation gives a clue as to which of the assumptions are not satisfied.

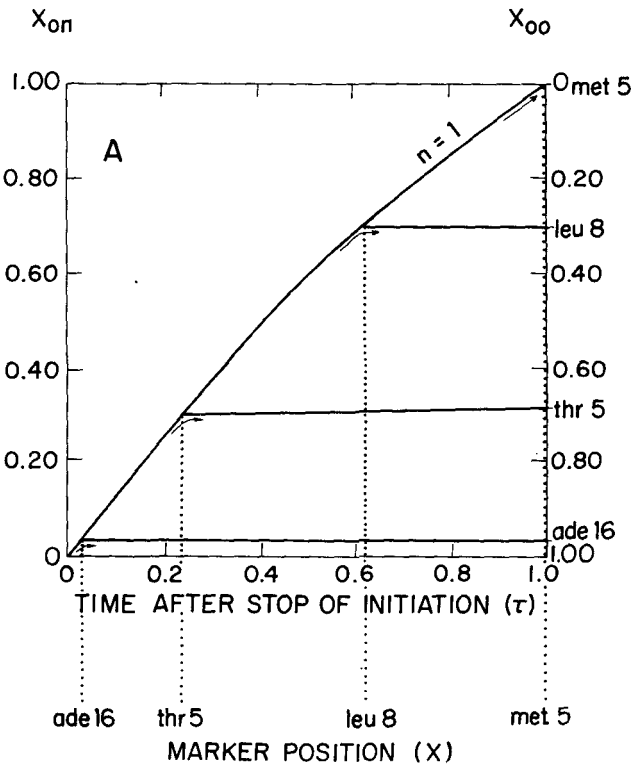
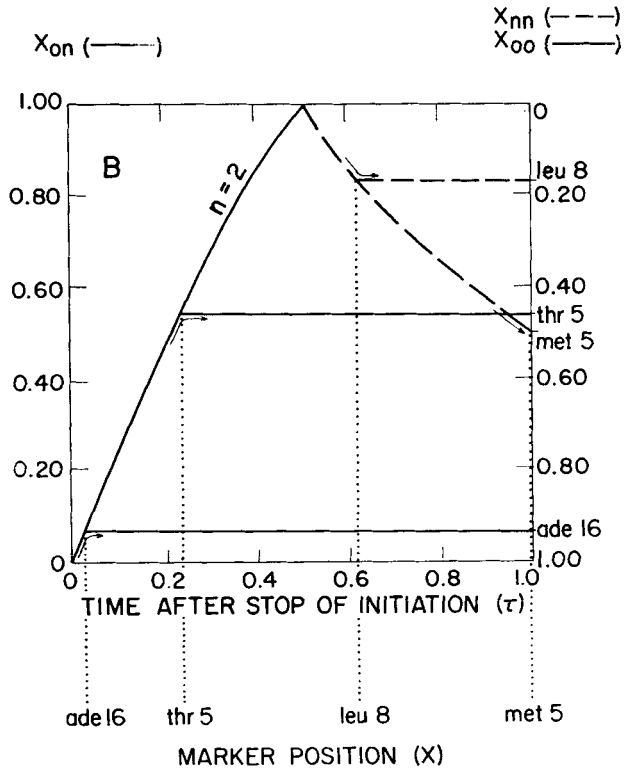


FIGURE 15.—Further explanation of Figure 14 with some specific markers. (A) $n = 1$. Observe that each marker transfers to the hybrid peak along a common curve until the time (τ) after the transfer reaches a point equivalent to the distance between the origin and the marker position (X) and no further transfer occurs. (B) $n = 2$. Note that markers in the latter half of the chromosome become all hybrid at $\tau = 0.5$; then a fraction becomes doubly labelled until the time reaches a point equivalent to the marker position and no further transfer is observed.

Figure 15—Continued on next page.


X_{on} (——)
X_{nn} (– – –)
X_{oo} (——)
B
n=2
1.00
0.80
0.60
0.40
0.20
0
0
0.2
0.4
0.6
0.8
1.0
leu 8
0.20
0.40
thr 5
met 5
0.60
0.80
ade 16
1.00
TIME AFTER STOP OF INITIATION (τ)
ade 16
thr 5
leu 8
met 5
MARKER POSITION (X)