TRIPARENTAL MATINGS IN ESCHERICHIA COLI

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THE present work was carried out with the purpose of establishing whether a single female cell (F^-) of *E. coli* K-12 could mate with two male cells (Hfr) at the same time, leading to a triploid merozygote. The degree of triploidy postulated would depend, in fact, on the type of males used, that is, on the common portion of the particular group of markers transmitted by each of them (JACOB and WOLLMAN 1957).

LEDERBERG (1947) investigated the possibility of triparental crosses in *E. coli* K-12, but at that time mating types had not yet been recognized in this organism (LEDERBERG, CAVALLI-SFORZA and LEDERBERG 1952; HAYES 1953; CAVALLI-SFORZA, LEDERBERG and LEDERBERG 1953), nor conjugation features established (WOLLMAN and JACOB 1955; LEDERBERG 1957). The experiment tested a *ménage à trois* of two females with one male, and present knowledge would lead one to expect a negative outcome, as was actually observed.

Regarding other microorganisms, triparental recombination has been recently discovered in yeasts by POLSINELLI (1960).

Occurrence of triploid or partially triploid zygotes by mechanisms other than a triparental cross is known in a variety of organisms. We are not going to summarize them here, as it is not our purpose to discuss patterns of segregation.

In connection with the problem investigated, was the question of whether a fertilized F^- acquires some kind of immunity against further matings. Some of the experiments described in this paper may furnish an indirect answer also to this latter question.

MATERIALS AND METHODS

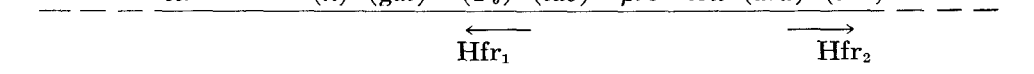
Strains:

439: F^- *leu pro str*
682: Hfr_I *leu* + +
683: Hfr_1 + *pro* +
U 187: Hfr_2 + *pro* +
684: Hfr_2 *leu* + +

only markers with a bearing on the experiments described in this paper have been cited.

(Numbers refer to L. L. CAVALLI-SFORZA's list of stocks, except for U 187, kindly supplied by E. CALEF, a lysogenic derivative of a strain received from E. LEDERBERG as W 3980.)

Position of markers on the linkage map of *E. coli* K-12 (imagining a portion of the circular map (JACOB and WOLLMAN 1957) stretched out):



Pedigrees of strains:

682: recombinant from a cross 622 (Hfr_1 *met*) × 439 (F^- *leu pro gal*) selected on EM† *gal leu.*

683: recombinant from a cross 622 × 439 selected on EM *gal pro.*

684: recombinant from a cross U 187 × 392 (F^- *his leu*‡) selected on MM *leu.*

† Minimal eosine-methylene blue medium without any other carbon source than the sugar added as required (LEDERBERG).

‡ Same *leu* mutation as 439'.

Media: Difco Penassay, as broth or agar, was used as a complete growth medium. DAVIS' formula (DAVIS and MINGIOLI 1950) was followed for minimal medium (MM), supplemented when required.

Crossing procedure: Both female and males were grown in broth to logarithmic phase in a rotator, centrifuged, and resuspended in fresh Penassay broth. Crosses were performed with gentle shaking in a water bath at 37°C with a volume varying from 1.5 ml to 4.5 ml in 100 ml Erlenmeyer flasks to give efficient aeration. For interruption of conjugation, vibration by a rotating eccentric rubber wheel was employed. Tenfold dilutions were made at given times in 1.8 ml of cold saline, whether interrupted or not, further diluted if necessary, and kept at ice temperature till plating was performed.

Plating on selective minimal media was done inoculating a top layer of 4 ml of MM without any supplementation (diffusion from the bottom layer was sufficient) in order to decrease plate recombination. Plates, and tubes containing 4 ml of melted agar, were previously kept at 40°C–45°C. Titration on complete medium was performed in a top layer of soft agar. Incubation was at 37°C.

EXPERIMENTAL RESULTS

Occurrence of triparental recombinants: A mating mixture (*ménage à trois*) was prepared as follows: a streptomycin resistant F^-, having a double requirement for leucine and proline (439), plus a streptomycin sensitive, leucine requiring Hfr_1 (682), plus a streptomycin sensitive, proline requiring Hfr_2 (U 187). Four possible types of crosses could take place in such a mixture. Of these, the first three are known to occur, the fourth one was under investigation:

(1) 439 × 682: recombinants from this cross could be selected on MM *str leu.*

(2) 439 × U 187: recombinants from such a cross could be selected by plating on MM *str pro.*

(3) 682 × U 187: this type of progeny could be selected on MM.

(4) 439 × 682 × U 187: plating on MM *str*, the only cells able to grow should necessarily have received a contribution from each of the three parents.

Numbers of colonies grown on these different media from the same mating mixture described above are given in Table 1.

leu and *pro* markers were chosen since they are introduced at high frequency by both of the two males used (JACOB and WOLLMAN 1957). This was confirmed

TABLE 1

Frequencies of recombinants from the four possible types of crosses occurring in a "ménage à trois"

Mating mixture viable cells/ml	439: 1.2×10^8 682: 2.2×10^8 U 187: 0.82×10^8	F⁻ Hfr_1 *leu* Hfr_2 *pro*
Selective medium and origin of recombinants	Time of contact (minutes) 0	30
MM *leu str* (439 × 682)	$<10^2$	2.8×10^6
MM *pro str* (439 × U 187)	$<10^2$	1.1×10^6
MM (682 × U 187)	10^4*	7.3×10^6
MM *str* (triparental)	$<10^2$	1.1×10^4

Time 0 minutes: interrupted immediately after mixing on a tenfold dilution, of which 0.1 ml were plated.

Time 30 minutes: diluted without interruption. Inocula of 0.1 ml of a 10^{-3} dilution, except for MM *str*, where 0.1 ml of a 10^{-1} dilution were plated.

Controls of spontaneous reversion for each marker and for each strain: $<0.3 \times 10^2$.

* Platings on MM always gave some plate recombination, probably due to residual growth.

by platings on MM *str leu* and MM *str pro* (Table 1), showing for types (1) and (2) crosses a recombination frequency of about one percent which was the usual value, in our experimental conditions, for a normal F⁻ × Hfr cross.

From the platings on MM, it may be noted that the recombination frequency of the Hfr × Hfr cross was found to be unexpectedly high. This will be discussed later.

As for colonies grown on MM *str* (Table 1), which appear at a much lower frequency (about 10^{-4} of the original mixture), they must have arisen, as said before, through a contribution from all three parents.

The possibility of recombination between nonallelic markers of only one of the two males and the female was excluded, for *leu* marker by pedigree and for *pro* by allelism tests.

Having thus established the occurrence of triparental recombinants, the following questions were formulated:

(a) Is a triparental cross conditioned by the presence of different Hfr's (in our case Hfr_1 and Hfr_2) or is it possible also with males of the same type? [Note—Hfr_2: Strain U 187, actually, as shown by subsequent experiments of interrupted mating, seemed to have an abnormal Hfr_2 type, introducing *leu* only at about 40 minutes (but still retaining a high frequency for *gal* and *lambda*).]

(b) What events lead to the triparental mating? A first trivial possibility, namely mating of the second male with a segregant from a cross of the female with the first male, is ruled out by the structure of the experiment. In fact, triparental recombinants are already present in the mixture after 30 minutes of contact (and earlier; see Table 3). We know from data of WOLLMAN, JACOB and

HAYES (1956) and of TOMIZAWA (1960) that segregation does not take place for at least 80 minutes after time of mixing. So we are left with two other possibilities:

(1) three cells (two male and one female) can mate simultaneously, forming a triplet (one-stage process).

(2) A female mates with one male; after transfer of the male material, the fertilized female (merozygote) can receive additional genetic material from other male cells (two-stage process).

These two hypotheses are not necessarily mutually exclusive. In both cases, formation of a partially triploid zygote is postulated.

Absence of an effect of Hfr type: To answer the first question, the same female (439) was mixed in turn with the four compatible pairwise assortments of four different Hfr's—two Hfr_1 and two Hfr_2. Results are summarized in Table 2. It can be seen that a triparental cross is established independently of whether or not the Hfr's are of the same type, as recombinants on MM *str* occur in the four mixtures at about the same frequency.

Kinetics of triparental crosses: Concerning the main question, whether triploid zygotes were formed by a one-stage or a two-stage process, the kinetics of appearance of triparental recombinants, with regard both to entry of markers and to conjugation itself, could supply some critical information. A combined experiment of interrupted and noninterrupted mating was performed with the same mating mixture. Controls were run as usual of the two "legitimate" types of crosses (Hfr × F^-) occurring in the mixture. These, for interrupted samples, gave the actual time of entry, for the given experiment, of each one of the two markers considered.

In Figure 1 are plotted numbers of colonies grown on the different selective media from the interrupted samples. The appearance of recombinants is at about ten minutes for *str*^{r} *pro*$^+$ progeny (from 439 × 682 crosses = C 1), and at about 20 minutes for *str*^{r} *leu*$^+$ progeny (from 439 × 683 crosses = C 2); according to the sequence expected from Hfr_1.

str^r *pro*$^+$ *leu*$^+$ progeny (from triparental crosses = M) follow closely the kinetics of the later marker, beginning to appear as soon as this one enters.

TABLE 2

Effect of Hfr types on the occurrence of triparental recombinants

Selective medium	Viable cells/ml	Mating mixtures (I) 439:1.6×10^8 682:1.6×10^8 U 187:1.9×10^8	(II) 439:1.6×10^8 684:3.3×10^8 683:1.7×10^8	(III) 439:1.6×10^8 682:1.6×10^8 683:1.7×10^8	(IV) (439:1.6×10^8 684:3.3×10^8 U 187:1.9×10^8	F− Hfr *leu* Hfr *pro*
MM *str*		1.96×10^4	1.8×10^4	2.05×10^4	0.63×10^4	
MM *leu str*		1.45×10^6	2.15×10^6	1.96×10^6	1.74×10^6	
MM *pro str*		0.88×10^6	2.0×10^6	0.98×10^6	1.19×10^6	

Contact for 30 minutes.

Time 0 minutes: $<10^2$ on each medium.

Time 30 minutes: as in Table 1.

Platings on MM *leu str* and on MM *pro str* served a control of recombination frequencies of the two types of biparental crosses Hfr × F^- occurring in each mixture.

In Table 3, numbers of recombinants from noninterrupted samples are given for the same three types of crosses as in Figure 1. It can be seen that in this case triparental progeny are already present in the mixture at ten minutes. A gradual beginning was always observed both for interrupted and noninterrupted experiments, and also in Hfr × F⁻ crosses, probably due to lack of synchronization. In addition it will be noticed that 683 is somewhat less efficient than 682 in conjugation.

These results may assist our problem as follows: firstly, they rule out definitely the possibility of repeated mating in the formation of triparental progeny. Secondly, they suggest that a simultaneous mating of three cells can occur. The synchrony between the kinetics of M and C_2 in Figure 1 seems to support the idea that when the later marker (*leu*+) begins to be inoculated into the F⁻, the first one (*pro*+) is already present in a fairly high proportion of zygotes. In other words, if injection by the two males could not be simultaneous, we should expect a lag between time of entry of the later marker and time of appearance of triparental progeny. These considerations, together with the early presence of triparental recombinants in noninterrupted samples of Table 3, support a one-stage process.

To test the second possibility, the following experiment has been performed (Figure 2):

The F⁻ was allowed to mate separately with each of the two Hfr's for 30 minutes, after which time conjugation was interrupted, the second Hfr added, and the rate of appearance of triparental progeny observed from these two mixtures, and from a third one, in which the three parents had been mixed together at the same time as in previous experiments.

Results show that in the two mixtures in which previous contact had occurred between the F⁻ and one of the Hfr's, triparental recombinants reach higher values sooner than in the case where mixing had been simultaneous. Values

TABLE 3

Kinetics of conjugation of a "ménage à trois"

Selective medium	Time of contact (minutes) 0	10	20	30	40	50	60
MM *str* (M)*	<20	1.3×10^3	2.8×10^3	5.2×10^3	4.3×10^4	4.3×10^4	6.2×10^4
MM *str leu* (C_1)*	<20	7.5×10^4	1.1×10^6	2.5×10^6	4.2×10^6	7.0×10^6	7.8×10^6
MM *str pro* (C_2)*	<20	4.7×10^4	6.5×10^5	1.2×10^6	1.8×10^6	4.4×10^6	7.0×10^6

* Symbols as in Figure 1.

Mating mixture: F⁻ = 439 : 3.9×10^8
Hfr *leu* = 682 : 3.4×10^8
Hfr *pro* = 683 : 4.3×10^8
} viable cells/ml

Time 0 minutes: interrupted on a tenfold dilution, of which 0.5 ml were plated (this was obviously the same 0 minutes sample of Figure 1).

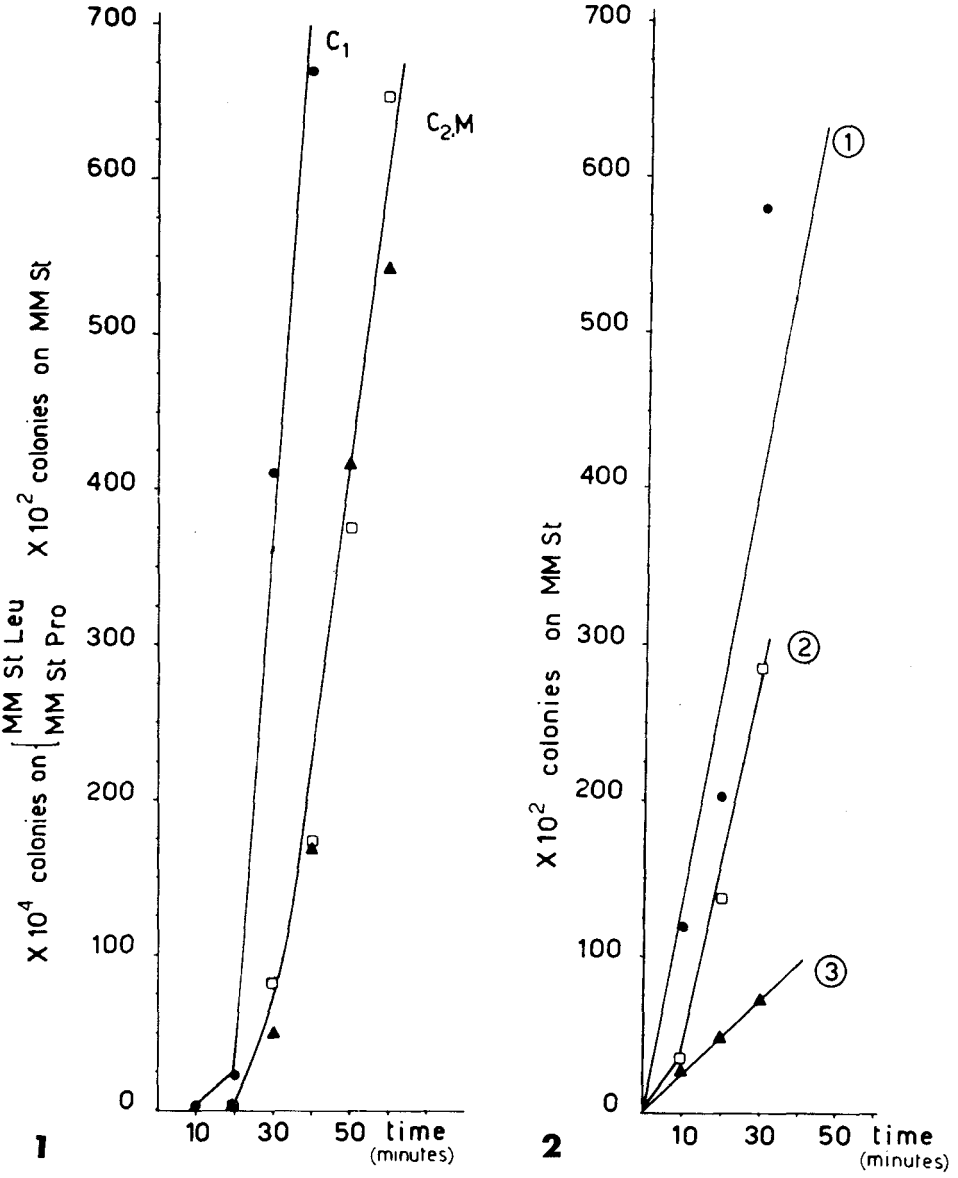


FIGURE 1.—Mating mixture:

F⁻ = 439 : 3.9×10^8
Hfr_1 *leu* = 682 : 3.4×10^8 } viable cells/ml.
Hfr_1 *pro* = 683 : 4.3×10^8

Samples interrupted on a tenfold dilution.

● = C_1, recombinants between 439 and 682 (platings on MM *str leu*) = entry of *pro*$^+$.

□ = C_2, recombinants between 439 and 683 (platings on MM *str leu*) = entry of *leu*$^+$.

▲ = M, triparental recombinants (platings on MM *str*).

A single line has been drawn to connect both □ and ▲ points since their kinetics seem the same, although ▲ frequencies are 1/100 of □ ones.

varied with the Hfr secondarily added, which can be related to the slower rate of conjugation of 683, seen in Table 3. At 60 minutes, approximately the same frequencies are reached in the three mixtures.

In analyzing these data, we can assume that in mixtures 1 and 2 there is already a high number of diploid zygotes at time zero minutes of the experiment. These would be ready for the second "stage" of mating (with the second Hfr), thus increasing, in early times, the rate of formation of triparental zygotes.

From these results we can conclude that both the two mechanisms postulated are probably involved in the formation of triparental progeny.

Effect of F⁻ in Hfr × Hfr crosses: We noted from data of Table 1 that the proportion of recombinants originating from Hfr × Hfr matings in a triparental mixture was higher than expected. Usual values for recombination frequency between two Hfr's are about 10^{-3} or less (CAVALLI-SFORZA, LEDERBERG and LEDERBERG 1953). We confirmed this for our strains, and simultaneously found that the frequency increased when a female was added to the Hfr × Hfr mixture (Table 4). This happened without the female taking part in recombination but for the few triparentals which were scored by plating on MM *str*. The latter, as we have seen, occur with a frequency a hundred times lower, and are therefore not involved.

Nevertheless, colonies grown on MM were scored again by streaking on streptomycin; and again only one percent of them was found *str*^{r}.

No "mating-activating substance" could be demonstrated in the cell-free supernatants of the female or of the triparental mixture treated as in crossing experiments. A similar effect (only slightly reduced in size) in increasing recombination frequencies between males was observed adding UV killed females (survival 10^{-7}) to Hfr × Hfr mixtures (Table 5). It is known that UV killed females cannot participate in a cross. It was thought, therefore, that the F⁻ might act in some way as an "attraction centre". For instance, a more effective pairing of the two Hfr's when a female is present could be accounted for by a difference in the surface properties between the males and the females

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FIGURE 2.—● = Mixture (1): 439+683 were previously incubated together for 30 minutes at 37°C, after which time conjugation was interrupted, and 682 added = time 0 of the experiment. Before adding 682, the interrupted mixture was titrated for recombinants between the preincubated parents (diploid zygotes) on MM *pro str*. These were 0.87×10^6.

□ = Mixture (2): 439+682 treated as 439+683 in mixture (1). 683 added at time 0 of the experiment. Recombinants between 439 and 682 after preincubation and interruption (time 0 of the experiment): 0.92×10^6.

▲ = Mixture (3): 439+683+682 mixed contemporaneously at time 0 of the experiment.

At 60 minutes the same level of about 2×10^5 was reached in the three mixtures.

Concentration of strains in the three mixtures:

F⁻ = 439 : 1.9×10^8
Hfr$_1$ *leu* = 682 : 1.4×10^8
Hfr$_1$ *pro* = 683 : 0.9×10^8
} viable cells/ml.

TABLE 4

Effect of F⁻ on recombination frequencies of Hfr × Hfr crosses

Selective medium	Viable cells/ml	(I) 682: 1.5×10^8 U 187:1.65×10^8 alone	(I) +439:0.84×10^8	(II) 684: 2.1×10^8 683:1.25×10^8 alone	(II) +439:0.84×10^8	(III) 682: 1.5×10^8 683:1.25×10^8 alone	(III) +439:0.84×10^8	(IV) 684: 2.1×10^8 Hfr *leu* U 187:1.65×10^8 Hfr *pro* alone	(IV) +439:0.84×10^8 F⁻
MM		5.1×10^5	6.8×10^6	2.7×10^5	4.6×10^6	1×10^5	1×10^7	1.35×10^6	4.05×10^6
MM *str*			3.6×10^4		3.9×10^4		1.7×10^5		1.3×10^4
MM *str leu*			5.9×10^6		4.0×10^6		4.3×10^6		2.2×10^6
MM *str pro*			2.9×10^6		5.2×10^6		3.7×10^6		2.0×10^6
Increase factor due to F⁻ in r.f. of Hfr × Hfr			13		17		100		3
Hfr × Hfr in triplets (qr)			2.15×10^{-2}		1.4×10^{-2}		3.6×10^{-2}		1.1×10^{-2}
Triparentals (qr^2)			4.3×10^{-4}		4.6×10^{-4}		20×10^{-4}		1.5×10^{-4}

Contact for 30 minutes. Dilutions without interruption. Platings on MM reveal Hfr×Hfr progeny both in mixtures with and without F⁻ added. Platings on MM *str* from F⁻ containing mixtures give frequencies of triparental matings. Platings on MM *str leu* and MM *str pro* give the two biparental Hfr×F⁻ recombination frequencies. In the two bottom lines relative frequencies are calculated for Hfr×Hfr and triparental progeny in the mixtures with F⁻ added (see discussion).

TABLE 5

Effect of UV killed F⁻ on recombination frequencies of Hfr × Hfr crosses

Hfr's	Alone	+ F⁻ (alive)	+ F⁻ (UV killed)
682 × 683	4×10^4	1.4×10^6	8.3×10^5
682 × U 187	8×10^4	3.2×10^6	1.2×10^6

Contact for 30 minutes. Dilutions without interruption. Platings on MM.

(MACCACARO and COMOLLI 1956; ØRSKOV and ØRSKOV 1960; SNEATH and LEDERBERG 1961), both Hfr's being attracted by the F⁻. The correlation which seems to exist between the degree of increase in recombination frequency of Hfr × Hfr in presence of F⁻, and the frequencies of triparental recombinants, respectively, in the four different mixtures of Table 4, might support this idea.

DISCUSSION

We have seen that the kinetics of markers injection and of conjugation, in a triparental cross, support the hypothesis of a role of simultaneous mating of three cells in the formation of triparental recombinants (one-stage process). On the other hand, the process can also take place in two stages, as supported by the experiment of Figure 2. In this case, mating had been allowed between only one of the males and the female for a time (30 minutes) which usually is sufficient for the majority of pairs to form, and, after that, mating was interrupted. We can assume that at this time most competent F⁻ cells were converted into merozygotes, separated from the fertilizing male. Addition of a second male would result (A) in a decreased outcome of triparental recombinants if the zygotes had become immune to further matings; or (B) in a faster rate of appearance of triparental progeny if zygotes are not immune, for, in this case, they will be ready for the "second stage" of the process. What was actually found is in agreement with alternative (B).

No evidence of interference against triparental matings was found from other sources either. In the absence of any complication, the expected relative frequency of triparental matings could be estimated as the product of the relative frequencies of the two biparental Hfr × F⁻ crosses. Actually it should be a little smaller, since biparental progeny can arise from pairs as well as from triplets (or larger clumps). One could calculate a "coincidence" value, i.e. the ratio of the observed to the expected frequency of triparentals. (The relative frequency of recombinants was calculated as the ratio of the total number of recombinants to the F⁻ cells input.)

If we calculate this "coincidence" from the data of Tables 1–4, we find, for triparental recombinants, an average value of nearly 1. The range of individual "coincidence" values is from 3 to 0.25, and this variation could be justified by the magnitude of the experimental errors involved.

The inference could be made, therefore, that on the average, there is neither

immunity nor other phenomena making triparental matings more or less probable than expected.

Turning to the problem of Hfr × Hfr recombination frequencies, we brought evidence that, in the presence of a female, males can mate between themselves more actively than in a plain Hfr + Hfr mixture. We explained this on the hypothesis that, when a female is present, Hfr × Hfr recombinants arise mostly from triplets (or larger clumps) containing at least one F^- cell, a more effective contact being established between the males by means of attraction exercised by the female. In these conditions, Hfr × Hfr recombination frequencies reach values which are roughly as high as those of Hfr × F^- crosses. Let us verify this on data of Table 4. Having established that, on the average, there is no interference against triparental matings, we may compare relative frequencies of Hfr × Hfr progeny with relative frequencies of triparental ones. Both these types of recombinants arise from triplets or larger clumps. Relative frequencies of Hfr × Hfr progeny (calculated as the ratio: concentration of recombinants on MM/sum of concentrations of parental Hfr's in the mating mixture) and those of triparental progeny (recombinants on MM *str*/parental F^- in the mating mixture, the F^- being in this case the limiting factor) are given in the last two lines of Table 4, respectively. If we call r the probability of recombination *after effective contact has been established* between the cells, and if r is the same for any recombinational event, i.e. Hfr × Hfr or Hfr × F^-, the two values just mentioned above should be equal to qr and qr^2, respectively, q being a factor accounting for the proportion of clumps in which both Hfr's and one F^- are represented. If q is high, e.g. between 0.5 and 1—and several considerations make it likely—then one would expect that the two frequencies of Hfr × Hfr, and Hfr × Hfr × F^-, are approximately given by r and r^2. In fact, the latter value (see Table 4) is found to be approximately the square of the former.

The idea is thus confirmed that the low value of recombination frequency usually found in Hfr × Hfr crosses depends, mostly, on a difficulty in the first stage of mating, namely effective contact between cells; and that once contact has been established, recombination efficiency is about the same as in Hfr × F^- crosses.

Another possible explanation is that the F^- cells "devirilize" (SNEATH and LEDERBERG 1961) part of the Hfr cells; devirilized Hfr's might then more easily mate with the residual normal Hfr cells. Experiments are under way which may throw light on this possibility.

SUMMARY

Occurrence of triparental recombinants between two Hfr and one F^- has been demonstrated in *E. coli* K-12. These are found with a frequency of about 10^{-4} in a mating mixture (*ménage à trois*) in which progeny from normal Hfr × F^- crosses appear at about 10^{-2}. The possibility of triparental recombination is independent of the Hfr type, occurring both with different and with similar Hfr. Formation of triparental zygotes is probably accomplished both by a simul-

taneous mating of three cells and by further matings of biparental zygotes with a second male. The latter mechanism implies a lack of "immunity" of an already fertilized F^-. An effect of the female in increasing Hfr × Hfr recombination frequency has been observed and explained on the assumption that females act as attraction centers.

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