MUTATION AS CELLULAR PROCESS

A Ciba Foundation Symposium

Edited by
G. E. W. WOLSTENHOLME
and
MAEVE O'CONNOR

J. & A. CHURCHILL LTD.
104 GLOUCESTER PLACE, LONDON
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The Ciba Foundation

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The Foundation's house at 41 Portland Place, London, has become well known to workers in many fields of science. Every year the Foundation organizes six to ten three-day symposia and three to four shorter study groups, all of which are published in book form. Many other scientific meetings are held, organized either by the Foundation or by other groups in need of a meeting place. Accommodation is also provided for scientists visiting London, whether or not they are attending a meeting in the house.

The Foundation's many activities are controlled by a small group of distinguished trustees. Within the general framework of biological science, interpreted in its broadest sense, these activities are well summed up by the motto of the Ciba Foundation: *Consocient Gentes*—let the peoples come together.

Preface

This meeting was held as a result of Professor Charlotte Auerbach's suggestion, first made to Mr. A. V. S. de Reuck in 1966, that molecular biologists, geneticists and others would benefit from an exchange of information about the secondary processes involved in mutation. Whether a mutagen acts on a given gene and whether the product of this reaction develops into a mutation clone or organism depends largely on cellular metabolism. Yet workers on mutation were only beginning to realize the importance of cellular events for the mutation process, and people studying cellular processes did not always appreciate the importance of their findings for mutation research. The symposium recorded here therefore brought representatives of the relevant different disciplines together to attempt to analyse the problem of mutation and reduce it to simpler levels.

Professor Auerbach gave much invaluable advice throughout the organization of the meeting and her help at every point was greatly appreciated. Dr. Kimball, who acted as Chairman, carried out this task with apparent ease, tact and excellent time-keeping, even though he had to cope with several last-minute changes in the programme. These changes arose because the blizzard which hit the East Coast of the United States in February 1969 prevented three members of the symposium from reaching London at the time expected. Two, Dr. L. Grossman and Dr. W. L. Russell, arrived after journeys of epic proportions, but after three frustrating days Dr. Evelyn Witkin finally had to give up her strenuous attempts to get to the meeting.

The editors wish also to thank Dr. A. Loveless for his generous help during the early stages of publication.

CHAIRMAN'S OPENING REMARKS

R. F. KIMBALL

As you all know, this symposium was initiated by Professor Charlotte Auerbach. Its subject reflects her view that mutation is not just an isolated event, a quantum event, or a simple chemical reaction, but a process in which cellular functions are intimately involved. This view I share, and therefore it is a pleasure and an honour for me to be asked to chair this symposium.

This is not to say that quantum events and simple chemical reactions are not the starting points of processes leading to mutation. Most of us, however, work with cellular systems and here we cannot forget that the mutagenic treatments we give initiate a process which only after an appreciable time results in our detection of mutations. Cellular metabolism and cellular events are intimately involved in this process.

The two points of view, mutation as a simple molecular event and as a more complex cellular process, have been in existence for a long time and attention has shifted back and forth between them. Concentration on the initial atomic and molecular event leads to a certain simplicity that is especially attractive to those who come to the problem from physics and chemistry. Concentration on the cellular process aspect is more congenial to those of us who come to the problem from biology and are more immediately aware of the complexities of biological systems. Here we must be very careful, however, that this awareness of complexity is not converted to a worship of it and a consequent resistance to the attempt to analyse the problem and reduce it to simpler levels. It is just this analysis and reduction that should be one of the main purposes of this symposium.

Let me start with a very crude overall view of mutation as a cellular process. We can recognize at least the following main steps: (1) initiation, (2) fixation, (3) detection. With many mutagens, initiation necessitates molecular alteration of the chromosome. In general, however, this alteration—an alkylated base for example—will not itself be self-replicating but must be converted to some self-replicating change, such as a base-pair transition. There are many possible variations of these first two steps. In some variations, such as a spontaneous mistake by the replicating system,

the two steps might be reduced to one, but with most induced mutations initiation and fixation are certainly separate. We have learned much in the last few years about the molecular aspects of those processes that can eliminate initial damage before it can be fixed, but we still understand all too little about the molecular aspects of fixation itself. Much evidence and some theory suggest that fixation normally occurs within the same cell cycle in which the initial event occurred, often associated with DNA replication, but apparent replicating instabilities suggest that sometimes fixation may be delayed for several or many cell generations.

By detection, I mean all those events, processes, and procedures that are involved in detecting a mutation once it has been fixed. This requires among other things that the mutant gene be expressed, i.e. that it make its gene product. In modern terminology transcription and translation must occur. Even if this happens, however, we must be able to recognize the effects of the gene product by some *test*, which may depend for its success on cellular metabolism. Among other things, a successful test may require that the mutant gene be *segregated* free of non-mutant ones. Techniques for doing this with germ cells have been part of the standard methods of genetics for many years. With single cells, one has to be aware that the chromosome consists of more than one conserved strand and that mutations may be fixed in only one strand. Segregation will be further complicated if fixation can be delayed for several cell generations.

Because mutation is a cellular process, modifications of mutation yield are not necessarily the result of direct action on the mutation process *per se*, but may be the result of actions upon the cell that affect mutation yield quite indirectly. As a result, the interpretation of many experiments is made difficult or ambiguous. For example, a modifying treatment given before fixation or even before the initiation might modify the post-fixation process of expression as a consequence of an alteration in cellular metabolism or in the cell cycle. The time of treatment is not by itself a safe guide to the portion of the mutation process that is affected.

Despite the difficulties caused by such complexities, much progress has been made as better and better methods for analysing the molecular events in initiation and fixation have been developed and a greater understanding has been reached of the processes involved in detection. I hope that this symposium will help to accelerate this progress by bringing together people with divergent interests who can look at the mutation process from different points of view.

INFLUENCE OF THE HOST ON THE INDUCTION AND EXPRESSION OF MUTATIONS IN PHAGE KAPPA

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The process of induced mutation consists of several steps or phases, each of which could be influenced by cell components in the vicinity of the mutating gene. One way to study such influences is to use phage mutations which allow that the phage can be treated, e.g. irradiated, separately from the host cell. This has been done for ultraviolet-induced and to a smaller extent with X-ray-induced mutations in phage κ.

This temperate phage forms turbid plaques on a (red) lawn of *Serratia marcescens*, strain HY. Several types of plaque mutations appear after irradiation of the free phage (Kaplan, Beckmann and Rüger, 1963; Beckmann and Kaplan, 1965). U.v. induces mainly clear-plaque (*c*) types to the extent of a few per cent among survivors. The dose/mutation curve indicates a two-hit process (Ellmauer and Kaplan, 1959). A large fraction of these mutations is photoreversible (Winkler, 1965*c*). X-rays induce *c*-mutations and also the rare type *b* by a one-hit process, and type *e* by about three hits (Rüger and Kaplan, 1966). Only *c*-mutations will be considered here because they are the easiest to distinguish from the turbid wild-type plaques; they map in four regions of the κ-genome. U.v.-induced *c*-types are mainly mutated in region III which is responsible for the phage repressor (Steiger, 1966, 1968). The following problems have been studied: (1) The influence of irradiation of the host protoplasm on mutation induction in the phage genome by u.v. and X-rays, in particular the "indirect action" of the radiation; (2) The influence of the growth phase of the host; (3) The dependence of the expression of the mutations as pure or mosaic clones on host cell reactivation (HCR).

INFLUENCE OF IRRADIATION OF THE HOST CELL

Winkler (1963) found that u.v. irradiation of the host cells (HY) alone does not cause mutations in unirradiated phage κ. Therefore, mutagenic

products of u.v. in the host protoplasm stable enough to survive until infection cannot be responsible for the mutations observed in the phage.

In order to see whether u.v. produces short-lived mutagens, e.g. free radicals, within the cell which could induce mutations in the injected phage-DNA, we irradiated the phage following infection of the host cell. Since it was necessary to inhibit intracellular phage development until the end of irradiation, cells in stationary phase were used. They were starved in saline for 60 minutes and then allowed to adsorb unirradiated phage for 15 minutes; after washing they were irradiated with different doses of u.v. (mainly 254 nm, 20 erg mm^{-2} sec^{-1}). The results show (Fig. 1) that the yield of *c*-mutations as well as the inactivation was much higher with intracellular than with extracellular irradiation. The dose reduction was of the order of 2, perhaps for mutation a little higher. This increase could be due to the hypothetical short-lived u.v.-mutagens. However Winkler (1963) had already observed that u.v. irradiation of the host cells before infection increased the frequency of *c*-mutations in u.v.-irradiated phage by a factor up to 2 as compared to infection of unirradiated host cells. Thus, the increase observed with intracellular irradiation could also be due to this effect since the host cell had simultaneously received u.v.

To decide between these alternatives the effect of intracellular irradiation was compared with the results of an experiment in which both the free phage and the host received the same u.v. doses before infection. This was done with host cells starved as before. They were irradiated quickly immediately after addition of phage to the cells and before adsorption had become significant. This technique ensured that the intensity of u.v. irradiation of the phage (which was lower due to shielding by the cells) was nearly the same as with intracellular irradiation. The results show that the yield of mutations as well as the inactivation is about the same as with intracellular irradiation (Fig. 1, points X). From this it can be concluded that the effect of intracellular irradiation is not more than the sum of the u.v. effect on the free phage plus that due to irradiation of the uninfected cells. Thus, neither short nor long-lived mutagenic radiation products from the host protoplasm are markedly involved in u.v. mutation of this phage.

The same was found to be true for X-rays. The hypothesis that X-rays induce mutations indirectly, via radicals etc., has long been discussed. We (Rüger and Kaplan, 1966) had already shown that an "indirect" effect of X-ray products of water did not significantly induce mutations in extracellular phage *κ* as the mutation frequency was the same in the presence and absence of protecting substances such as broth. We therefore X-irradiated intracellular phage and found the mutation frequency to be no

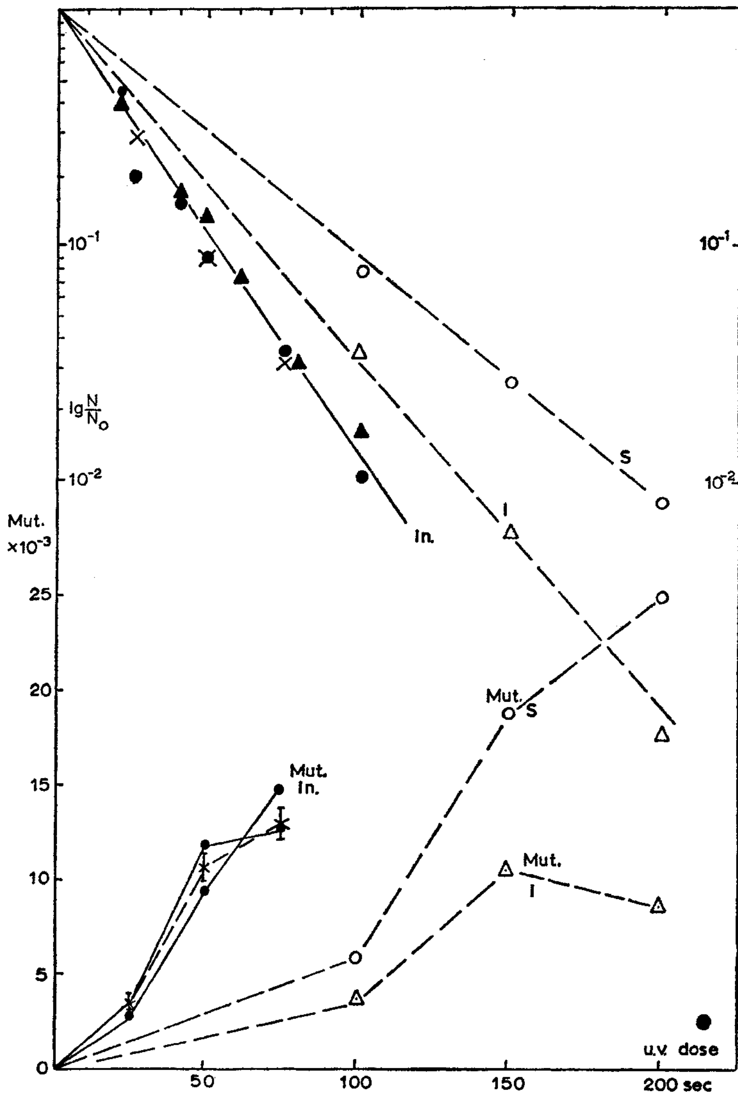

FIG. 1. Survival ($\lg N/N_0$) and frequency of *c*-mutations in phage ϰ as a function of the u.v. dose. Irradiation of extracellular (○, △) or intracellular (in. ●, ▲) phage using stationary (s; ○, ●) or logarithmic growing (l; △, ▲) cells of strain HY of *Serratia marcescens* as host for preadsorption. X = Irradiation of free ϰ and stationary host cells with the same doses before infection. All platings with HY. Stationary cells were grown overnight in Difco Nutrient Broth (NB) and resuspended in buffered saline. Log cells were grown overnight in NB, then diluted (10^{-2}) into fresh NB, grown for 2½ hours, resuspended in buffered saline and used immediately. All incubations at 30°C.

higher than in free phage irradiated in broth. However the inactivation was much greater, a fact which is not yet understood (Table I). This suggests that the *c*-mutations which are induced by X- or u.v. irradiation are mainly or solely due to direct hits in the phage DNA.

TABLE I

INACTIVATION AND MUTATION OF κ-PHAGE AFTER X-IRRADIATION* IN EXTRA- AND INTRACELLULAR STATES

	Extracellular	*Intracellular*
Plaque survival	$2 \cdot 8 \times 10^{-1}$	3×10^{-4}
Plaques scored	18 440	57 769
c-mutants	16	54
Fraction of mutants among survivors ($\times 10^{-3}$)	$0 \cdot 87 \pm 0 \cdot 22$	$0 \cdot 94 \pm 0 \cdot 13$

* 55 kV; 0·3 mm Al; dose 234 krd; phage suspended in 10 times usual concentration of Difco Nutrient Broth.

The question remains, what causes the increase in mutations which is observed after u.v. irradiation of the host cells? A plausible explanation seems to be that u.v. inhibits host cell repair (HCR) of premutations as well as potentially lethal damage in the phage DNA. Winkler (1965*a*) found that inhibition of HCR by either caffeine or host mutation (*hcr*⁻ mutants) increases both u.v. effects. An increase in u.v. inactivation as a result of host-irradiation is known to occur in different temperate phages. It is usually attributed to inhibition of HCR (see review by Rupert and Harm, 1966).

INFLUENCE OF THE GROWTH PHASE OF THE HOST

Where mutation induction in cells is influenced by differing physiological conditions this could be due to differences either in the quality of the genome, e.g. its replication phase, or in the condition of the surrounding protoplasm, or both. With extracellularly-irradiated phage only the condition of the protoplasm can be responsible for a differing mutation yield of the phage.

To discover whether such influences operate in the κ system, host cells in stationary and in logarithmic growth phase were inoculated from the same u.v.-irradiated phage suspension. In log phase cells the inactivation was greater than in stationary cells but the mutation yield was significantly lower (Fig. 1). For the same extent of inactivation, 1·4 times the dose is required when the host cells are in stationary phase as when they are in log phase, but for mutation the factor is only about 0·6 or 0·8.

It is interesting that this influence of growth phase on inactivation disappeared with intracellular irradiation (Fig. 1). This may be a hint that

HCR is involved. It seems reasonable to assume that HCR can act on the phage DNA for a longer time in resting than in log phase cells since the latent period of phage production was found to be 80 minutes in stationary cells, but only 65 minutes in log cells. Also u.v. is much less effective in preventing colony formation when irradiation is given to stationary phase cells, and the survival curve has a much broader shoulder.

To test the influence of HCR stationary and log phase cultures of the mutant *hcr*$^-$42 (Winkler, 1965*a*) were used as hosts. The irradiated phage was preadsorbed, the remaining free phage removed by washing, and the complexes were plated on *hcr*$^+$ strain HY. No significant difference in phage survival was observed. Indeed, in some of the experiments a very small reverse difference was found (Fig. 2). On the other hand, the mutation induction was again much lower in log than in stationary host cells. When *hcr*$^-$42 and HY, both in stationary phase, were compared as hosts, the extent of inactivation and the yield of mutants was much smaller for HY. This agrees with the earlier findings of Winkler (1965*a*). The dose reduction factors are 12 to 15 for inactivation and about 3 for mutation.

What could be the reason for the much lower mutation induction in log as compared to stationary host cells? Since HCR is excluded two possibilities may be considered:

(1) To produce a mutation from a premutation in the DNA, e.g. from a pyrimidine dimer, an error in replication has to occur. The conditions in log cells may provide less chance for such errors to occur than those in stationary cells, e.g. they may influence the exactness of action of the replicase.

(2) As in other cases of differing sensitivity to radiation, a repair mechanism could be involved. This repair must of course be different from HCR, it must act preferentially on premutations as compared with lethal lesions, and it must be more active in log than in stationary cells.

The second hypothesis of a "log phase mutation repair" is perhaps more plausible than the first because it could explain the weak reactivation of plaque formation observed in some of the experiments with *hcr*$^-$42 when there is also a weak repair of otherwise lethal damage.

A type of repair acting mainly on premutations in phage ϰ was discovered by Winkler (1965*b*) in his two u.v.-sensitive mutants, *hcr* 91 and 614, and by Steiger and Kaplan (1964) in the *Serratia* strain CN. None of these hosts gives any mutations with u.v.-irradiated ϰ-phage, although they plate "old" *c*-mutants as clear plaques very well. They also do not allow X-ray-induced mutations of ϰ (Winkler, 1965*b*; Rüger and Kaplan, 1966). This mechanism for "extinguishing" all u.v. and X-ray-induced premutations

in ϰ phage resembles the EXR mechanism of *E. coli*. As Witkin (1967) found, the *exr*⁻ strains of this bacterium do not yield u.v. mutations in the bacterial genome. She proposed that post-replication gaps in the DNA strand opposite to the premutated strand are closed later by repair replication. This produces errors, i.e. mutations, in *exr*⁺ strains but no errors, i.e. wild-type, in *exr*⁻. Perhaps the non-mutating strains contain an apparatus reversing different types of premutationally changed DNA bases (e.g. pyrimidine dimers as well as certain X-ray products) to the wild-type state.

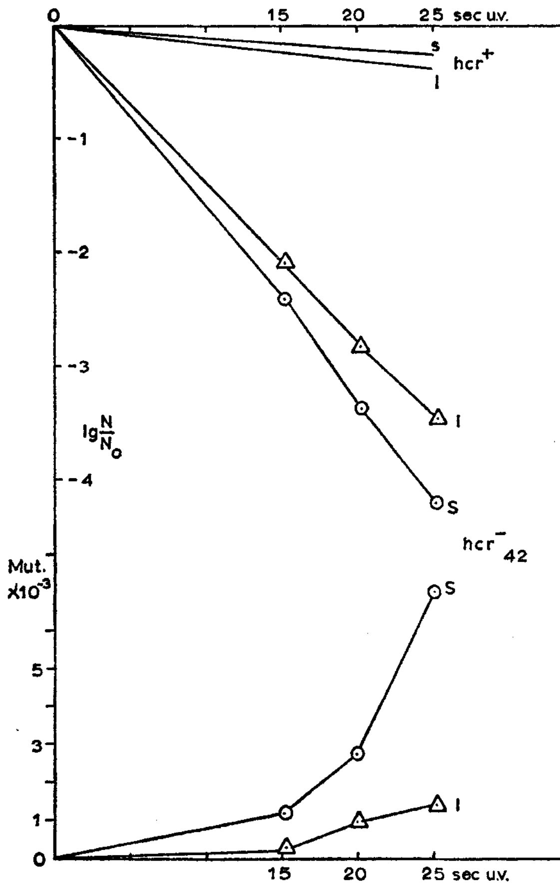

FIG. 2. Survival (lg N/N_0) and frequency of *c*-mutations in phage ϰ as a function of u.v. dose. Irradiation of extracellular ϰ, preadsorption for 15 min on host strain *hcr*⁻42 in stationary (s) or logarithmic growth (l) phase, then washing to remove free phage and plating with strain HY. Above: survival in strain HY (*hcr*⁺) for comparison.

INFLUENCE OF HCR ON U.V.-INDUCED MOSAICS AND PURE MUTANT CLONES

It was reported earlier (Kaplan, 1966) that most u.v.-induced *c*-plaques of κ contain only mutant phages. Several hypotheses exist (listed e.g. by Nasim and Auerbach, 1967) to explain the finding of pure mutant clones from two-stranded DNA in different organisms: (1) the premutation is located in both strands; (2) a lethal hit in the non-premutated strand inhibits the replication of this strand alone; (3) a repair replication in the strand opposite to the premutation transfers the mutation to this strand too ("mutating repair"); (4) one strand only, the master strand, gives the information to both daughter DNA molecules ("simplex" hypothesis of Barricelli, 1965).

The lethal-hit hypothesis predicts an increase in the fraction of pure clones among all clones containing mutants with increase in the u.v. dose, since relatively more strands with such hits would occur at higher doses. A weak increase was indeed observed earlier in u.v.-induced *c*-mutation of phage κ (Kaplan, 1966). Therefore, a "purification" of the mutant clones could be caused, at least partially, by such hits. But mutating repair could also be involved since the host strain HY used at that time has a repair potential (HCR). A case where mutating repair seems to play a role was demonstrated in hydroxylamine-induced mutation in phage T4 by Bautz Freese and Freese (1966).

To test the influence of HCR for the κ system the clone composition of u.v.-induced *c*-mutations was studied using the hosts *hcr*⁻42 and HY (*hcr*⁺). Phage κ was u.v. irradiated and preadsorbed onto each strain. Both types of complexes were then plated on HY and many of the *c*-plaques thus obtained were picked, and the virus was diluted and replated. The results (Table II) show that the defect of HCR in *hcr*⁻42 compared with

TABLE II

PURE AND MOSAIC *c*-MUTANT PLAQUES INDUCED BY U.V.

No. of expts.	U.v. -dose (erg mm^{-2})	Survival	*c*-plaques picked	Mosaics* (with wild type among *c*-plaques)
(A) Host strain HY (*hcr*⁺), plated on HY				
2	4800	10^{-4}	331	6 (1·8%)†
2	3600	10^{-3}	236	8 (3·4%)
(1966)	5400	10^{-4}	94	1 (1·6%)
(1966)	3600	10^{-3}	95	9 (9·5%)
(B) Host strain *hcr*⁻42, plated on HY				
4	600	10^{-5}	95	13 (13·7%)
3	350	10^{-3}	110	7 (6·4%)

* Wild types and mutants of all mosaics listed were stable in later post-cultures.

† Besides the six mosaics with wild type, four plaques contained two stable mutant types: three with about 0·1% *c*-type besides *l*-type and one with 82% *c*- and 18% *l*-type.

hcr+ has increased the proportion of mosaics among all *c*-plaques from 1·8 per cent to 13·7 per cent at the high doses, and from 3·4 to 6·4 per cent at the low doses. The latter difference is not significant but the first is ($P = 1 \times 10^{-5}$).

The influence of the dose in *hcr*+ was smaller in this new series than in the earlier one, and alone was not significant, but the pooled results of both series show statistical significance ($P = 0 \cdot 008$). In *hcr*−42 the dose effect even seems to be reversed but this is not significant ($P = 0 \cdot 11$). When the distribution of the clone types (percentage of mutants in a clone) was considered the sample seemed to have a deficit of clones with few (< 50 per cent) mutants. Special experiments with mixed infections (by 50 per cent wild types plus 50 per cent of 30 u.v.-induced *c*-types) showed that clones with few mutants often produce plaques looking like wild type. Therefore in the new u.v. series many plaques appearing to be wild type were picked and replated. Several clones were found which contained few mutants but they were only a minority of those appearing as *c*-plaques. The clone distribution obtained in these experiments was added to those from *c*-plaques in order to obtain a corrected distribution (Fig. 3).

A comparison of the distributions obtained with the two hosts shows that active HCR is correlated with a relative increase of completely pure clones (with 100 per cent mutants) from 45 per cent in *hcr*−42 to 81 per cent in *hcr*+ ($P = 10^{-7}$ for the difference). This increase is paralleled by a decrease in clones with few wild phages (99 to 50 per cent mutants): 33 per cent in *hcr*−42 and 4 per cent in *hcr*+ ($P = 3 \times 10^{-3}$). One can assume that both these clone types descended from premutated phage DNA of which the first daughter DNA was mutated, either by mutating repair or by the master strand mechanism. The wild-type genomes in the nearly pure (99 to 50 per cent mutant) clones would be produced mostly in later generations, e.g. by replication of the still premutated parental DNA without a copy error or by such DNA where the premutation had later been removed by repair. Since HCR converts nearly pure to completely pure clones it seems that it is acting as a mutating repair mainly in late generations of the vegetative phage. This interpretation is supported by the result (Fig. 3) that more clones with only few mutants (< 6 per cent) were found in *hcr*+ (12 per cent) than in *hcr*− (0 per cent), though the difference is not significant ($P = 0 \cdot 06$). Such clones could arise when mutating repair again yields mutant DNA *late* in the growth of the vegetative phage clone which consists in this case mainly of wild type.

The conclusions are that HCR is not responsible for the predominance of pure or nearly pure mutant clones induced by u.v. in phage κ. It seems

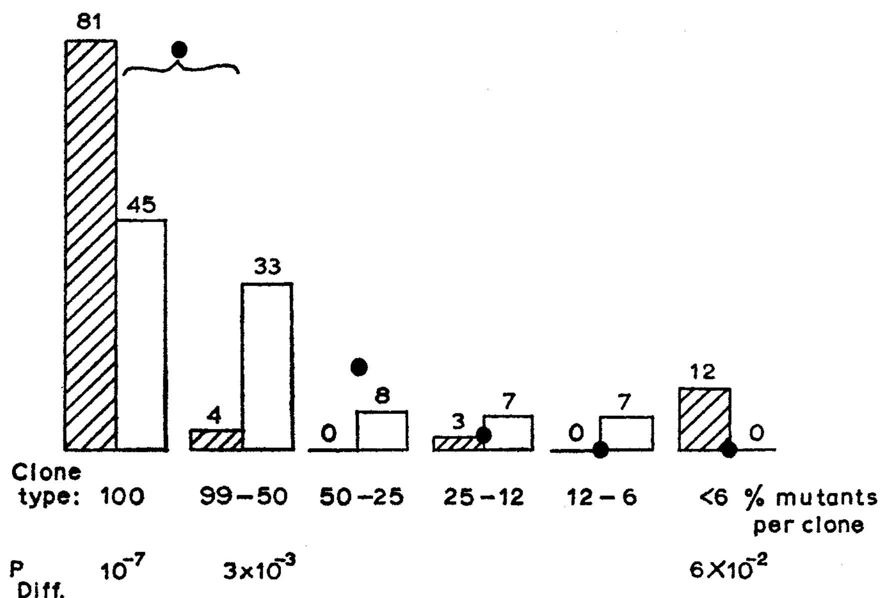

FIG. 3. Distribution (%) of frequencies of clones with various percentages of mutants (clone types) after u.v.-irradiation of phage ϰ, using strains HY (*hcr*$^+$) or *hcr*$^-$42 as preadsorption hosts and plating on HY.

U.v. doses: 4800 erg mm^{-2} (survival 3×10^{-4}) for HY; 600 erg mm^{-2} (survival 3×10^{-5}) for *hcr*$^-$42.

▨ in *hcr*$^+$

□ in *hcr*$^{-42}$

●: Frequencies expected according to the simplex model (Barricelli, 1965) with M = 0·8.

P_{Diff}: Probability of obtaining the difference observed between *hcr*$^+$ and *hcr*$^-$ by random variation only.

to be only an additional "purifying" factor, acting mainly late in clone growth as a mutating repair mechanism. Its main effect is to "extinguish" part of the u.v. premutations. The main cause of the clone purity could be a different type of mutating repair, e.g. the one indicated by the non-mutating strains or by the effect of log growth phase. But the master strand ("simplex") mechanism could also be responsible. If the formula given for this hypothesis by Barricelli (1965) is used for calculating the expected clone-type distribution, the data obtained with *hcr*$^-$42 fit the hypothesis, assuming a copying error chance of M = 0·8 (Fig. 3). For *hcr*$^+$ an additional effect of HCR, e.g. the one proposed, has to be assumed to fit the results better.

SUMMARY

(1) When phage ϰ was u.v.-irradiated inside the host cell both inactivation and the yield of *c*-mutations were higher than with phage

u.v.-irradiated extracellularly. When host cell and phage received the same u.v. doses before infection the mutagenic effects were about the same as with intracellular irradiation. Thus, the mutagenic effects of intracellular irradiation are nothing more than the sum of the effects on the phage plus those on the cell. The increase in mutagenic effects produced by irradiation of the host is probably due to a weakening of HCR by u.v. Intracellular X-irradiation gave the same *c*-mutation yield as extracellular irradiation but a much higher inactivation. Therefore neither type of irradiation induces mutations in the phage via mutagenic products from the protoplasm. Indirect effects may play a role in X-ray inactivation.

(2) When cells in the logarithmic growth phase were used as hosts for u.v.-irradiated phage the inactivation of phage was greater than with cells in the stationary phase but the mutation yield was lower. With strain $hcr^{-}42$ again fewer mutations appeared in log host cells but the inactivation was the same or even a little less. The decrease of mutation in log cells may be due to either a lower chance of errors occurring in copying premutated DNA or to a repair system differing from HCR and acting mainly on premutations. Such a repair was already indicated by host strains which "extinguish" all u.v.- as well as X-ray-induced premutations.

(3) Most u.v.-induced *c*-mutant plaques contained only *c*-phages. The small proportion which had some wild type as well as mutant type was a little higher at a lower u.v. dose. Thus, the "purification" of mutant clones may only partly be due to recessive lethal hits. The proportion of mixed clones was also slightly higher with the host $hcr^{-}42$ than with hcr^{+}. The distribution of clone types (assessed by picking *c*- as well as wild-type plaques) fits the "simplex" model of Barricelli in $hcr^{-}42$. Thus, HCR is not the main cause of clone purity. In hcr^{+} an additional mechanism producing mutant genomes instead of wild-type seems to be present, which may be due to (mutating) HCR acting late in intracellular clone multiplication.

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