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Population replacement in *Culex fatigans* by means of cytoplasmic incompatibility*

1. Laboratory experiments with non-overlapping generations

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Bidirectional cytoplasmic incompatibility in the Culex pipiens *complex appears to provide a mechanism for the replacement of a wild population by a strain refractory to filaria or a strain made partly sterile by a translocation. As a preliminary test of the feasibility of the replacement process, various ratios of strains with the cytoplasm of either Delhi or Paris, which are bidirectionally incompatible, were tested in laboratory cages. Where one strain was marked with the ruby-eye gene, this strain always declined in frequency in the next generation. In experiments in which the Paris strain was marked with a male-linked translocation complex, after 2–4 generations of breeding there was complete elimination of either the Paris or the Delhi type depending, as expected, on the relative frequencies of the two types with which the population began. In one experiment a type with Paris cytoplasm devoid of the translocation was found. This type increased in frequency in succeeding generations. The possible causes of origin of this type and its relevance to the practical use of the replacement principle are discussed.*

If crosses between two strains of a species are sterile or yield sterile or partially sterile progeny, but matings within the strains are fertile, it is to be expected that whichever strain initially predominates in a mixed population will entirely displace the other one unless other powerful selective forces intervene. This is because, with random mating, most individuals of the predominant strain will mate with their own type, whereas most individuals of the minority strain will cross-mate and these cross-matings will yield few or no progeny at the F_1 or F_2 generations. Thus the direction of selection is expected to be frequency-dependent, the advantage lying with the predominant type. The relevance of this principle to the genetic control of insects was perceived by Serebrovskij (1). Curtis (2) proposed that, in a disease vector species, a gene for refractoriness to the pathogen might be linked to a translocation that is viable when homozygous. If sufficient homozygotes were released to constitute a majority in the wild population, replacement of the wild type by the refractory translocation homozygote would be expected because of the semi-sterility of the translocation heterozygotes. This approach to a vector problem would be unaffected by density-dependent regulation of the population. It would result in a population that would tend to obviate the re-establishment, in the target area, of a wild-type population emanating from small numbers of wild-type immigrants, because these would constitute a minority and would therefore be weeded out by natural selection. By contrast, density-dependent regulation and immigration (especially in combination) are likely to be very serious hazards for any procedure aimed at population suppression.

Previously, Laven (3) had pointed out the possibility, as regards *C. fatigans*, of protecting an area from the effects of immigration by releasing a strain that was refractory to disease transmission and bidirectionally cytoplasmically incompatible with the surrounding population. He proposed the release of the refractory strain as a follow-up to the successful eradication of the native population in the area. It would appear, however, that elimination of the local wild type and replacement by an incompatible type could advantageously form part of the

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same operation according to the principles described above.

Whitten (5, 6), Curtis & Hill (7), and Foster et al. (8) have each proposed population replacement by various genetic mechanisms that give totally sterile crosses or hybrids. This would be superior to the use of a single translocation because of the greater speed and certainty of the replacement process. Furthermore, there would be no risk of recombination of the " transport system " and the gene that it was desired to transport. Whitten (5) suggested several types of gene that might be advantageously introduced into a pest population—e.g., insecticide-sensitive alleles and temperature-sensitive mutants, as described by Suzuki (9). Laven & Aslamkhan (10) proposed replacing a wild *C. fatigans* population by a strain cytoplasmically incompatible with the target population and carrying a male-linked translocation complex. The idea was that, after the wild strain had been eliminated by means of cytoplasmic incompatibility, the process of population suppression would be completed as a result of the partial sterility of the translocated replacing strain.

In studies of *Drosophila*, in the laboratory, population replacement by means of compound chromosomes has been demonstrated by Foster et al. (8), Childress (11), and Fitz-Earle et al. (12) with experimental designs based on either overlapping or non-overlapping generations. However, in the case of a single translocation homozygote with a recessive position effect, Robinson & Curtis (13) did not succeed in achieving replacement even with an initial 9 : 1 ratio in its favour.

Population replacement in a pest species has not been previously reported. This paper describes the first part of an experimental investigation in *C. fatigans*.

According to Mattingly (14), this species poses a serious public health problem because of its recognized role in the transmission of Bancroftian filariasis—a large and increasing worldwide problem. Furthermore, the control of *C. fatigans* is becoming more difficult because of its resistance to DDT, as has been pointed out by Brown & Pal (15). Cytoplasmic incompatibility in the *C. pipiens* complex appears to offer a " ready-made " system for population replacement with the advantage that it is possible to produce strains with cytoplasm bidirectionally incompatible with the target populations but with the genome of the target strain. Hence, these strains are presumably well adapted to life under the natural conditions prevailing locally. The experiments were carried out with the Paris and Delhi cytoplasms, which were reported by B. S. Krishnamurthy and H. Laven (unpublished observations, 1972) to be bidirectionally incompatible. Since it would be laborious to follow the changes in frequency of these two cytoplasmic types by test-mating samples from experimental populations, genetic markers—either an eye colour mutant or a translocation—were used to mark one of the two cytoplasms. The procedure assumes that the marker will always remain associated with the cytoplasm in which it entered the mixed population. The validity and limitations of this assumption are discussed later in the light of the results obtained.

A strain of *C. fatigans* refractory to *Wuchereria bancrofti* is not at present available, but the question is under active study in several laboratories. Two series of experiments intended as models of replacement by such a refractory strain were carried out, one of them specifically to investigate the feasibility of population replacement by a translocation-carrying strain with only about one-third of the fertility of the target strain.

MATERIALS AND METHODS

The following strains were used.

Delhi (De), derived from collections of *C. fatigans* from villages in Delhi Union Territory, India, in 1970. The strain was colonized and has been maintained in the laboratory of the WHO/ICMR Project on Genetic Control of Mosquitos since that time.

IS31B: Paris cytoplasm (Pa) [a] with Delhi genome introduced by a two-stage back-crossing programme with the Bangkok strain. The latter is compatible with both Delhi and Paris strains; the Delhi genome includes a male-linked translocation complex induced by two successive irradiations (B. S. Krishnamurthy & H. Laven, unpublished observations, 1974).

In preliminary, unsuccessful experiments strains carrying the ruby-eye (*ru*) recessive autosomal marker described by Iltis et al. (16) were used. The gene has been back-crossed into the Delhi strain and made homozygous; the strain is designated as (De)*ru*/*ru*. The gene was also crossed into a strain with Paris cytoplasm and Delhi genome *via* a strain

[a] In some unpublished communications from this Unit this cytoplasm has been referred to as " D3 ". However, this designation was originally devised for a strain with Paris cytoplasm and Freetown genome. Since the Freetown genome has now been replaced, it seems clearer to revert to the designation Pa for Paris cytoplasm as used by Laven (4).

with Bangkok cytoplasm and Delhi genome and then made homozygous; this strain is designated as (Pa)*ru*/*ru*. The strains homozygous for normal eye colour are referred to as (De)+/+ and (Pa)+/+.

The laboratory was maintained at 27±2°C and 70% relative humidity. Cages measuring 30×25×20 cm were used to accommodate up to about 500 mosquitos. Raisins were provided continuously and, after a 3-day mating period, day-old chicks were placed in the cages for one night as a blood source. A bowl of water was provided 4 days later for oviposition. Egg rafts were transferred individually the morning after laying to tubes 7.5×2.5 cm containing water and larval food. Data were obtained only from the rafts laid after the first blood meal. The day after collection, hatched rafts were examined under the microscope (magnification: ×50). Rafts from matings within the Delhi and IS31B strains could readily be distinguished by the fact that almost 100% of the eggs in the former hatched (" normal hatch "), whereas about 65% of the embryonated eggs in the latter remained unhatched (" partial hatch "). Unhatched rafts were left for a second day for further hatching and these were classified as above. Unhatched rafts were examined for evidence of an incompatible mating (i.e., a characteristic mixture of unhatched embryonated and unembryonated eggs). Occasionally, completely unembryonated rafts were found. These are thought to have been laid by uninseminated females and they were excluded from the data presented. Normal-hatch, partial-hatch, and incompatible rafts were usually qualitatively distinct. However, in doubtful cases, 15 unhatched eggs were taken as the upper limit for a normal hatch and 15 hatched eggs as the upper limit for incompatible rafts.

After the rafts had been classified, first-instar larvae were counted from all the tubes and transferred into an enamel rearing tray (30 × 25 cm). About 1 000 larvae per tray were reared. As indicated below, the number of larvae taken from normal and partial-hatch rafts were either equal or three times as great from the former as from the latter. Thus the number of larvae cultured from normal and partial-hatch rafts was proportional either to the number of each type of raft or to the number of larvae produced by the two types of raft (taking into account that, in partial-hatch rafts, only about one-third of the eggs hatch).

The larvae were reared on a diet of powdered dog biscuit and yeast, with a change of water on alternate days. Pupae were sexed, as far as possible, by size. Males were placed in cages for emergence and females were placed in groups of five in stoppered tubes. To ensure virginity of the females, only those tubes were used in which all five emerged adults were females. Four days after collection of the last male pupae, the males and virgin females were mixed in a ratio of approximately 1:1. The purpose of mixing the sexes several days after emergence was to ensure that all males were sexually mature at the time of mixing, thus avoiding the possibility that a minority of early-maturing males might take a disproportionate share of the matings.

In the experiments with the ruby-eye stocks, individuals with ruby and normal (black) eyes were scored by examining pupae on a cavity slide at a magnification of 30 ×.

RESULTS

Experiments with the ruby-eye marker

The results of replacement experiments using the *ru* gene as a marker for either the De or the Pa cytoplasms are shown in Table 1. The expected numbers of incompatible *versus* hatching rafts and ruby-eyed *versus* black-eyed pupae were calculated from the Hardy Weinberg ratio, on the basis of the parental frequencies and the total numbers of rafts and pupae, i.e., on the assumptions of random mating, equal survival, and fecundity of the strains, and of the complete incompatibility of the De and Pa cytoplasms. In all cases, the proportion of ruby-eyed pupae was far below expectation and the direction of selection was not frequency-dependent, but was always against the strain with the *ru* gene. Therefore the progeny were not retained for a second generation of breeding.

Table 1 shows also the results of mating competition tests in which, unlike the replacement experiments, only one type of female was used. These indicated greatly reduced competitiveness of the (Pa)*ru*/*ru* strain, and it seems that this effect was more marked in competition for (De)+/+ females. This may be explained by the apparent subnormal activity of the ruby-eye strains, which might reduce the ability of the males to compete with normal-eyed males for normal-eyed females. In addition, the *ru* gene had a deleterious effect on larval survival. When counted numbers of the first-instar larvae of (Pa)*ru*/*ru* and (De)+/+ were reared together, the proportions surviving to the pupal stage were 55.5% and 78.8%, respectively; comparable figures for (De)*ru*/*ru* and (Pa)+/+ were 38.0% and 61.5%.

Table 1. Observed and expected results in experiments with the ruby-eye marker

Experiment No.	Ratio among parents	Rafts				Pupae			
		No. incompatible		No. hatched		Ruby eyes		Black eyes	
		observed	expected	observed	expected	observed	expected	observed	expected
					Replacement experiments				
A1	Male 4(Pa)*ru/ru* : 1(De)+/+ Female 4(Pa)*ru/ru* : 1(De)+/+	34	36.8	81	78.2	220	402.0	207	25.0
A2	Male 2(Pa)*ru/ru* : 1(De)+/+ Female 2(Pa)*ru/ru* : 1(De)+/+	77	80.0	103	100.0	178	347.3	256	86.7
A3	Male 1(Pa)*ru/ru* : 1(De)+/+ Female 1(Pa)*ru/ru* : 1(De)+/+	33	60.5	98	60.5	674	446.0	218	446.0
A4	Male 2(Pa)+/+ : 1(De)*ru/ru* Female 2(Pa)+/+ : 1(De)*ru/ru*	10	24.4	45	30.6	10	51.8	249	207.2
A5	Male 2(De)*ru/ru* : 1(Pa)+/+ Female 2(De)*ru/ru* : 1(Pa)+/+	46	43.5	52	54.5	154	237.8	143	59.2
					Mating competition tests				
A6	Male 1(Pa)*ru/ru* : 1(De)+/+ Female 1(De)+/+	14	66.5	119	66.5				
A7	Male 1(Pa)*ru/ru* : 1(De)+/+ Female 1(Pa)*ru/ru*	103	77.0	51	77.0				

These data on the effects of the *ru* gene provide at least a partial explanation of the very large deficits of ruby-eyed pupae in the replacement experiments, and they demonstrate that the *ru* marker is unsuitable for the present purpose or for use in the field in the manner suggested by Laven (3).

Experiments using the translocation of the IS31B strain as a neutral marker

Apart from the ruby-eye marker, no other marker gene was available in this laboratory, and the IS31B strain was therefore used for subsequent experiments. The relative numbers of normal-hatch and partial-hatch rafts were taken as measures of the relative numbers of De×De and IS31B×IS31B matings that had occurred, and also as measures of the relative numbers of De and IS31B larvae at the beginning of the next generation. In this series of experiments, partial sterility due to the translocation was used only as a neutral marker to indicate the frequency of the Paris cytoplasm and it was prevented from influencing the outcome of the experiments by the device of culturing the same number of larvae from each partial-hatch raft and from each normal-hatch raft. These experiments were intended as a model for replacement by a filaria-refractory gene which, it is assumed, would not have a strong adverse effect on the fitness of the mosquito.

Each experiment was begun by mixing about 500 adults in specified ratios. In each case, the proportion of incompatible rafts was high at the first generation but declined towards zero in subsequent generations of inbreeding, indicating elimination of one of the cytoplasmic types from the population. The results are shown in Fig. 1.[a] At generation 0, the initial proportions of adults of the two strains introduced are shown and, at subsequent generations, the proportions of normal-hatch and partial-hatch rafts, after deducting the incompatible rafts, are presented. Experiments B1, B2, and B3 showed the expected elimination of whichever type was initially in the minority. Experiment B4 showed that elimination of the De strain could be achieved starting with this strain in the majority among the females, provided that the ratio among the males was strongly biased against it.

Using the same assumptions of random mating and equal fitness as above, the expected proportions of each type of raft were calculated and compared with those observed. In 8 cases out of 11, the χ^2 values were not significant. The cause of the significant deviations from the expected values in the other three cases is not known with certainty.

Experiment B4 proceeded close to expectation up to generation 3, but elimination of the De type was completed unexpectedly early at generation 4.

[a] The detailed data were presented in unpublished document WHO/VBC/74.481.

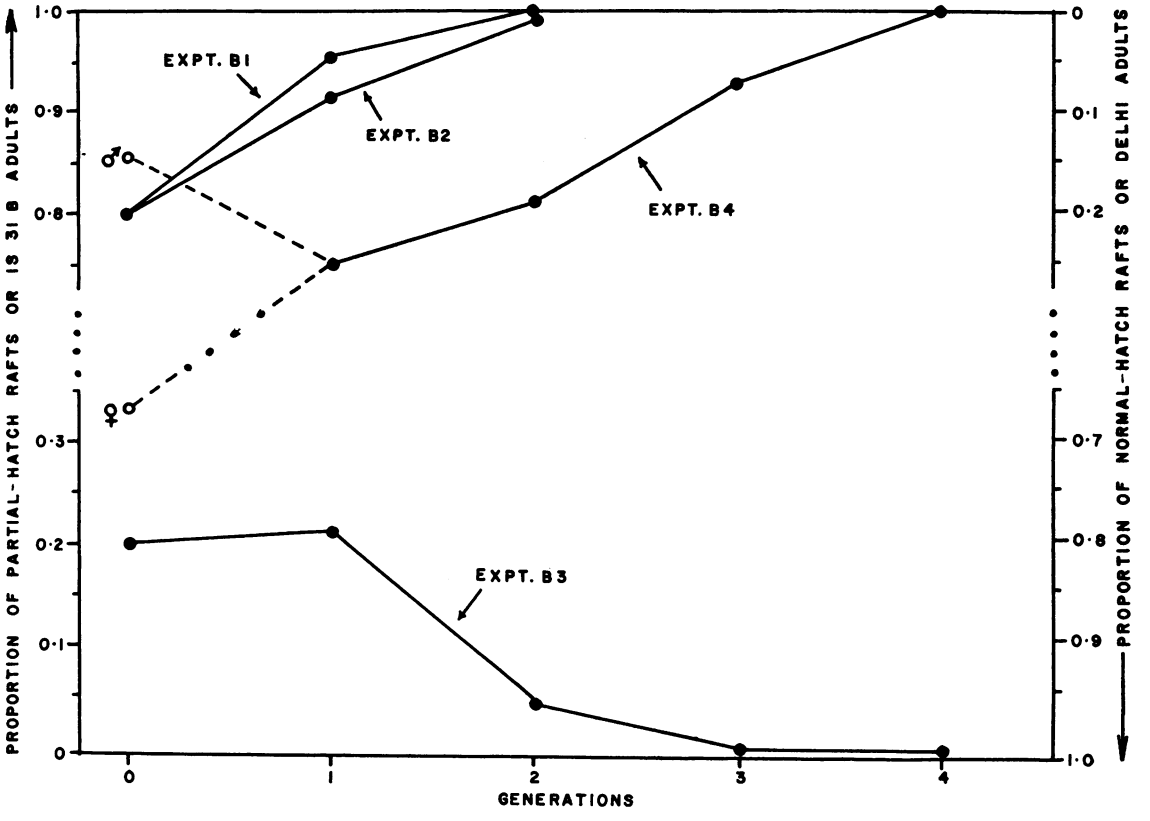

Fig. 1. Experiments using the translocation of the IS31B strain as a neutral marker. At generation 0, the proportions of IS31B and Delhi adults from which the populations started are plotted. At subsequent generations, the proportions of normal-hatch and partial-hatch rafts are plotted, after deduction of the incompatible rafts from the totals.

Experiment on replacement by a partially sterile strain

In this experiment, after classification of the rafts, three times as many larvae were cultured from normal-hatch rafts as from partial-hatch rafts. Thus the numbers of larvae cultured were proportional to the total numbers produced from the two types of raft and the experiment was intended as a model of an attempt to replace a fully fertile wild population by a partially sterile translocated type, as proposed by Laven & Aslamkhan (10). As shown in Table 2, the first-generation rafts conformed to expectations. However, at the second generation, there were many more normal-hatch rafts than had been expected. It was thought that IS31B had been established in an insufficently high proportion to overcome its selective disadvantage owing to reduced fertility. Therefore an additional release of IS31B males and females was made in the ratio 4 : 1 with the products of generation 2 of the experimental population. Subsequently the proportion of incompatible rafts declined to zero, but that of normal-hatch rafts rose until, at generation 5, it approximately equalled that of the partial-hatch rafts. At generations 4 and 5, samples of the normal-hatch rafts were reared as single-raft cultures and were test-mated with the De or IS31B stocks. All these tests showed that the normal-hatch rafts had Pa cytoplasm and the males no longer carried a translocation. The origin of this new type is discussed below, but it is clear from the results that it was increasing steadily in numbers because it was the most favoured type, being compatible with the majority cytoplasmic type and three times as fertile as the IS31B strain.

DISCUSSION

Frequency-dependent selection of the divergent type leading to complete population replacement is

Table 2. Observed and expected results of an experiment on replacement by a partially sterile strain

Generation	No. normal hatch		No. partial hatch		No. incompatible	
	observed	expected	observed	expected	observed	expected
Started with 5 IS31B males and females to 1 De male and female						
1	6	4.8	108	120.1	59	48.1
2	21	2.7	87	95.1	22	32.2
Release of 4 IS31B males and females to 1 male and female from generation 2						
3	14		141		8	
4	19[a]		65		6	
5	46[a]		53		0	

[a] A total of 19 single rafts were cultured, reared, and test-mated. All were found to be of the Pa cytoplasmic type.

demonstrated in Fig. 1. The number and duration of releases required to initiate the replacement process are small in relation to the numbers of sterile males likely to be required to eradicate a population and, though some females must be released to initiate replacement, the numbers of these could be kept small, so that the temporary increase in the biting rate would be hardly detectable.

The experiment on replacement by a partially sterile strain, summarized in Table 2, showed the generation of a genotype with Pa cytoplasm and no translocation in the males, and this prevented replacement of a fertile strain by a partially sterile one. Two hypotheses may be put forward for the origin of this genotype:

(*a*) A cross-over between the *M*, male-determining, gene and the translocation, yielding non-translocated males. These, on mating with normal females of the IS31B stock, would produce normal-hatch rafts with Pa cytoplasm.

(*b*) Partial compatibility in an IS31B female × De male mating, which would yield males with Pa cytoplasm and no translocation.

In current studies [a] of both these possibilities, a marker gene is being used so that cross-overs or cases of partial compatibility can be unequivocally distinguished from contamination of one of the strains or a sexing error in making up the test matings. If a male-linked translocation recombined at appreciable frequency with the *M* locus, it would be a serious handicap to attempts to use the strain for genetic control because, as emphasized by the results in Table 2, following a cross-over in a wild population or mass rearing colony, there would be strong natural selection in favour of the recombinant type.

Barr (17) demonstrated partial compatibility in several crosses in the *C. pipiens* complex, including one involving Pa cytoplasm. If partially compatible matings occurred in the wild population after release or in a mass rearing colony that contained some De type contaminants, they would lead to recombination of the cytoplasmic transport system with the gene or chromosome to be transported into the population. This would be fatal to an attempt to eradicate by replacing a wild population by a partially sterile translocation strain. In the case of attempts to replace by a filaria-refractory gene, the effect of a low level of partial compatibility would depend on the relative fitness of the refractory and susceptible alleles, the speed at which the replacement operation could be carried out, the immigration rate of filaria-susceptible individuals into the treated area, and other factors.

The danger of recombination of the native genome and the released cytoplasm, as a result of occasional partial compatibility, would be minimized by ensuring that the females of the replacing strain had mated with their own type of male before release. A test of this system has been initiated as part of a series of experiments on population replacement in field cages containing populations with overlapping generations.

[a] These experiments, demonstrating compatibility of some De males with Pa females, are summarized in: SUBBARAO, S. K. ET AL. *Journal of communicable diseases*, **6**: 80-82 (1974).

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RÉSUMÉ

SUBSTITUTION DE POPULATION CHEZ *CULEX FATIGANS* À L'AIDE DE L'INCOMPATIBILITÉ CYTOPLASMIQUE: 1. EXPÉRIENCES DE LABORATOIRE SUR DES GÉNÉRATIONS DISTINCTES

L'incompatibilité cytoplasmique bidirectionnelle, au sein du complexe *Culex pipiens*, se révèle comme un mécanisme permettant de remplacer une population sauvage par une souche réfractaire à la filariose ou par une souche rendue partiellement stérile par translocation. Une étude préliminaire de la faisabilité de ce processus de substitution a eu lieu au laboratoire en utilisant dans des proportions variables des souches de *C. fatigans* à cytoplasmes, Delhi ou Paris, incompatibles.

Lorsqu'une souche était porteuse du gène marqueur *ru* (*ruby-eye*), la densité de sa population a toujours décru dans la génération suivante, ce qui est attribué principalement à l'effet nocif du gène sur l'aptitude à la concurrence sexuelle.

Des expériences ont été effectuées avec la souche Delhi et la souche IS31B, qui possède un cytoplasme Paris et un génome Delhi. Ce dernier porte un complexe de translocation lié au sexe mâle qui provoque une stérilité partielle de la descendance lorsque les accouplements ont lieu entre individus de cette souche. Après 2-4 générations, on a assisté à l'élimination complète du type Paris ou du type Delhi selon la proportion relative de ces types dans la population au début de l'expérience. Par ailleurs, on a constaté que l'élimination de la souche Delhi était possible lorsque cette souche était prédominante parmi les femelles à condition que les mâles de souche IS31B soient de loin les plus nombreux au départ. On pourrait mettre ce phénomène à profit lors d'un programme de lâcher sur le terrain afin de minimiser l'accroissement temporaire de la population d'insectes agressifs nécessaire à la mise en route du processus de substitution.

Au cours d'une autre expérience, des mâles à cytoplasme Paris mais dépourvus de translocation sont apparus dans la population de *Culex* et leur proportion s'est rapidement accrue dans les générations succesives en raison de leur fécondité supérieure à celle des mâles IS31B. L'apparition de ce type est peut-être due à une compatibilité partielle fortuite entre des femelles IS31B et des mâles Delhi. Des recherches sont en cours pour étudier les moyens de pallier ce risque.

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