MECHANISM OF GENETIC RECOMBINATION DURING BACTERIAL CONJUGATION OF *ESCHERICHIA COLI* K-12. I. HETEROGENEITY OF THE PROGENY OF CONJUGATED CELLS

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IN the course of bacterial conjugation the donor cells of *Escherichia coli* K-12 (Hfr) transfer part of their chromosome to the recipient cells (F^-). As a result, merozygotes are formed, i.e., cells containing a complete chromosome of F^- and the transferred part of the Hfr genome. The state of the merozygotes is unstable and they split off stable recombinant structures which are segregated and multiplied. The recombinant structure is hybrid; it inherits part of the F^- genotype (the greatest one as a rule) and part of the Hfr genotype. The analysis of recombinants, the measurement of their segregation rate gives information about the fate of the merozygotes from the moment of the donor DNA insertion till the moment of pure recombinant formation.

First LEDERBERG (1957) and then ANDERSON (1958), using a micromanipulation technique of selection of single conjugating pairs, investigated the progeny of individual zygotes and showed that in many successive generations the same zygotes are able to segregate recombinants of different genotype. According to LEDERBERG's data, out of 75 investigated exconjugants, 20 gave two to four different recombinant genotypes (~26%). ANDERSON's data are somewhat different. He found on the average over 2.5 different recombinant genotypes in every exconjugant clone. Besides, the number of generations in which the segregation of new recombinant types takes place was evaluated. This was found to amount to nine generations.

These results require a definite recombination model taking into account the possibility of many recombination events in the course of a zygote development. We will come back to this question.

In 1960 the problem was studied anew by TOMIZAWA. He observed the segregation of *Lac*$^+$ and *Lac*$^-$ markers during the postconjugation period. His data lead to the assumption that a mixed progeny (the appearance of mosaic clones) is observed only during three generations owing to the segregation of nonfertilized nuclei of the exconjugant cell in the course of its division. In other words TOMIZAWA concluded that the merozygote which is the direct product of conjugation gives rise only to one recombinant type.

But TOMIZAWA's conclusions are not at all convincing. In contrast to previous investigators he used only one genetic marker. Naturally, the segregation rate of a single marker may be higher than that of a complex diploid structure. Besides,

TABLE 1

Characteristics of bacterial strains

Strain	Mating type*	Genotype†	Source
HfrH	O-*thr-leu-pro*. . . F	thi^-Sm^s	HAYES (1953)
CC23	Type of HfrH	$met^-try^-Sm^s$	F. JACOB's collection
P10	O-*thi-isol-Sm*. . . F	$thr^-leu^-T6^sSm^s$	Isolated from C600 (APPLEYARD 1954), F. JACOB's collection
P10 Sm^r	Type of P10	$thr^-leu^-T6^sSm^r$	Isolated from P10 by selection of spontaneous mutants
AB313	O-*mtl-Sm-ser/gly*. . . F	$thr^-leu^-thi^-T6^s\lambda^+Sm^r$	TAYLOR and ADELBERG (1960)
PA309	F^-	$thr^-leu^-try^-his^-arg^-thi^-Sm^r$	F. JACOB's collection
PA309–2	F^-	$thr^+leu^+try^-his^-arg^+thi^-Sm^s$	Product of conjugation CC23 × PA309
PA309–3	F^-	$thr^-leu^-try^-his^-arg^+thi^-Sm^s$	Product of conjugation CC23 × PA309
PA309–5	F^-	$thr^-leu^-try^-his^+arg^+thi^-Sm^s$	Product of conjugation CC23 × PA309
PA260	F^-	$thr^-leu^-his^-ser/gly^-thi^-T6^sSm^s$	JACOB and WOLLMAN (1961)
PA260–1	F^-	$thr^+leu^+his^-ser/gly^-thi^-T6^sSm^s$	Product of conjugation CC23 × PA260
PA260–1 $T6^r$	F^-	$thr^+leu^+his^-ser/gly^-thi^-T6^rSm^s$	Isolated from PA260–1 by selection of spontaneous mutants

* The following symbols are used: *arg*—arginine, *thi*—thiamine, *his*—histidine, *isol*—isoleucine, *gly*—glycine, *leu*—leucine, λ^+—lysogenicity for phage λ, *met*—methionine, *mtl*—mannitol, O—starting point of chromosome transfer during conjugation, *pro*—proline, *ser/gly*—serine or glycine, Sm^s/Sm^r—sensitivity and resistance to streptomycin, *thr*—threonine, *try*—tryptophan, $T6^s/T6^r$—sensitivity and resistance to phage T6.

† Only those markers are listed which were used in the work.

the technique of his experiment did not discriminate between real genetic segregation in the fertilized nucleus and the apparent one caused by the polynuclear structure of bacterial cells. The inconsistency of published data induced us to start the present work.

It was necessary to verify that successive recombination events actually take place in the individual zygote causing heterogeneity of its progeny. And if so, it was important to find the mechanism of recombination leading to these consequences.

We investigated the multiple progeny of exconjugants and unlike ANDERSON we used a statistical approach for investigation of the progeny of each zygote. The results obtained are in general agreement with those of LEDERBERG and ANDERSON.

MATERIALS AND METHODS

Bacteria. Strains of *E. coli* K-12 used in our work are listed in Table 1. Strains HfrH, CC23, PA309 were obtained from F. JACOB. Strain AB313 was obtained from J. SCAIFE.

Phage. Wild-type coliphage T6 was used for counterselection of Hfr cells besides streptomycin (250 μg/ml). A phage suspension of high titer ($\sim 10^{12}$/ml) was prepared by the procedure of ADAMS (1950).

Culture media, conjugation procedure, preparation of Hfr cells labelled with radiophosphorus and the method of measurement of "radioactive suicide" were described by BLINKOVA, BRESLER and LANZOV (1965).

EXPERIMENTAL RESULTS

Analysis of the progeny of single zygotes: In the *E. coli* chromosome, three different loci were chosen, all situated at a rather long distance from each other ($\sim$25 min). These were *thr-leu, try* and *his.* Every one of them can be independently integrated into the recombinant after being transferred during conjugation. *his* was the distal locus, measuring from the initial point O.

Cells Hfr H and PA 309 were grown and conjugated on the Tris-H or AP medium. Conjugation was interrupted after 80 to 120 min by 1000-fold dilution of the suspension with phosphate buffer and treatment with a blender for 1.5 min ($\sim$3000 rev/min). Then the suspension was suitably diluted (so that no more than 20 to 30 colonies would grow on a Petri dish) and plated on selective agar devoid of histidine. In this case, those of the merozygotes were selected which had obtained during conjugation all the characters: *thr-leu, try* and *his.* For counterselection of Hfr cells streptomycin was used. After 2 days growth of his^+Sm^r recombinant clones, those containing the active *thr-leu* marker were selected by the LEDERBERG's method of replicas (1952). Replicas were grown for 8 to 10 or 24 hours. The recombinant clones on the initial plate that were found to be thr^+leu^+ were taken with a platinum loop and resuspended in a small volume of complete synthetic medium supplied with streptomycin. Test tubes with cell suspensions were shaken for 2 hours on a vibrational shaker for attaining homogeneity. Then the suspensions were suitably diluted and plated on selective agar devoid of histidine so that the number of colonies would not exceed 100 to 150

TABLE 2

Genotypes in the progeny of single merozygotes. 533 colonies were investigated; $n^*/n \sim 30\%$

Genotypes	No. of merozygotes leading to these genotypes	Genotypes	No. of merozygotes leading to these genotypes
$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{-}thr^{+}\text{-}leu^{+}$	40	$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{-}thr^{+}\text{-}leu^{+}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	6
$his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	33	$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	11
$his^{+}try^{-}thr^{+}\text{-}leu^{+}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	29	$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{-}thr^{+}\text{-}leu^{+}$	3
$his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{-}thr^{+}\text{-}leu^{+}$	25	$his^{+}try^{-}thr^{+}\text{-}leu^{+}$ $his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	1
$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	11	$his^{+}try^{-}thr^{-}\text{-}leu^{-}$ $his^{+}try^{+}thr^{-}\text{-}leu^{-}$ $his^{+}try^{-}thr^{+}\text{-}leu^{+}$ $his^{+}try^{+}thr^{+}\text{-}leu^{+}$	6

per plate. After 2 days growth at 37°C the genotype of the plated cells was determined by the replica method.

Table 2 shows the results of a typical analysis of over 500 recombinant clones. It is clear that the table contains all the possible genotypic combinations which could be screened by this method. Although the method is limited since it is possible to observe no more than four different recombinant genotypes, the number of the mixed clones containing simultaneously 2, 3 or 4 different genotypes (we shall designate the number of multiple clones by n^*) is over 30% of the total number of clones investigated (we shall designate the total number by n). The presence of three and four genotypes in the progeny of one zygote as well as the high probability in general of the appearance of mixed progeny suggests a series of successive recombination events in the course of the development of each merozygote.

Segregation of nonfertilized nuclei is of no consequence for this experiment since the corresponding cells are not viable and cannot give clones on selective agar. The method of statistical study of single merozygotes progeny used by us has its advantages and drawbacks. In contrast to the micromanipulation technique, it allows one to get statistically significant figures. On the other hand, one must be careful to eliminate sources of possible errors: (1) The possibility of triparental conjugation. Though it is by two orders of magnitude less probable than the biparental (FISHER-FANTUZZI and DIGIROLAMO 1961) it can occur if the conjugation lasts a long time. This possibility was excluded by choosing the ratio of donor to recipient cells in the range 1:20 to 1:100. (2) The possibility of aggregation of recipient cells, i.e. the possibility of simultaneous conjugation of several

F⁻ cells sticking together could cause in principle the formation of overlapping clones, which would be taken for one.

The following controls were performed to exclude this possibility: (a) Before conjugation F⁻ cells were homogenized in a blender for various time periods. After conjugation, the heterogeneity of their progeny was studied by the method described. The results revealed no difference from data obtained in standard conditions. (b) A mixture of two or three cultures of F⁻ strains which were genetically different derivatives of PA309 (strains PA309–2, PA309–3 and PA309–5) was plated on agar, and clones were subjected to genetic analysis by the same procedure as in the main experiment. The analysis showed that the probability of different cells sticking together is negligible (less than 0.5%).

Similar sources of errors are discussed in the papers by WITKIN (1951) and IYER (1965).

Our results confirm in general LEDERBERG's and ANDERSON's data, i.e. that the multiplicity of the exconjugant progeny is a real fact and does not depend on segregation of nonfertilized nuclei.

Kinetic investigation of merozygote segregation: This experiment differs from the preceding only in one respect: the multiplicity of the progeny was investigated after the conjugating cells were allowed to grow and divide in a liquid medium during different time periods. Experiments were conducted on Tris-H medium with the conjugating pair: HfrH × PA309. The results are shown in Figure 1. In the lower part of the figure the yield of his^+Sm^r recombinants is plotted against

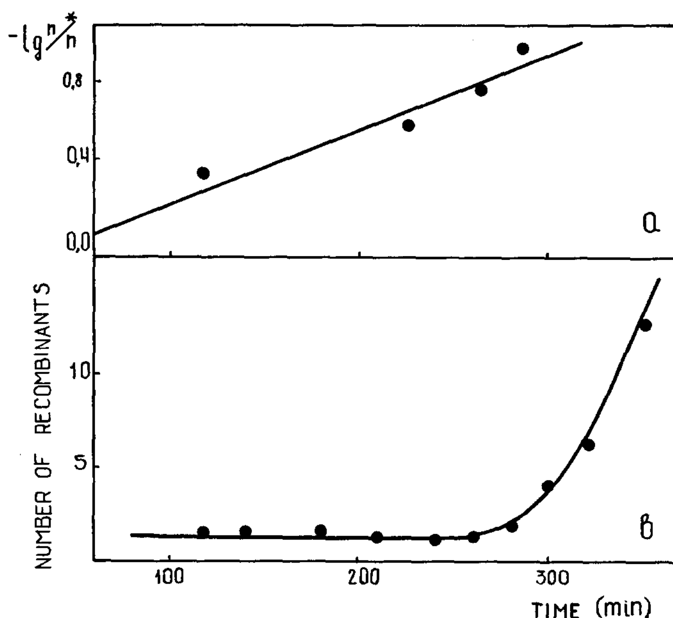

FIGURE 1.—Kinetics of decay segregation in merozygotes. a. Relative number of diploid cells yielding heterogeneous progeny, versus time. b. Yield ($\times 10^{-5}$) per ml of his^+Sm^r recombinants during the postconjugation period.

the time which elapsed from the moment of chromosome transfer. We see that the recombinants formed do not begin to copy and divide until a period of the order of three generation times elapsed. This situation is well known (JACOB and WOLLMAN 1961). The curve in the upper part of the figure is a plot of the logarithm of the relative number of mixed clones (ratio to the total number of clones analyzed) versus the time from the moment of chromosome transfer. Each point of this curve is the result of analysis of 60 to 100 his^+Sm^r recombinant clones. We see that this function is a straight line crossing the origin. This means that at the time 0, i.e. when the donor chromosome fragment enters the recipient cell, the ratio $n^*/n = 1$, i.e. all merozygotes formed are potentially able to give multiple progeny. Then as merozygotes grow and divide, their progeny is segregated and approximately by the 10th generation only pure recombinant types remain. These results are in quantitative agreement with ANDERSON's data.

The second conclusion which follows from these experiments is that the diploid structure or merozygote must be reduplicated many times and gives rise to several recombinant types. Indeed, if the diploid structures were not repeated during many generations, how could it happen that various combinations of the same genetic loci originally belonging to the donor cells were found in the mixed progeny of exconjugants. The only alternative to the multiplication of merodiploids would be the concept of copy choice. In this scheme, the recombinants are formed in every generation as a result of copying different parts of the same initial diploid structure, the merozygote. At present, direct experiments with phage (MESELSON and WEIGLE 1961; KELLENBERGER, ZICHICHI and WEIGLE 1961), transformation (BODMER and GANESAN 1964; FOX and ALLEN 1965) and conjugation (SIDDIQI 1963; BRESLER and LANZOV 1967) refute this hypothesis. The recombination obviously proceeds according to the breakage-reunion mechanism. We designed an experiment, using our experimental model, which allows us to exclude the mechanism of copy choice.

Analysis of the multiple progeny after the radioactive suicide of the donor DNA fragment: The HfrH cells grown on Tris-H medium with labelled phosphorus of high specific radioactivity (50 to 70 mC/mg) were conjugated with unlabelled PA309 cells in Tris-H medium. The conjugation proceeded for 80 min, then the suspension was diluted 1:500 with fresh Tris-H medium supplied with streptomycin, treated in a blender and aerated for a long time at 37°C. At definite time intervals samples of the suspension were diluted with protective glycerol-casein medium and frozen in liquid nitrogen. Simultaneously the yield of his^+Sm^r recombinants was measured. The kinetic plot is completely similar to that of Figure 1b.

If the donor DNA fragment is really the template from which recombinants are copied in successive cell generations then the destruction of this fragment by "radioactive suicide", should give a pure progeny. If the progeny proves to be heterogeneous, it means that the genetic structures, reduplicated before freezing and containing therefore nonradioactive phosphorus, must be diploid and able to maintain the heterozygous state even though the initial template was destroyed by radioactive decay.

TABLE 3

Heterogeneity of the progeny of exconjugants before and after the radioactive suicide of the donor DNA fragments

Time (minutes)	n^*/n (percent) Before P^{32} decay	After P^{32} decay
215	30	32
255	22	20.5

The analysis of samples taken at various moments after conjugation and preserved in frozen state the time required for "radioactive suicide" (time of the order of 20 to 30 days, characterized by the decay of 70 to 90% P^{32}) is presented in Table 3. It shows that P^{32} decay does not produce pronounced changes in the multiplicity of the progeny. As a control of the efficiency of "radioactive suicide" of DNA fragments transmitted during conjugation, the kinetics of inactivation of *thr*$^+$-*leu*$^+$ markers by P^{32} decay was measured (Figure 2). For this purpose samples of the suspension were taken 30 min after the beginning of conjugation, preserved in frozen state and finally plated.

Thus the results of this experiment show that diploid cells are copied and remain heterozygous during many generations.

Complexity of the progeny of cells which underwent recombination for one of the genetic characters: This series of experiments was aimed to prove that recombinants for one genetic marker remain heterozygous for others, or, in other words, that the same cells undergo subsequently many recombination events.

For this purpose the experimental scheme was changed in one important respect. As was mentioned before, the primary selection of exconjugants had been made for the *his*$^+$ marker. Evidently this is a dominant character and therefore both true recombinants *his*$^+$ and heterozygous cells *his*$^+$/*his*$^-$ are selected with equal success. In the new series *Sm*^{r} was chosen as the marker for primary selec-

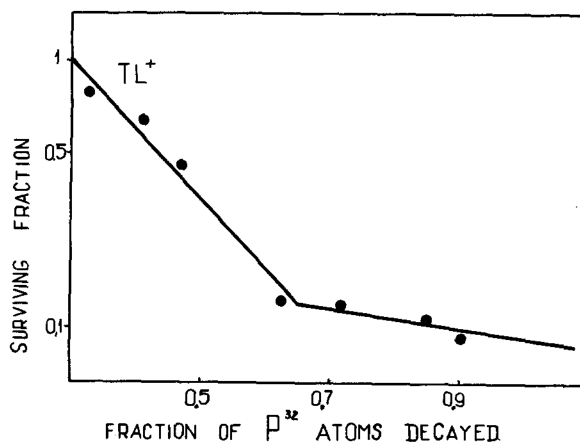

FIGURE 2.—Kinetics of "radioactive suicide" of recombinants *thr*$^+$-*leu*$^+$*Sm*^{r}.

tion. This is a recessive character (LEDERBERG 1951). Consequently, on a medium supplied with streptomycin only true Sm^r recombinants survive and heterozygotes die out. The question we are asking is whether clones formed after conjugation by Sm^r recombinants contain a simple or a mixed progeny.

In order to solve this problem, strains P10 Sm^r and PA260–1 $T6^r$ were submitted to conjugation. Experiments were conducted on AP medium. For counter-selection of Hfr cells, phage T6 was used. Conjugation lasted for 80 to 100 min and then phage T6 was added to the suspension (with a multiplicity 300:1 in respect to the Hfr's) for 3 to 5 min. Then the mixture was diluted with fresh AP medium and aerated for some hours. Aliquots were taken at different moments and plated on streptomycin-containing medium. The first Sm^r recombinants which were obtained after 120 to 160 min of phenotypic expression were analyzed for multiplicity of their genotypes. The procedure was as described in the first section, the only modification being that in this case *ser/gly* and *his* served as additional markers. As control an experiment was conducted simultaneously according to the previous program with initial selection for the dominant his^+ marker and subsequent selections for Sm^r and *ser/gly* markers.

The results of both experiments are represented in Table 4. In both cases the progeny proved to be mixed. Similar results were obtained when the conjugation pair: AB313 × PA260–1 $T6^r$ was used.

Hence, cells which were true recombinants for the Sm^r marker remained essentially heterozygous with respect to other characters (*ser/gly*, *his*).

DISCUSSION

First we will briefly sum up the experimental results: (1) Each exconjugant F^- is able to segregate more than one type of recombinant. (2) As the exconjugants F^- undergo division this capacity is gradually lost. (3) Segregation of new recombinant types occurs owing to reduplication and multiplication of heterozygous structures of exconjugants. (4) In the course of division, exconjugants F^- become homozygous for one of the characters but remain diploid according to others.

Now we will formulate a recombination model explaining these data. First, we shall take into account that isotope-labelling experiments performed with λ phages proved that the recombinant chromosome is composed of fragments of both parental chromosomes (MESELSON and WEIGLE 1961; KELLENBERGER, ZICHICHI and WEIGLE 1961; IHLER and MESELSON 1963; MESELSON 1964). The breakage and reunion of both parental chromosomes occurs at the level of double stranded DNA. In case of bacterial conjugation there are the data of OPPENHEIM and RILEY

TABLE 4

Heterogeneity of the progeny of true recombinants for the Sm^r^ *marker*

	Initial selection for his^+	Initial selection for Sm^r
n^*/n (percent)	17.5	13.5

(1966), which show that recombination occurs also by incorporation of double DNA strands into the chromosome of the recipient cell. It can be argued that in transformation only one of the DNA strands is incorporated into the recipient cell genome (Fox and ALLEN 1965; BRESLER, KRENEVA, KUSHEV and MOSEVITSKII 1964). But in transformation a small DNA fragment containing only few cistrons is integrated into the genome. The probabilities of recombination in this case are tens of times greater than in conjugation. This is a manifestation of the fact that the recombination processes are essentially different. We know from the work of PRITCHARD and others that abnormally high recombination probabilities are observed for very close genetic loci (PRITCHARD 1960). This is explained by the formation of small regions of efficient pairing in various points of the chromosome. It is quite probable that these regions of efficient pairing are denatured areas of DNA where synapsis can be established at the level of single DNA strands. But the recombination of parental chromosomes on a large scale must proceed by another mechanism resulting in much lower probability values. We will assume that double-stranded DNA fragments are incorporated into the genome of maternal cells during conjugation.

The second circumstance which must be taken into account is the existence of stable merodiploid strains of *E. coli* F^-. These strains had been discovered by LEDERBERG *et al.* (1951) and recently were studied in detail by CURTISS (1964) and CAMPBELL (1965). They differ from the ordinary strains because their genome contains a deletion affecting evidently the enzymatic system responsible for recombination ("recombinase" system). Bacteria with defective recombinase are mainly multiplied as diploids but they always segregate few stable haploid recombinants. The decay of merozygotes into haploid recombinants continues for common strains, as we have seen, some ten generations. The rate of decay of the merozygotes in the stable diploid strains is tens of times lower. Nevertheless there is a profound analogy between normal strains and these exceptional diploids.

Consequently the model in question assumes that the fragment of the donor chromosome exists in combination with the recipient cell chromosome for many generations, is copied when the cell is reduplicated, and is gradually used in recombination events. The first stage of the fragment's existence in the recipient cell is a state of synapsis before true integration occurs. In the paper of OPPENHEIM and RILEY (1966), it was shown by means of isotope labelling and density gradient centrifugation that the fragment of donor DNA is bound only by hydrogen bonds to the recipient chromosome during this first period. Also no reduplication of the fragment takes place; obviously it does not contain a replicator (JACOB and BRENNER 1963). Then a new event occurs—the fragment of DNA is integrated into the chromosome, becomes a part of its replicon, and is copied during the chromosome reduplication. We may anticipate that this integration occurs by the same mechanism that is well proved for the integration of episomes, namely for the integration of prophage and F'-factor (CAMPBELL 1962; SCAIFE and GROSS 1964). The donor DNA fragment forms a closed loop by means of some complementary regions, sticks to homologous regions of the chromosome, and is inserted by crossing over. Therefore a region of the chromosome becomes

diploid, it contains a series of genes duplicated in tandem. In this state the donor fragment becomes covalently bound to the chromosome (OPPENHEIM and RILEY 1966). The reverse process of elimination of these additional genes by a similar crossover phenomenon (like the liberation of prophage and F′-factor) proceeds with a definite probability. This leads to true recombinants since the elimination of the tandem region can occur in any place. This suggestion explains very well the probabilities of different recombinant types segregated by the stable diploid strains of CURTISS (1964).

According to this hypothesis, the decay of heterozygotes because of formation of recombinants must proceed according to first order kinetics. At the same time the number of heterozygotes must increase because of exponential growth. This gives

$$\frac{dn^*}{dt} = \frac{n^*}{\tau} - \frac{n^*}{T} = n^* \left(\frac{1}{\tau} - \frac{1}{T}\right) ,$$

hence

$$n^* = n_0 \, e^{-t(1/T - 1/\tau)} ,$$

where τ is the period of generation of heterozygotes, while T is the period of their decay by recombination. If the total population of cells grows exponentially, i.e. $n = n_0 \, e^{t/\tau}$ the relative amount of diploid cells will be $n^*/n = e^{-t/T}$. This is exactly what the experiment gives (Figure 1). In time $t = 0$ (the time of transfer of the donor DNA fragment) $n^* = n_0$, i.e. all the exconjugants are heterozygous. If, as usual, $T \geqslant \tau$ the absolute amount of heterozygous cells grows even though their relative concentration in the population decreases. The special merodiploid strains found by LEDERBEG fit this scheme well. These are cells with large values of T. While in normal cells it is possible to trace the diploid structures for several (up to ten) generations, in the special strains these can subsist for hundreds of generations.

The details of recombination events which we observed (Table 2) are not surprising. We see that the yields of different recombinants are approximately equal in cases of double recombinations, and they decrease in a drastic way if fourfold recombination is required for the realization of the corresponding genotype. Owing to large distances between the genetic loci used, the probabilities of recombination between any pair of points do not differ practically from their limiting value (0.5). This fits well with the general picture of recombinant types we observed.

SUMMARY

By means of statistical analysis of the progeny of single merozygotes it was shown that: (1) Every exconjugant segregates more than one type of recombinant cells; (2) when growth and division of exconjugants proceeds, this capacity is lost; (3) the segregation of many recombinant genotypes is due to the reduplication and the multiplication of heterozygous structures of exconjugants; (4) during any division the exconjugants may become homozygous for some of the markers but remain heterozygous according to others.—A genetic model, which

is in accord with all these findings, assumes that a male chromosome fragment exists in combination with the exconjugant cell chromosome, is copied when the cell is reduplicated, and is gradually used in recombination events.

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