SPECIFICITY OF INDUCTION OF T4 r II MUTANTS BY ULTRAVIOLET IRRADIATION OF EXTRACELLULAR PHAGES¹

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CHEMICAL mutagens as 5-bromouracil (BENZER and FREESE 1958), 2-6 diaminopurine (FREESE 1959a), nitrous acid (FREESE 1959c), and proflavine (BRENNER, BARNETT and BENZER 1958) induce specific r II mutations in phage T4. In each instance, analysis of locations of mutants within the r II region has demonstrated that some specific sites were highly prone to mutate and that certain of these sites were not reflected as such in the spontaneous spectrum.

When mutability spectra derived from sets of various mutagen-induced r II's were compared to the spontaneous spectrum and to each other (BENZER 1961) each spectrum appeared unique, but most spectra overlapped to some extent. One may conclude that various chemical mutagens have specific actions upon the genetic material.

Extensive studies by FREESE (1959b,c, 1961) upon base analogue reversion induction of r II mutants of varying origin have supported the hypothesis of WATSON and CRICK (1953) that a base pairing error can lead to mutation. FREESE's studies (1959c, 1961) have led to the construction of mutagenic selection rules in which there are two basically different modes of molecular mutation: transitions and nontransitions. T4 r II mutants have been bipartitioned into analogue revertable and analogue nonrevertable classes. The former is due to base pair transitions in which the only permissible substitutions are purine for purine, or pyrimidine for pyrimidine. Most mutants induced from the standard type by base analogues and nitrous acid typify the transition class. The second, base analogue nonrevertable class, nontransitions, characterizes most spontaneous, proflavine, and heat-low pH induced mutants. BRENNER, BARNETT, CRICK and ORGEL (1961) suggest that single base additions and deletions may also be induced by proflavine and should be considered in the above theory of mutation, in the nontransition class.

This communication will present evidence for a specific mutagenic action of UV upon extracellular phage and will demonstrate that most UV-induced mutants resemble spontaneous or proflavine induced mutants inasmuch as they cannot be induced to revert with base analogues, and that UV induces three

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classes of *r*II mutants, transitions, revertable nontransitions, and nonrevertable nontransitions.

1. EXPERIMENTAL

(a) *Bacteria*

- (i) *Escherichia coli* B (BENZER) for assay of *r* and *r*⁺ phage, *Escherichia coli* BB (BENZER) for growth of high titer *r* stocks only, *Escherichia coli* K-12 (λ) (BENZER) for assay of *r*⁺ phage.

(b) *Phage*

- (i) T4Br⁺, standard type, and *r*II mutants *n*24, *r*131, and H11 were obtained from DR. S. BENZER.
- (ii) T4BrII deletion mutants *r*164, H88, A105, *r*196, *r*187, and *r*638 were obtained from DR. E. FREESE. These deletion mutants delineate segments 1–11 (BENZER and FREESE 1958).

(c) *Media*

- (i) Broth, used for all routine dilutions, growth tubes, preparation of phage stocks, and to grow plating bacteria contained, per liter ion-free water, 13 g Bacto tryptone, 8 g NaCl, 1 g glucose.
- (ii) Buffer, employed for dilutions of phage stocks prior to UV irradiation contained, per liter ion-free water, 10⁻⁶ g gelatin, 0.25 g MgSO₄·7H₂O, 5 g NaCl, 6.8 g KH₂PO₄, 8.7 g K₂HPO₄.
- (iii) Agar plates and soft agar were prepared according to CHASE and DOERMANN (1958).

(d) *Analogues and dyes*

- (i) Chromatographically pure 2-aminopurine and 5-bromodeoxyuridine were obtained from the California Foundation for Biochemical Research, New York.
- (ii) Pure food dyes were the product of the McCormac Food Co., New York.

(e) *UV irradiation*

- (i) Standard UV irradiation conditions: source, single 15 inch Sylvania germicidal low pressure mercury lamp; distance from material, 67 cm from lower edge of lamp tube; temperature, 4°C; phage stocks diluted at least 100 fold into chilled buffer were irradiated in dim yellow light with shaking to a survival of 0.001 percent (70 seconds) as four ml aliquots contained in the bottom of a 100 × 15 mm plastic petri dish, covered until irradiation began, with a pyrex top coated with aluminum foil.

(f) *Abbreviations*

- (i) (AP) 2-aminopurine, (BD) 5-bromodeoxyuridine, (DNA) deoxyribonucleic acid, (UV) ultraviolet irradiation, (B) *E. coli* strain B, (K) *E. coli* strain K-12 (λ), (MR) multiplicity reactivation.

(g) Incubation

- (i) All plates, growth tubes, and plating bacteria were incubated at 37°C.

(h) Isolation and preparation of r mutant stocks

- (i) After 18–24 hours incubation mottled or *r* plaques on B were stabbed with a sterile needle and transferred to sterile broth tubes which contained one drop of chloroform. These *r* isolates were replated on B, single well-isolated *r* plaques were stabbed and used to inoculate aerobic exponential phase BB cultures at low multiplicities. BB cultures thus infected yielded *r* stock cultures of high titer ($5\text{--}10 \times 10^{10}/\text{ml}$) upon clearing after 12 hours incubation.

(i) Experimental techniques

- (i) To assay T4 r^+ stocks for their *r* phage content and to obtain spontaneous and UV-induced *r* mutants, a simplified rapid plating technique has been devised. Aliquots of approximately diluted phage, bacterium B, and 450 ml soft agar at 46°C were mixed to make a final volume of 500 ml. Immediately two ml aliquots were rapidly spread over each of 250 agar plates by a Cornwall automatic pipettor. In this manner the time required to spread 250 plates was always less than ten minutes. Up to 1,000 plates per day containing an average of 200 plaques per plate could be prepared, incubated, and scanned for *r* and mottled plaques.
- (ii) Soft agar employed for the conventional agar layer technique of ADAMS (1959) was prepared in batches of 1,000 13 × 150 mm tubes each containing two ml, and frozen at –15°C. Tubes were withdrawn as required, autoclaved prior to use, and equilibrated at 46°C.
- (iii) To obviate technical accidents bacteria B and K were color-coded with one drop per ten ml culture of McCormac's food dye. Suitable control experiments indicated that these dyes did not affect phage assays or bacterial growth.
- (iv) Deletion mutants which delineate segments 1–11 (BENZER and FREESE 1958) were color-coded with food dyes when used in spot tests to determine segmental locations of all *r*II mutants. This color coding eliminated tiresome labeling of agar plates and gave clear warning of confluence or splashing of drops which did occasionally occur.
- (v) Intersegmental crosses (BENZER 1961) were performed by mixing in the well of a dry, ethanol-sterilized porcelain spot test plate one drop of chilled exponential phase B (at $10^8/\text{ml}$) and one drop of each of the two *r*II mutants to be crossed (at a titer of $1\text{--}2 \times 10^8/\text{ml}$). After resting 5–10 minutes at room temperature (25°C) to permit adsorption, a loop sterilized in boiling water was used to transfer a small aliquot of the mixture onto agar medium freshly seeded with 20K:1B in soft agar. Spot test plates were incubated 18–24 hours. To correct for the background of *r*⁺ phage, each parental phage was separately spot tested as above.
- (vi) Routine techniques for handling phage were, with the above exceptions, those of ADAMS (1959).

Legend to Tables 1 and 2. Segments 1-11 are defined by BENZER and FRIESE (1958). Mutant sites are not arranged in order; numbers of mutational sites are of *rII* stocks chosen as representative of that site. UF and SF sites connected by a slash are upon the same chromosome. The letters are identical by recombination spot tests. Key to tables: a=2-aminopurine revertable; b=5-bromouracil revertable; ab=revertable by both analogues, X=spontaneous reversion frequency $0 \rightarrow 1 \times 10^6$, y=spontaneous reversion frequency $0.005 \rightarrow 1 \times 10^6$, z=1-100 $\times 10^6$ per 10^6 . UV mutation frequencies were 8.2 times greater than spontaneous. All deletion mutants of the SF group and some deletions of the UF group are not included in Tables 1 and 2 as their large span prevented accurate mapping. Text selections 1(i,v; i,v) describe methods of localization of mutants to and within segments.

TABLE 2
Mutability spectrum of Cistron B. See legend below Table 1

Segment	UV site	No. and type	S site	No. and type
8	UF-101	19(17y,1yb)	SF-219	15y
	14	2(1y,1z)	687	3y
	41	4(2z,1y,1ya)	149	1yb
10	UF-47	1y	SF-10	3(1y,1yab,1x)
	62	1y		
	106	1y		
	80	1y		
	111	1y		
	178	1y		
	45	segment 10 deletion		
7,9,11	UF-65	10(5y,5yb)	SF-211	4y
	95	1y	94	4(2y,1yb,1yab)
	133	3(2y,1yb)	599	2(1y,1x)
	163	1y	661	1y
	29	1y	298	2(1y,1x)
	1	7(4y,3x)	626	2y
	84	1y	308	1y
	100	1y		
	171	1y		
	119	1x		
	115	8(6y,1yb,1x)	632	1yb
	170	1y		
	117	deletion over sites 100,171		
	108	deletion over sites 119,115		

TABLE 3

Comparison of UF and SF mutants regarding spontaneous and base analogue induced reversion

	Spontaneous reversion index*				Base analogue induced reversion		
	<i>x</i>	<i>y</i>	<i>z</i>	<i>hfr</i>	BD	AP	Both
SF mutants	22	76	3	17	5	2	8
(total 118)	(18.5)	(64.5)	(2.5)	(14.5)	(4.3)	(2.3)	(6.7)
UF mutants	25	104	5	10	13	3	0
(total 144)	(17.5)	(72.0)	(3.5)	(7.0)	(9.0)	(2.1)	

* Spontaneous reversion indices: *x* is 0 *r*⁺ per 10⁸, *y* is 0.005 to 1 *r*⁺ per 10⁶, *z* is 1 to 100 *r*⁺ per 10⁶, *hfr* (high frequency of reversion) is 100 to 100,000 *r*⁺ per 10⁶. Phage belonging to the *hfr* class could not be mapped due to their high *r*⁺ content. See text 1(c,i), 1(f,i), and Tables 1-2. Numbers in parentheses are percentages.

(a) *Spontaneous r mutants derived from r⁺ stocks*

- (i) Four to five hour single r⁺ plaques on B were stabbed and employed to infect aerobically growing B at a titer of $5-10 \times 10^8$ per ml, and at multiplicities lower than 0.1. Separate r⁺ stocks obtained in this fashion were diluted, plated on B, and scanned to determine the frequency of r mutants present in the stock. r mutants were isolated from that r⁺ stock which manifested the lowest content of r mutants. The r⁺ phage stock from which all spontaneous and UV-induced mutants were isolated contained one spontaneous r mutant per 2,400 total phage (r frequency = 4.1×10^{-4}). In one of 15 experiments, 1.69×10^5 plaques on B were scanned, of which 38 plaques were pure r type and 32 plaques were mottled (contained r and r⁺ phage).
- (ii) Of 723 r mutants of spontaneous origin, 118 proved to be unable to grow on K, were labeled SF-1 . . . , and were classified as rII. Of these 118 rII mutants, 88 were mappable, 13 were deletions of various lengths, and 17 reverted to r⁺ type at frequencies greater than one per 10^4 .
- (iii) The frequency of spontaneous mutation to rII was thus $118/723 \times 1/2,400$ or 6.7×10^{-5} .

(b) *UV-induced mutants*

- (i) Extracellular r⁺ phage were UV irradiated under standard conditions 1 (e,i) and surviving phage were plated on B at multiplicities of total phage per bacterium ranging from 0.05 to a maximum of 0.20. From 2.04×10^5 plaques on B, 685 different r mutant phage were collected, of which 305 were pure r, and 380 were mottled (r⁺ and r). The frequency of mutation to the r phenotype under these conditions was 3×10^{-3} . UV irradiation of extracellular phage increased r mutation frequency from 4.1×10^{-4} to 3×10^{-3} , or by a factor of about 7.3 times.
- (ii) Of 685 r isolates, 144 were unable to grow on K, were designated rII, and their origin indicated by the prefix UF. Of these 144 rII mutants, 11 were deletions and ten were unmappable due to their high frequency of reversion to r⁺ (greater than one per 10^4). Four of the 11 deletions spanned relatively short map regions and are depicted in Tables 1 and 2. The remaining seven deletions covered larger regions including most of cistron B (3) or most of both cistrons (4) and are not included in Tables 1 and 2 as their extent could not be accurately determined.
- (iii) The frequency of UV induced rII mutants was $144/685 \times 1/336$ or 6.2×10^{-4} . Thus UV irradiation increased rII mutation frequencies from 6.7×10^{-5} to 6.2×10^{-4} or by a factor of 9.3.

(c) *Multiplicity reactivation*

- (i) Because UV-irradiated phages were plated at multiplicities up to 0.2 on B the contributions of MR and marker rescue due to multiply infected bacteria containing r markers preexisting in the irradiated phage stock have been assessed. The frequency of r mutants before irradiation was 4.1×10^{-4} (r)

while the highest observed multiplicity was 0.2 (m). Thus frequency of r -containing multicomplexes in the irradiated phage suspension at the time of plating was:

$$2r(1 - (1 + m)e^{-m}) = 1.64 \times 10^{-5} \quad (1)$$

where the frequency of bacteria multiply infected was:

$$1 - (1 + m)e^{-m} = 2 \times 10^{-2} \quad (2)$$

Thus, if every multicomplex containing at least one r phage were to survive and produce an r or mottled plaque, 5.4×10^{-3} of the observed r and mottled plaques could be due to selection for multicomplexes containing preexisting r mutants.

- (ii) Similar maximal corrections may be employed for the r II mutants. The r II spontaneous frequency is 6.7×10^{-5} (r). The frequencies of multicomplexes containing at least one r II, according to equation (1) are 2.7×10^{-6} , and 4.4×10^{-3} of the observed r II UV mutants would be due to selection for surviving multicomplexes containing at least one preexisting r II.
- (iii) The multiplicity (0.2) chosen for the above calculations was the highest observed; generally multiplicities of 0.1 were employed. Also, it was assumed above that every multicomplex receiving a preexisting r mutant survives. Clearly these corrections yield maximal estimations of selection for preexisting r phages.
- (iv) The survival level of UV irradiated phages (10^{-5}) was also determined by plating on B at multiplicities up to 0.2. Thus the true survival value for single phages is not clear and may be less, due to MR occurring in some of the 2×10^{-2} multiply infected bacteria.
- (v) Employing maximal corrections for rescue of preexisting r mutants due to multiple infected bacteria and knowing the frequency of spontaneous r mutants, one obtains an estimate of the proportion of the background of spontaneous r mutants among the UV r mutants, which is:

$$\frac{4.1 \times 10^{-4} \text{ (spontaneous } r \text{ frequency)} + 1.64 \times 10^{-5} \text{ (MR } r \text{ frequency)}}{3 \times 10^{-3} \text{ (UV } r \text{ frequency)}}$$

or 14.5 percent. Similarly, one obtains for r II mutants an estimate of 11.3 percent background due to preexisting r II mutants. Thus, of the 144 r II mutants examined, 16 could be ascribed to the background spontaneous r II mutants. Correcting for spontaneous r and r II background the extent of UV induction, (1) of r mutants is 6.2 (rather than 7.3) times spontaneous levels, (2) of r II mutants is 8.2 (rather than 9.3) times spontaneous levels.

(d) Spontaneous reversion frequencies of SF and UF mutants

- (i) Thirty ml cultures of bacterium BB in exponential growth at a titer of $1-2 \times 10^7$ per ml were infected with 300–900 r phage. The infected culture was subdivided into three aliquots and incubated with aeration. Each phage stock thus prepared was assayed on bacterium B (total phage titer) and on bacterium K (r^+ phage titer). Ratios of r^+ to total phage were averaged for all three cultures of similar origin. The average r^+ content of these stocks was termed the spontaneous reversion index (after BENZER and

FREESE 1958). The distribution and frequencies of spontaneous reversion frequencies of UF and SF mutants is presented in Tables 1–3. As a group, the UF mutants did not clearly differ from the SF mutants regarding spontaneous reversion indices. Both sets contain nonreverting mutants of short extent and deletion mutants of sufficient span to include several point mutations within their length. BENZER (1961, personal communication) has indicated that UV-induced mutants derived from ultraviolet irradiation of phage-bacterium complexes yielded a homogenous group of *rII* mutants most of which would spontaneously revert at frequencies of 10^{-8} (group Y). Although many UF mutants fit this description (104/144, 72 percent), the frequencies of nonreverting small span mutants (as UF-119) are quite high (25/144, 17.5 percent) and also appear to be induced by UV. A possible explanation for the origin of these mutants is given in the discussion to this communication. Contamination of the UF mutants with mutants of spontaneous origin cannot account for all these mutants since the expected background of spontaneous *rII* mutants of all types was about 11.3 percent, or 16 per 144.

(e) *UV and spontaneous mutational sites*

- (i) Representatives of all mutational sites of the UF group were crossed by spot tests to site representatives of the SF group by method (1i,v). Sites which were identical by recombination spot tests are indicated by dashed lines in Tables 1–2. Of 43 SF mutational sites, 15 were identical with the 53 UF mutational sites.

(f) *Mutational hot spots*

- (i) Two sites of the UF and the SF groups (UF-42, UF-101; SF-328, SF-219) were characteristic of BENZER's spontaneous mutational hot spots *r131* and *H7*, respectively (BENZER 1961). The UF group contained four other sites of high mutability, one in segment 2 (UF-13) and three in segments 7, 9, 11 (UF-65, UF-1, UF-115). All these sites were represented by identical but far less mutable sites in the spontaneous spectrum, except UF-1. Since the frequency of UV-induced mutation to *rII* is 8.2 times greater than spontaneous frequencies, these four UV sites appear to be UV hot spots of mutation. Possibly UF sites 101 and 42 are UV, as well as, spontaneous hot spots.

(g) *Base analogue reversion induction*

- (i) Every UF and SF mutant was tested in duplicate for BD and AP reversion induction by the spot test method of FREESE (1959a). Results are presented in Tables 1–3. UV mutants differ in no definite manner from spontaneous mutants regarding reversion induction by base analogues except that most analogue revertable UV mutants respond to BD and not AP.

DISCUSSION

Evidence has been presented that UV irradiation of extracellular phages leads to the production of specific *rII* mutants. Under UV irradiation four sites appeared as mutational hot spots. Three of these sites were also detected in the spontaneous spectrum, but the frequency of occurrence of spontaneous mutants at these sites was markedly lower.

UV irradiation apparently induces three classes of *rII* mutants: transitions, detectable by being base analogue revertable; spontaneously revertable non-transitions, which are not base analogue revertable; and stable nontransitions.

Neither UV nor spontaneous hot spots are homogenous. At the same sites one notes analogue revertable, spontaneously revertable but analogue nonrevertable, and nonreverting mutants. This indicates that more than one kind of change can occur at a hot spot site to yield an *rII* mutant. Presumably additions and deletions of single bases, as in proflavine mutation (BRENNER *et al.* 1958) or transversions, or transitions or additions and deletions of multiple bases may all give rise to UV mutagenesis.

Recent studies by MARMUR and GROSSMAN (1961) suggest that one of the primary effects upon DNA of UV at biological doses is the formation of inter-chain crosslinks and of intrachain crosslinks between thymine residues. If these events represent the bulk of the initial extracellular mutagenic action of UV then three interrelated specific actions of UV upon DNA may be reasoned. First, most UV mutagenic effects would be localized upon areas of the DNA molecule where frequent local nonrandom groupings of adenine-thymine base pairs exist. Second, if not all base pair alterations represent a critical functional alteration (*vide infra*) then some base pair changes will not be recognized as they will be "mutagenic" only at the DNA level and not mutagenic at the level of function of the altered *rII* product. Third, thymine dimerization of the interchain or intrachain variety can, *a priori*, lead to more than one kind of base pair change; transitions and nontransitions (transversions, additions, and deletions). Thus, heterogeneity of mutants belonging to the same hot spot site can be expected, and indeed, is observed.

Due to other effects of UV irradiation as cytosine residue alteration and DNA backbone breakage (see MARMUR and GROSSMAN 1961), UV mutants arising as a consequence of these perturbations would be widely distributed throughout the *rII* region. That this is the case is evident when the general appearance of the UV mutational spectrum is noted in Tables 1-2.

Numerous small nonreverting mutants (group Z) are a feature evident in the UV spectrum. Intrachain thymine dimerization could restrict certain short segments of DNA as loops during replication so that the loops would not be copied. In this event true deletions of all sizes would result. FRESCO and ALBERTS' studies (1960) upon accommodation of noncomplementary bases in DNA indicate that this mechanism is structurally feasible.

A comparison of mutants arising from UV irradiation of extracellular phage to those from irradiation of complexes would be of interest. Thymine dimerization

which can occur between and within chains of a DNA molecule could, in irradiated complexes, also occur between precursor thymidylic acid molecules and DNA thymine. In this instance UV-thymidylic acid might act in much the same manner as proflavine and assume an intercalated position between two DNA thymines thus resulting in an addition of a base pair as suggested for proflavine by BRENNER *et al.* (1961).

At present a more precise interpretation of the nature of UV mutants will not be attempted in light of the anomalous behavior of *hfr rII* mutants, which do not clearly fit any molecular model for mutagenesis, and due to uncertainties regarding the genetic nature of r^+ revertants obtained from *rII* mutants. Work on the nature of the r^+ revertant phage phenotype, only recently initiated in this laboratory, tentatively indicates that some spontaneous r^+ revertants appear to be the result of mutation at other sites in the *rII* region and are not due to reversion to the original r^+ standard type phage.

A comparison of mutagen specificities determined by analysis of mutational spectra is at first order a comparison of the functional properties of the *rII* product. Obviously, the *rII* product is nonfunctional for any *rII* mutant. All observed mutational sites are critical, but some other, unobserved, mutational sites may exist; these may be termed noncritical. Imagine the *rII* product as a progression of nucleotides (or amino acids). For the product to function normally it is not theoretically necessary to specify one unique sequential arrangement of elements. Rather, it is possible that certain regions upon the progression must be rigidly specified while other different regions may, within limits, tolerate some variability, while still permitting normal function.

Thus, one may explain the existence of mutational hot spots in several non-exclusive ways. A hot spot may be a DNA site at which mutations recur with high frequency due to certain topographic features of the DNA as nonrandom base pair clustering. In addition, hot spots may also represent the extent to which variation in gene product sequence is tolerated. For example, one may assume the most conspicuous spontaneous hot spot of segment 8 to represent a DNA site at which *any* change whatsoever in its information content results in the production of a nonfunctional product. Other less marked hot spots, as in segment 5, may indicate that most DNA changes at that site produce a nonfunctional product while some other DNA changes are unobserved as they produce a functional, but sequentially altered *rII* product. Mutationally silent areas as in segment six may be genetic regions whose resultant sequential product can be altered, but this altered product region is noncritical to *rII* product function. Thus one may be examining the gene through the looking glass of its product due to the possibility that there may be DNA sites which can be *changed*, but not *mutated*.

The above considerations do not detract from the observations of mutagen specific spectra but merely suggest that other *rII* regions in noncritical areas may exist which could be detected by other means.

UV irradiation markedly stimulates genetic recombination in phage lambda (JACOB and WOLLMAN 1955) and phage T4 (EPSTEIN 1958). As genetic recombination may represent a property of the phage DNA molecule which is not

complicated by problems of gene product function ("the looking glass effect"), a search for recombinational topography over the *r*II region may prove of interest. Experiments recently reported (FOLSOME 1961) do, indeed, show that the UV mutational hot spot of segment 2 was also a region over which recombination was UV stimulated to a considerably greater extent when compared to UV stimulated recombination about the mutationally silent areas of segment 4. Further analyses of these observations are in progress.

SUMMARY

- (i) Ultraviolet light irradiation of extracellular T4Br⁺ phage increased the frequencies of *r* mutants 6.2 times over spontaneous levels and increased the frequencies of *r*II mutants 8.2 times over spontaneous levels.
- (ii) 144 UV *r*II mutants have been localized in the *r*II region by qualitative recombination spot tests and their map distribution was compared to 88 spontaneous mutants. Fifteen of 53 UV and 43 spontaneous mutational sites are identical. Four distinct UV mutational hot spots were noted.
- (iii) The majority of the UV mutants appear to be nontransitions (single base pair transversions, additions, or deletions) since only nine percent of the UV mutants were transitions (base analogue revertible), 72 percent reverted only spontaneously at levels of one per 10⁸. The balance comprised small span deletions or nonreverting mutants, and high frequency of reversion mutants.
- (iv) Possible modes of origin of the different types of UV mutants are discussed.

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