GENETIC HETEROZYGOSITY IN UNREPLICATED BACTERIOPHAGE λ RECOMBINANTS

RAYMOND L. WHITE¹ AND MAURICE S. FOX

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT

Bacteriophage crosses using density-labeled parents have been carried out under conditions restricting DNA synthesis. The parental material and genetic contributions to progeny manifesting recombination within a genetic interval sufficiently short to exhibit high negative interference have been examined. The unreplicated products of recombination isolated as phage particles appear to contain long continuous heteroduplex regions which are heterozygous for the closely linked markers. Recombination between closely linked markers seems to be the consequence of the removal of base-pair mismatches that are present within the heteroduplex regions. This localized reduction of heterozygosity within the heteroduplex regions that join the parental components of recombinant DNA molecules can account for high negative interference.

IN a previous paper (WHITE and FOX 1974), we have suggested that recombination between closely linked markers in phage λ occurs by a two-step process. First a heterozygous heteroduplex overlap region is formed, presumably by a breakage and joining reaction, usually including most of the closely linked markers under consideration. Secondly, regions of base-pair mismatch present in these nonrecombinant heterozygotes are reduced to homozygosity or "repaired" by some unspecified process. Furthermore, it has been shown that the heterozygous heteroduplex overlaps have only one of the two possible chemical orientations of the DNA (Figure 1 b). Here we wish to describe in more detail the genetic structure of the unreplicated products of the recombination events as revealed by examination of genotypes present among the progeny of individual phage products of a genetic cross.

Crosses, involving closely linked markers, are carried out under conditions severely restricting DNA synthesis (McMILIN and RUSSO 1972). The parental phage contain differential density labels and progeny phage mature without replication. The buoyant density of recombinant phage reflects their relative material contribution from each of the parents. In order to examine the parental genetic contributions, the "two-strand genotype" of the unreplicated recombinant phage is deduced from the genotypes found among the progeny of single infections. Although it is apparent from previous experiments that infection with heterozygous DNA frequently does not yield mixed or heterozygous bursts of

¹ Present address: Department of Biochemistry, Stanford University School of Medicine, Stanford, California 94304.

FIGURE 1.—See text.

FIGURE 2.—Cesium formate density gradient profile of progeny from a cross of heavy *cl60* *Pam3* by light *cl+* *Pam80* in FA77 (*su-*, *tsDNA*) at 41.5°.

- Total phage titer Q_1 (su^+)
- Total phage titer WD5021 (su^-)
- Titer clear plaques on WD5021 (su^-)
- Titer turbid plaques on WD5021 (su^-)
- Titer mottled plaques on WD5021 (su^-)

progeny phage (DOERFLER and HOGNESS 1968; SPÄTZ and TRAUTNER 1970), some information can be gained by examination of those bursts which do yield progeny of several genotypes.

The results of these experiments allow several generalizations: (1) The amount of parental DNA contributed to recombinants is not sharply defined; in fact, very few phage progeny either singly or doubly recombinant between the closely linked markers seem to have received less than 15% or more than 85% of their DNA from either one of the parental DNA molecules. As can be seen in Figures 2 and 3, showing the density distribution of the progeny of heavy-by-light crosses, recombinant phage are more or less uniformly distributed between these limits. However, as a consequence of the specificity of orientation of the heteroduplex overlap (Figure 1), the heterozygote classes found among recombinants at the opposite ends of the density distribution are strikingly dissimilar.

(2) Among phage recombinant for the closely linked selected markers, the incidence of heterozygosity for outside markers is high.

(3) The pattern of heterozygosity at the site of a selected marker is substantially different when the primary infection (single burst) is carried out on per-

FIGURE 3.—Cesium formate density gradient profile of progeny from a cross of heavy *cl60* *Pam3 Ram5* by light *cl+* *Oam29 Pam80* in FA77 (*su*⁻, *tsDNA*) at 41.5°

- Total phage titer Q_1 (*su*⁺)
- Total phage titer WD5021 (*su*⁻)
- Titer clear plaques on WD5021 (*su*⁻)
- Titer turbid plaques on WD5021 (*su*⁻)
- Titer mottled plaques on WD5021 (*su*⁻)

missive bacteria than among single bursts arising from infection of nonpermissive bacteria.

MATERIALS AND METHODS

Bacterial and phage strains used are listed below:

E. coli bacterial strains:

Designation	Relevant Phenotype	Source
Q ₁	SuII ⁺	E. SIGNER
QD5003	SuIII ⁺	E. SIGNER
M107	SuI ⁺	E. SIGNER
WD5021	Su ⁻	E. SIGNER
CA85	Su ⁻	E. SIGNER
CA85 (λ imm434c ₁₇ Oam29)	Does not complement λ Oam mutants, does complement λ Pam, λ Ram mutants	Derived from CA85 by high multiplicity infection with λ imm434c ₁₇ Oam29
CA85 (λ imm434c ₁₇ Pam80)	Does not complement λ Pam mutants, does complement λ Oam, λ Ram	By infection with λ imm434c ₁₇ Pam80
LS446a	Su7	LARRY SOLL via IRA HERSKOWITZ
FA77	Su ⁻ , tsDNA	K. McMILIN
MR93	Su ⁻	E. SIGNER
MR93/ λ	/ λ maltose ⁻	From MR93 by selection with λ vir.
WD5021 (λ imm434Qam73)	Does not complement Qam mutants	I. HERSKOWITZ

Salmonella bacterial strain

D21	grows phage P22	D. BOTSTEIN
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λ phage strains: cI857 Oam29, cI60, Oam29, Pam80, Pam3, Ram5, λ imm434c₁₇Oam29, and λ vir were obtained from E. SIGNER; cI60Pam3 from I. HERSKOWITZ, and cI857Oam29Pam3 from N. KLECKNER. Derivative combinations of these strains were constructed by standard phage techniques.

P22 phage strains: Wild type, labeled with (²H, ¹⁵N) and c2h (¹H, ¹⁴N) were obtained from J. EBEL-TSIPIS.

Culture media: λ Broth—10 g of Difco Bacto-Tryptone, 2.5 g of NaCl, and 1 liter of H₂O, supplemented with 0.1% yeast extract and 0.3% maltose. Trypticase Bottom Agar—10 g of Baltimore Biological Labs Trypticase, 5 g of NaCl, 10 g of Difco Bacto-Agar, and 1 liter of H₂O. Trypticase Top Agar—same as trypticase bottom agar except 6.5 g of agar/liter. 10 $\times$ M9 salts in D₂O without NH₄Cl—3 g of KH₂PO₄, 0.5 g of NaCl, 5.8 g of Na₂HPO₄, and 100 ml of D₂O. M9Cl contains 1/10 volume 10 $\times$ M9 salts, 0.2% glucose, 2 μ g/ml thiamine, 10⁻³ M MgSO₄ and 1.5% charcoal clarified casamino acids. M9-D₂O—10 ml of 10 $\times$ M9 salts in D₂O, 2.5 ml of ¹⁵N H₄Cl (20 mg/ml), 1 ml of 20% glucose, 0.1 ml of thiamine (2 mg/ml), 0.1 ml of 1 M MgSO₄, 0.5 ml of proline (4 mg/ml), and 90 ml of D₂O. All component solutions made up in D₂O and sterilized separately by heating 20 min in a steamer. SM—5 gm of NaCl, 2.4 gm of Tris:HCl, and 1 liter of H₂O. After sterilization, one ml of 1% gelatin and 1 ml of 1 M MgSO₄ (pH 8) were added per 100 ml of solution.

Solutions for density gradients: D₂O Buffer—100 ml of D₂O, 0.1 ml of 1 M Tris:HCl (pH 8.4) and 0.1 ml of 1 M MgSO₄. Cesium Formate in D₂O—70 g of cesium formate and 30 ml of D₂O buffer.

Chemicals: Deuterium Oxide (99.9% D_2O): Bio-Rad Laboratories, Richmond, Calif. Cesium Formate: Varlacoid Chemical Co., Elizabeth, N.J. $^{15}NH_4Cl$: Prochem Limited, U.K. Lysozyme: grade I, Sigma Chemical Co., St. Louis, Mo.

Plating bacteria and phage titration: Overnight cultures were grown in M9Cl from a single colony inoculum at 37° with shaking. For plating, the overnight cultures were diluted 1/100 into lambda broth containing 0.01% yeast extract and 0.3% maltose. The culture was incubated at 37° with shaking to a cell density of $5-8 \times 10^8$ as determined by microscope count of cells in a Petroff-Hauser counting chamber. These cells were kept on ice and usually used on the same day they were grown. Phage suspensions to be titered were diluted through SM buffer and a 0.05-0.2 ml aliquot added to 2-3 drops of plating bacteria in a Wasserman tube at 37° . After 10-15 minutes incubation, 2 ml of Trypticase top agar was added and the mixture layered over 50 ml of Trypticase bottom agar in a plastic petri dish. The overlaid plates were usually incubated overnight at 37° .

Phage stocks: Standard phage stocks were prepared by a confluent lysis-agar layer method. Petri dishes containing a thin layer of Trypticase bottom agar were prepared on the same day they were used. These plates were overlaid with a mixture of 2 ml Trypticase top agar, 0.5 ml λ broth, 0.4 ml of a Q1 culture grown as described before for plating bacteria, but without maltose, and 0.1 ml of a 1 ml λ broth suspension of 1-3 isolated single plaques picked with a capillary tube. After 5-8 hours of incubation at 37° , the top layer was slithered off into a centrifuge tube, a few drops of chloroform added and the mixture vortexed vigorously for a full minute before centrifugation in a Sorvall SS-34 rotor at 10,000 RPM for 10 minutes. The supernatant was removed and titered, usually yielding $1-4 \times 10^{10}$ phage/ml.

All 2H , ^{15}N substituted phage stocks were made by infection of strain LS446a and growth in M9- D_2O medium. Aliquots of LS446a adapted to growth in M9- D_2O medium were kept frozen at -20° in M9- D_2O with 5% dimethyl sulfoxide added. These cultures were allowed to thaw, diluted 1/100 into M9- D_2O , grown to $3-4 \times 10^8$ at 37° with shaking, pelleted at 5,000 RPM for 10 minutes and resuspended in 1/5 volume of 0.01 M $MgSO_4$ in D_2O . The supernatant was conserved and heated to 37° . The resuspended pellet was incubated at 37° for 1 hour and infected with a multiplicity of 3-5 of a phage suspension that was either very high titer such that a small inoculum could be used, or had been previously dialyzed against 0.001 M $MgSO_4$ in D_2O . After 30 minutes adsorption at 37° (about 80% of the phage were adsorbed) the conserved M9- D_2O supernatant was added and incubation with shaking continued for 90-120 minutes at which time chloroform was added and bacterial debris removed by centrifugation. The lysate usually contained phage at about 2×10^9 . Lysates were concentrated either by pelleting at 17,500 RPM in a Sorvall SS-34 rotor followed by overnight resuspension in SM or centrifugation in a CsCl block gradient in a Beckman SW27 rotor for 2 hours at 26,000 RPM. The block gradient consisted of a layer of 3 ml 65% w/v CsCl of refractive index 1.3720 under 10-20 ml of lysate. The phage were recovered by drop collection. With both methods, the phage were further purified by equilibrium centrifugation in a CsCl density gradient. Three drop fractions were collected, and the three heavy peak fractions pooled and dialyzed once against λ broth with 0.5 M NaCl followed by dialysis against λ broth with maltose and yeast extract.

Construction of multiple mutants: Phage strains carrying multiple mutations were constructed by mixed infection of Q_1 plating bacteria with appropriately marked parents followed by 1/10 dilution of the infected cells into SM. This dilution was irradiated with UV from a GE 15 watt germicidal lamp with a dose sufficient to reduce the survival of free phage to 10^{-3} . After irradiation the infective centers were diluted 1/10 into λ broth with yeast extract and maltose and incubated at 37° for 90-120 minutes. Chloroform was added, and the lysate diluted and plated on Q_1 . The genotypes of the resulting plaques were determined as described below. The appropriate spot from the Q_1 spot plate was streaked on a Trypticase bottom agar petri plate and overlaid with Trypticase top agar containing 0.1 ml Q_1 plating bacteria. A single plaque was picked from this plate and used as an inoculum for a plate stock.

Genotyping of plaques: Genotypes of phage plaques were determined by either picking into 1 ml SM with a capillary tube and spotting on plates seeded with indicator or stabbing the plaque with a toothpick and sequentially stabbing a set of seeded indicator plates. For example, the progeny of a cross between *cl60 Pam3 Ram5* and *c+ Oam29 Pam80* were genotyped in the

following manner: the progeny particles were plated on Q_1 ; each plaque to be genotyped was stabbed with both ends of a toothpick. One end was sequentially stabbed into plates seeded with plating bacteria of strains WD5021, Q_1 and CA85 (*imm434c₁₇Oam29*); the other end into M107, QD5003, and CA85 (*imm434c₁₇Pam80*). These plates were incubated overnight at 37°. The *cl* allele was determined from the Q_1 plate; a turbid spot denoted *c*⁺, a clear spot *cl*60. The *R* allele was also determined from the Q_1 plate by inverting the plate and adding 2–3 ml of chloroform to the lid (this procedure is somewhat more esthetic when glass petri dishes are used; however, when glass plates are used, a piece of folded aluminum foil is placed between the lid and the dish). After 2–4 hours at room temperature with chloroform, spots derived from *R*⁺ phage develop a marked halo; spots from *Ram5* phage plaques show no halo. The *Oam29* allele was determined from the plate seeded with CA85 (*imm434c₁₇Oam29*); plaques from phage containing the *Oam29*⁺ allele show growth (lysis of the lawn) and *Oam29* phage give no growth. The *Pam3* allele was determined from the plate seeded with QD5003. Phage carrying the mutant allele *Pam3* cannot grow on SuIII⁺ suppressor strains, the other *am* alleles used can grow; no growth on QD5003 defines *Pam3*, growth defines *Pam3*⁺. The characterization of the *Pam80* allele is somewhat more difficult. Plaques derived from phage carrying *Pam80* give no growth on indicator plates seeded with CA85 (*imm434c₁₇Pam80*); however, *Pam3* shows only a very weak response on this indicator. Indicator plates seeded with M107 provide the necessary discrimination; *Pam80* shows very little growth on this indicator and *Pam3* mutants grow very well. The double mutant, *Pam3 Pam80*, is defined by absence of growth on QD5003, M107, and CA85 (*imm434c₁₇Pam80*). Spot testing on plates seeded with WD5021 (*Δimm434Qam73*) provided the necessary discrimination for detecting the presence of *Qam73*⁺ or *Qam73*.

Phage crosses with restricted DNA synthesis: Crosses performed in the *tsDNA* strain FA77 (McMILIN and RUSSO 1972) were executed according to the following regimen. Bacterial cultures were inoculated from a single colony into λ broth with yeast extract (.01%) and maltose (0.3%) and grown at room temperature with shaking to a cell density of 4×10^8 as determined by Petroff-Hauser count. Before adding to the phage suspension, the bacteria were incubated with shaking 15 minutes at 41.5°. Simultaneously, a phage suspension in λ broth plus yeast extract and maltose containing, unless otherwise noted, 4×10^9 /ml of each parent phage to be crossed, was also heated 15 minutes at 41.5°. After this preincubation, an equal volume (either 0.25 or 0.5 ml) of the bacterial suspension was added to the phage and incubation continued for 60–70 minutes at 41.5°. To terminate incubation, 3.0 ml of chloroform saturated D₂O buffer was added. After 10 minutes with chloroform the lysate was centrifuged 10 minutes at 5,000 RPM in a Sorvall SS34 rotor to remove bacterial debris.

For equilibrium density-gradient analysis, 2.25 ml of the above lysate was added to 2.75 ml cesium formate solution in D₂O together with 0.05 ml ²H, ¹⁵N labeled Salmonella phage P22 and 0.05 ml unlabeled P22 in a Beckman cellulose nitrate tube. The refractive index was adjusted to 1.3710–1.3715 and the tubes centrifuged in a Beckman SW39, SW50L, or SW50.1 rotor for 40–48 hours at 27,000 RPM at 20°. After centrifugation the bottom of the tube was punctured with a needle and one drop fractions collected. This usually yielded 130–150 fractions. After collection either 1.0 or 0.5 ml of SM buffer was added to each tube, the fractions containing phage identified by spot test on Q_1 and titered on Q_1 (Su⁺) and WD5021 (Su⁻). The resulting plates were differentially scored for clear, turbid, and mottled plaques.

Single burst in liquid medium: Phage to be tested were absorbed at a total multiplicity of 10^{-3} to strain WD5021 plating bacteria at $2-4 \times 10^8$ at 37° for 10 minutes. Anti- λ serum at a final concentration such that K=1 was added for an additional 10 minutes. The infective centers were diluted into λ broth plus 0.01% yeast extract and 0.3% maltose to a final concentration of 1 phage-producing infective center/ml. This suspension was distributed into Wasserman tubes (0.1 ml/tube) and incubated at 37°. After 60 minutes, 0.3 ml of iced, chloroform saturated λ broth containing 20 μ g/ml lysozyme was added to each tube and incubation continued for 45 minutes at 37°. 0.1–0.5 ml Q_1 plating bacteria were then added to each tube. After 10 minutes adsorption at 37°, 2 ml Trypticase top agar was added and the contents plated on Trypticase bottom agar plates followed by overnight incubation at 37°. From plates with 20 or fewer plaques, all were picked and their genotypes determined. From plates with more than 20 plaques, the plaques selected for genotyping were not chosen at random. With some experience it became

possible to partially discriminate genotypes from the appearance of a plaque; for example, a plaque of genotype *Oam29* is smaller than a *Pam3* plaque is smaller than wild type. Since the plates were chloroformed before picking some discrimination of the *R* allele was sometimes possible. The object of this plaque picking was to determine qualitatively which genotypes were present.

An alternative method for examination of the progeny from single infections is to form infective centers of WD5021 (Su^-) as described above, plate before lysis on Q_1 (Su^+), pick the plaques that form into buffer and replate a small aliquot on Q_1 (Su^+). These plaques are picked and genotyped as before.

A third method is to form infective centers on the permissive strain Q_1 (Su^+), plate before lysis and pick resultant plaques into buffer. Since most of the plaques formed will be of parental genotype, these were screened by spot test to identify the plaques containing a nonparental combination of *am*⁺ alleles. This method identifies plaques resulting from infection with non-recombinant heterozygotes as well as true recombinants. Plaques containing a nonparental combination of *am*⁺ alleles were replated and progeny plaques genotyped.

Frequently three or more genotypes were found among the progeny of a single infection with a heterozygous phage. This is presumably due to recombination between the progeny genomes. The genotypes representative of the two-strand genotype of the input phage were defined on the basis of being (1) represented in the burst and (2) able to generate by recombination all other genotypes found. Occasionally, ambiguity still remained in which case the input genotype was chosen as the majority type present in the burst.

Control reconstruction experiments indicate that the permissive plating method of two-strand genotype determination allows the recovery of all genotypes (except *Oam29 Pam3 Pam80* triple mutant types which were not tested) yielded by a mixed burst from the progeny found in the resulting plaque.

That lysozyme and chloroform effectively complement *R*-gene-defective infective centers was shown by reconstruction single-burst measurements.

Some general parameters of the high-temperature crosses in strain FA77 should be noted. From an input multiplicity of about 20 phage per cell, the average burst size of the progeny ranges from 0.2 to 1.0. This implies that only a minority of the infecting phage genomes are matured. Furthermore, the level of unadsorbed phage is about the same as the output phage since no attempt was made to remove the unadsorbed phage. In a density gradient of the progeny of a heavy-by-heavy cross, the unadsorbed phage, due to their heavy coat protein, are found in fractions of much higher density than the progeny phage. In a heavy-by-light cross, however, the unadsorbed light phage run at the same density as light progeny phage.

RESULTS

Phage crosses are performed at high temperature by infection of nonpermissive *ts dnaB* bacteria with high multiplicities of phage parents one of which carries an isotopic density label. Both parents carry *amber* mutations in gene *P*, providing an additional restriction on λ DNA synthesis. It has been shown that phage matured under these conditions have undergone little DNA synthesis and are highly recombinant. Indeed, recombination has been shown to be necessary for maturation (STAHL *et al.* 1973) when DNA synthesis is blocked.

The results shown in Figures 2 and 3 were unexpected; the broad distribution of recombinant progeny is inconsistent with a simple double or single-strand break—reunion hypothesis. In either case, one would have expected recombinants to appear primarily at a discrete density representing a well-defined transfer of DNA to the recombinant progeny. In fact, the results described suggest long regions of interaction between the two recombining parents.

In spite of the apparent complexity, however, an attempt was made to genetically characterize the unreplicated, recombinant phage progeny.

In order to define the genotypes of both DNA strands of the recombinant phage, their segregation products were determined by examination of the genotypes present among the progeny of single infection. When permissive bacteria are used for the initial infection, plaques containing recombinant phage are identified by subsequent screening. When nonpermissive bacteria are used for the initial infection, only recombinant phage that are functionally wild type yield progeny. It is useful for these analyses to include markers in the genetic crosses that flank the region containing the selected markers and which may be scored independently. Markers in the *cI*, *R*, and *Q* genes were used. The presence of both parental alleles of a given site among the single-burst progeny defines the infecting particle as heterozygous at that site. The presence of only one allele in the burst provides less information since it is clear that some alleles may be lost during infection (WHITE and FOX 1974).

The results of a permissive single-burst analysis of a central fraction of phage progeny from the cross of heavy *cI60 Pam3* by light *cI+ Pam80* (Figure 2, Fraction 65) are presented in Table 1, column 1a. The single-burst analysis was carried out by examination of the progeny found in plaques formed on a permissive (*Su*⁺) indicator. The plaques selected for further examination, each containing only the progeny of a single infecting phage, were those shown by spot test to contain both *am*⁺ alleles. This test includes plaques derived from non-recombinant heterozygotes as well as plaques derived from bursts containing a high proportion of wild-type recombinants.

About $\frac{1}{3}$ of the selected plaques do not contain a high proportion of recombinants, but do contain phage of both parental genotypes. These infecting phage must have carried the *am*⁺ alleles in a nonrecombinant, heterozygous fashion. The remaining $\frac{2}{3}$ contain a high proportion of recombinants and are missing one or both of the parental *am* genotypes. Of the bursts containing wild-type recombinants, at least 75% also yield phage of one of the parental nonrecombinant genotypes. Most of these (80%) are also heterozygous for the unselected *cI* allele; among the bursts containing the *Pam3*⁺ allele it often appears linked to *cI60*. Table 1, column 1b, gives the results of an experiment similar to that described above except that only plaques having a mottled appearance were picked and tested. The plaques selected on this phenotypic basis also support the conclusion that the recombination associated heterozygotes are often sufficiently long to span the region between *C* and *Pam80*.

An analysis of the contents of single plaques formed on the permissive indicator was also performed with phage progeny from the multi-factor cross of heavy *cI60 Pam3 Ram5* by light *cI+ Oam29 Pam80* (Figure 3). The phage from a central fraction were plated on *Q*₁ (*Su*⁺) and a two-strand genotype defined for those phage whose progeny include phage carrying both the *Oam29*⁺ and *Pam3*⁺ alleles or both the *Pam3*⁺ and *Pam80*⁺ alleles (90/720), i.e. phage progeny resulting from an infecting particle with at least one wild-type allele from each parent. The results are listed in Table 2b.

TABLE 1

Compiled two-strand genotypes from cross heavy cI60 Pam3 by light cI⁺ Pam80 (Fraction 65, Figure 2)

I. Phage were plated on Q₁ (Su⁺) and plaques were selected on the basis of (a) those containing both Pam3⁺ and Pam80⁺ (81 out of 535 plaques examined), and (b) those exhibiting a mottled phenotype (45). II. Phage were adsorbed to WD5021 (Su⁻) and plated on Q₁ (Su⁺). (a) Plaques (89) tested without further screening; (b) Plaques (80) selected on the basis of mottled phenotype.

Two-strand genotype			I		II	
<i>cI</i>	<i>Pam3</i>	<i>Pam80</i>	a	b	a	b
<i>c60</i>	+	+				
<i>c60</i>	+	+	4		34	
<i>c</i> +	+	+				
<i>c</i> +	+	+	5		15	
<i>c60</i>	+	+				
<i>c</i> +	+	+	4	1	35	64
<i>c</i> +	+	+				
<i>c60</i>	<i>P3</i>	+	18	6	4	14
<i>c60</i>	+	+				
<i>c60</i>	<i>P3</i>	+	4		1	
<i>c60</i>	+	+				
<i>c</i> +	<i>P3</i>	+	2			
<i>c</i> +	+	+				
<i>c</i> +	<i>P3</i>	+	2			
<i>c60</i>	+	+				
<i>c</i> +	+	<i>P80</i>	13	14		2
<i>c60</i>	+	+				
<i>c60</i>	+	<i>P80</i>	1			
<i>c</i> +	+	+				
<i>c</i> +	+	<i>P80</i>	1			
<i>c60</i>	<i>P3</i>	+				
<i>c</i> +	+	<i>P80</i>	24	10		
<i>c60</i>	<i>P3</i>	+				
<i>c60</i>	+	<i>P80</i>	3			
<i>c</i> +	+	<i>P80</i>				
<i>c60</i>	+	<i>P80</i>		9		
<i>c60</i>	<i>P3</i>	+				
<i>c</i> +	<i>P3</i>	+		5		

One of the more prominent features observed is that although each of the three selected markers *Oam29*, *Pam3*, and *Pam80* is equally frequently heterozygous among the phage containing all three selected *am*⁺ alleles in some configuration, the frequencies vary markedly among phage with a wild-type recombinant strand; 60% of these (17/29) are heterozygous at *Pam80*, 40% (11/29) at *Oam29* (almost all of these were also heterozygous for *Pam80*), but none were found to be heterozygous for *Pam3*. DNA transfection experiments would suggest that we should have found some wild-type recombinants heterozygous for *Pam3* and their absence is not understood (WHITE and FOX, in preparation). A little more than 1/3 of the recombinant-containing bursts were homozygous for

all three selected markers. Again, most of the recombinant-containing bursts are heterozygous for the *cI* allele.

The *R* locus, although physically removed from the selected region by about 15% of the λ chromosome, is found to be heterozygous in 18% (16/90) of the bursts examined. Further, the plaques that are not heterozygous for the *Ram5* allele are as frequently homozygous *Ram5* as *Ram5*+. The *R* heterozygotes show the same frequency of heterozygosity at the *cI* locus as the other selected bursts, about 75% (12/16). It is clear from the genetic composition of the plaques that multiple-strand interruptions can occur on both strands of a given molecule, since many of the heterozygous bursts shown in Table 2b derive from phage multiply recombinant in both strands. The genetic structures suggested by these observations cannot be readily interpreted in terms of simple breakage and joining models, but appear to require multiple-strand interruptions of a composite type or events that result in rectification of limited regions of heteroduplex structures that include heterozygous elements (Figure 1).

It is possible to set an upper limit of the extent of localized loss of genetic information from heterozygotes due to events occurring either during the crosses or after permissive infection. Since the frequency of heterozygosity for the *cI* allele among the bursts described (Table 1, column Ia, and Table 2, column b) is found to be about 75%, events resulting in localized information loss at the *cI* locus could have occurred with a probability no greater than 25%.

A similar analysis may be made from the data in Table 2b with respect to localized marker loss from heterozygotes at the *Oam29* and *Pam80* loci. Among the bursts containing the *Pam3*+ and *Pam80*+ alleles, selected without regard to the character of the *Oam29* allele, the *Oam29* allele is found to be heterozygous in 56% (44/78) of the bursts. The *Pam80* allele, among bursts containing *Oam29*+ and *Pam3*+ is heterozygous in 50% of the bursts. An upper limit on localized (not extending to adjacent alleles) loss of genetic information at the *Oam29* and *Pam80* sites is set at about 50%. Further, by comparison with Table 1a, the presence of the *Oam29* marker does not visibly alter the frequencies of the recombinant and heterozygous classes found among the phage carrying both the *Pam3*+ and *Pam80*+ alleles. It should be noted that among the *Pam3*+ and *Pam80*+ selected bursts, the *Oam29*+ homozygous class (29/78) is significantly more frequent than the *Oam29* homozygous class (5/78). Similarly, among the *Oam29*+ *Pam3*+ bursts, the frequency of *Pam80*+ homozygotes (34/85) is greater than the frequency of *Pam80* homozygotes (10/85).

From examination of plaques derived from infection of *Su*⁺ cells by phage from central fractions of the gradient shown in Figures 2 and 3, the following conclusions are possible: (1) Phage progeny either singly or doubly recombinant in the *O,P* region are very frequently heterozygous for the *am* markers in the selected region. (2) Heterozygous phage progeny either recombinant or nonrecombinant for selected markers frequently (75%) manifest heterozygosity for the *cI* allele and less often (20%) show heterozygosity at the more distant *R* locus. (3) An upper limit on localized loss of genetic information from single strands of heterozygotes has been set at 25% for the *cI* locus and about 50% at the *Oam29* and *Pam80* loci. (4) The abundance of bursts (Table 1, column Ia) in

which there is evidence of loss of the *Pam3* allele without loss of its linked *cl60* allele suggests that events responsible for this loss operate on only a limited portion of the heteroduplex molecule.

The results of single burst experiments performed by a primary infection of Su^- bacteria differ markedly from results obtained with primary infection of Su^+ bacteria. Certainly some differences might have been expected since direct selection on Su^- bacteria requires function from wild-type *O* and *P* genes before replication can occur, and it is known that only one of the two DNA strands of the infecting phage is used for the synthesis of *O* and *P* messenger RNA (OGAWA and TOMIZAWA 1968; SZYBALSKI *et al.* 1970).

The contents of plaques derived from plating nonpermissive infective centers on the permissive indicator have been examined. The results of this analysis are shown in Table 1, columns II a and b. The pattern and frequency of heterozygotes found by direct selection on Su^- differs markedly from that found on Su^+ . Most (85%) of the recombinants selected on Su^- are homozygous for the *Pam*⁺ markers; those few that are heterozygous for one of the *Pam* markers are almost all (19/21) heterozygous for the *Pam3* allele. This is to be contrasted with the result obtained by infection of Su^+ with the same population of phage; most of the phage yielding a wild-type recombinant progeny strand were found to be heterozygous for one or the other of the *Pam* alleles and these were about as frequently heterozygous for *Pam80* as *Pam3*.

A similar comparison of Su^+ versus Su^- initial infection may be made for the recombinants formed in the cross heavy *cl60 Pam3 Ram5* by light *cl+ Oam29 Pam80*. The results obtained by permissive plating of a central fraction of the gradient from this cross (Figure 3, fraction 75) shown in Table 2b may be contrasted with the results of liquid culture single bursts on Su^- of phage from this same fraction shown in Table 2a. The titer of recombinants homozygous for the *am*⁺ alleles is similar in both cases. The classes heterozygous for *am* alleles, however, are not similar.

On Su^+ , none of the bursts containing a high proportion of wild-type double recombinants are heterozygous for the *Pam3* allele, many are heterozygous for both *Oam29* and *Pam80*, a few are heterozygous for *Pam80* alone, and only one heterozygous for *Oam29* alone. In contrast, on Su^- 20% of the bursts are heterozygous for *Oam29* alone, 15% for *Pam3* alone, only one burst for both *Oam29* and *Pam80*, and none are heterozygous for *Pam80* alone.

Results similar to these are found upon examination of the products of transfection with artificially constructed heteroduplex heterozygotes of genotype $O + P80$

$+ P3 +$ into either Su^+ or Su^- cells. In fact, the phage recombinants from these central fractions have a pattern of heterozygosity suggesting that they are composed of roughly equal proportions of phage with both orientations $\frac{+ P3 + r}{O + P80 1}$ and $\frac{+ P3 + 1}{O + P80 r}$. The primary products of recombination appear to be nonre-

combinant heterozygotes on which subsequent operations give rise to the ob-

TABLE 2

*Compiled two-strand genotypes of phage from cross of heavy cI60 Pam 3 Ram 5
by light cI⁺ Oam29 Pam80 (Fraction 75, Figure 3)*

(a) Single burst in liquid culture on WD5021 (Su⁻). Lysis by lysozyme and chloroform, progeny plated on Q₁ (Su⁺) and genotyped. (b) Genetic composition of those plaques formed on permissive indicator Q₁ (Su⁺), among 720 screened, that were found to contain O⁺ and Pam3⁺ or Pam3⁺ and Pam80⁺.

Two-strand genotype cI	Oam29	Pam3	Pam80	Ram5		a	b
c60	+	+	+	+	$(\frac{R}{R})$	9 (13)	3 (5)
c60	+	+	+	+			
c+	+	+	+	R	$(\frac{+}{R})$	1 (0)	0 (1)
c+	+	+	+	R			
c60	+	+	+	+	$(\frac{R}{R})$	1	0
c60	+	+	+	R			
c60	+	+	+	+	$(\frac{R}{R})$	3 (3)	0 (1)
c+	+	+	+	+			
c60	+	+	+	+	$(\frac{+}{R})$	0	1
c+	+	+	+	R			
c60	+	+	+	R	$(\frac{+}{R})$	2 (1)	0
c+	O	+	+	+			
c60	+	+	+	R	$(\frac{c+}{c60})$	1 (1)	0
c+	O	+	+	R			
c+	+	+	+	+	$(\frac{+}{R})$	1	0
c+	O	+	+	+			
c60	+	+	+	+	$(\frac{+}{R})$	3	1
c+	O	+	+	+			
c60	+	+	+	+	$(\frac{+}{R})$	1	3
c+	O	+	P80	+			
c60	+	+	+	R	$(\frac{+}{R})$	0	6 (1)
c+	O	+	P80	R			
c60	+	+	+	+	$(\frac{R}{R})$	2 (2)	0
c60	+	P3	+	R			
c+	+	+	+	+	$(\frac{+}{R})$	1	0
c60	+	P3	+	+			
c+	+	+	+	R	$(\frac{+}{R})$	1 (1)	0
c60	+	P3	+	+			
c60	+	+	+	+	$(\frac{R}{R})$	0	4 (1)
c+	+	+	P80	+			
c60	+	+	+	+	$(\frac{R}{R})$	0	1 (1)
c60	+	+	P80	+			
c60	+	P3	+	+	$(\frac{R}{R})$	0	4 (3)
c+	+	+	P80	+			
c60	+	P3	+	R	$(\frac{R}{+})$	1	2 (2)
c+	+	+	P80	+			
c60	+	P3	+	+	$(\frac{R}{+})$	2 (2)	2 (2)
c60	+	+	P80	+			

TABLE 2—Continued

<i>c60</i>	+	<i>P3</i>	+	+	$(\frac{R}{R})$	6 (8)
<i>c+</i>	<i>O</i>	+	+	+		
<i>c+</i>	+	<i>P3</i>	+	+	$(\frac{R}{+})$	2 (1)
<i>c+</i>	<i>O</i>	+	+	+		
<i>c60</i>	+	<i>P3</i>	+	+	$(\frac{R}{R})$	4 (4)
<i>c+</i>	<i>O</i>	+	<i>P80</i>	+		
<i>c60</i>	+	<i>P3</i>	+	<i>R</i>	$(\frac{+}{R})$	4 (1)
<i>c+</i>	<i>O</i>	+	<i>P80</i>	+		
<i>c+</i>	<i>O</i>	+	+	<i>R</i>		1
<i>c+</i>	<i>O</i>	<i>P3</i>	+	<i>R</i>		
<i>c+</i>	<i>O</i>	+	+	+	$(\frac{R}{R})$	1 (1)
<i>c+</i>	<i>O</i>	+	+	+		
<i>c+</i>	<i>O</i>	+	+	<i>R</i>		1
<i>c+</i>	<i>O</i>	+	<i>P80</i>	<i>R</i>		
<i>c60</i>	<i>O</i>	+	+	+		1
<i>c+</i>	<i>O</i>	+	+	+		
<i>c60</i>	+	+	<i>P80</i>	+		3
<i>c60</i>	+	+	<i>P80</i>	+		
<i>c+</i>	+	+	<i>P80</i>	+	$(\frac{R}{R})$	1 (1)
<i>c+</i>	+	+	<i>P80</i>	+		
<i>c60</i>	+	+	<i>P80</i>	+	$(\frac{R}{R})$	3 (1)
<i>c+</i>	<i>O</i>	+	<i>P80</i>	+		
<i>c60</i>	+	+	<i>P80</i>	+		1
<i>c60</i>	<i>O</i>	+	<i>P80</i>	+		

served recombinants (WHITE and Fox, in preparation). In these experiments the operations appear to occur in the recipient cells.

To examine the various heterozygous recombinant phage classes as a function of relative parental DNA contribution, the progeny from fractions of varying density (Figure 3) were analyzed by examining single bursts derived from cells nonpermissive (*Su*⁻) for the parental geotypes. It was found that heterozygote classes exhibit differences in frequency that are characteristic of the density species among which they are found (Table 3). Heterozygous recombinant phage from a heavy region of the gradient (Fractions 62, 64) are often heterozygous for *Pam3*. Similarly, heterozygous recombinant phage from a less dense region (Fractions 86, 87) are most often heterozygous for the *Oam29* allele. Recombinant heterozygotes from a central fraction are heterozygous for either *Pam3* or *Oam29*. These asymmetries are interpreted to be the result of an asymmetry in the formation of heteroduplex overlaps and are discussed in some detail in an earlier publication (WHITE and Fox 1974). The *cl*⁺/*cl60* heterozygote frequency also varies as a function of density and may reflect the fact that heterozygosity at the *cl* locus is more tightly linked to heterozygosity at the *Oam29* locus than to heterozygosity at the *Pam3* locus. Phage exhibiting heterozygosity at the *R* locus seem to be equally frequent among all density species.

TABLE 3

*Compiled two-strand genotypes of phage from cross of heavy cI60 Pam3 Ram5
by light cI⁺ Oam29 Pam80 (Figure 3)*

Single burst in WD5021 (su⁻). Lysis by lysozyme and chloroform, progeny plated on Q₁ (su⁺) and genotyped.

	<i>cI</i>	Two-strand genotype				Heavy by light cross; number found of each two-strand genotype in bursts from fraction			
		<i>Oam29</i>	<i>Pam3</i>	<i>Pam80</i>	<i>Ram5</i>	62,64 (51)	75 (47)	80 (50)	86,87 (51)
(Total bursts examined)									
1.	<i>c60</i>	+	+	+	+	14	9	15	4
	<i>c60</i>	+	+	+	+				
2.	<i>c60</i>	+	+	+	<i>R</i>	19	13	7	4
	<i>c60</i>	+	+	+	<i>R</i>				
3.	<i>c+</i>	+	+	+	+				3
	<i>c+</i>	+	+	+	+				
4.	<i>c+</i>	+	+	+	<i>R</i>		1		3
	<i>c+</i>	+	+	+	<i>R</i>				
Frequency of genotypes 1-4						0.65	0.49	0.44	0.27
5.	<i>c60</i>	+	+	+	+	2	1	1	1
	<i>c60</i>	+	+	+	<i>R</i>				
6.	<i>c60</i>	+	+	+	+	3	3	5	1
	<i>c+</i>	+	+	+	+				
7.	<i>c60</i>	+	+	+	<i>R</i>			3	3
	<i>c+</i>	+	+	+	+				
8.	<i>c60</i>	+	+	+	<i>R</i>		3		2
	<i>c+</i>	+	+	+	<i>R</i>				
9.	<i>c60</i>	+	+	+	+				1
	<i>c+</i>	+	+	+	<i>R</i>				
Frequency of genotypes 5-9						0.10	0.15	0.18	0.16
10.	<i>c60</i>	+	+	+	+		3	5	7
	<i>c+</i>	<i>O</i>	+	+	+				
11.	<i>c60</i>	+	+	+	+		1		1
	<i>c+</i>	<i>O</i>	+	+	<i>R</i>				
12.	<i>c60</i>	+	+	+	+				1
	<i>c60</i>	<i>O</i>	+	+	+				
13.	<i>c60</i>	+	+	+	<i>R</i>	1	2	4	2
	<i>c+</i>	<i>O</i>	+	+	+				
14.	<i>c60</i>	+	+	+	<i>R</i>		1		4
	<i>c+</i>	<i>O</i>	+	+	<i>R</i>				
15.	<i>c+</i>	+	+	+	+		1		2
	<i>c+</i>	<i>O</i>	+	+	+				
16.	<i>c+</i>	+	+	+	<i>R</i>			2	3
	<i>c+</i>	<i>O</i>	+	+	<i>R</i>				
17.	<i>c+</i>	+	+	+	<i>R</i>		1		1
	<i>c60</i>	<i>O</i>	+	+	<i>R</i>				
Frequency of genotypes 10-17						0.02	0.19	0.22	0.41
18.	<i>c60</i>	+	+	+	+	3			
	<i>c60</i>	+	<i>P3</i>	+	+				
19.	<i>c60</i>	+	+	+	+	3	2		
	<i>c60</i>	+	<i>P3</i>	+	<i>R</i>				

TABLE 3—Continued

20.	<i>c60</i>	+	+	+	<i>R</i>	1		2	
	<i>c60</i>	+	<i>P3</i>	+	+				
21.	<i>c60</i>	+	+	+	<i>R</i>	1	2	1	1
	<i>c60</i>	+	<i>P3</i>	+	<i>R</i>				
22.	<i>c</i> +	+	+	+	+		1		
	<i>c60</i>	+	<i>P3</i>	+	+				
23.	<i>c</i> +	+	+	+	+	2	1	1	2
	<i>c60</i>	+	<i>P3</i>	+	<i>R</i>				
24.	<i>c</i> +	+	+	+	<i>R</i>		1	1	
	<i>c60</i>	+	<i>P3</i>	+	+				
25.	<i>c</i> +	+	+	+	<i>R</i>	1		1	1
	<i>c60</i>	+	<i>P3</i>	+	<i>R</i>				
Frequency of genotype 18-25						0.22	0.15	0.12	0.08
26.	<i>c60</i>	+	+	+	+		1	1	2
	<i>c</i> +	<i>O</i>	+	<i>P80</i>	+				
27.	<i>c60</i>	+	+	+	<i>R</i>	1			1
	<i>c</i> +	<i>O</i>	+	<i>P80</i>	+				
28.	<i>c60</i>	+	+	+	<i>R</i>				
	<i>c</i> +	<i>O</i>	+	<i>P80</i>	<i>R</i>				
29.	<i>c</i> +	+	+	+	<i>R</i>				
	<i>c</i> +	<i>O</i>	+	<i>P80</i>	<i>R</i>				
30.	<i>c</i> +	+	+	+	<i>R</i>				1
	<i>c</i> +	<i>O</i>	+	<i>P80</i>	+				
Frequency of genotypes 26-30						0.02	0.02	0.02	0.08
31.	<i>c60</i>	+	+	+	<i>R</i>			1	
	<i>c</i> +	<i>O</i>	<i>P3</i>	+	+				
Frequency of genotype 31								0.02	

Among phage that give rise to *O,P* recombinants, *R* heterozygotes occur with a frequency of 22%. Most of these heterozygotes are also heterozygous for *cI*, preserving parental arrangement of the *cI* and *R* alleles. These observations are in agreement with those of Russo (1973) and support the suggestion that the heterozygous region present in primary recombinants is often continuous throughout the *cI-R* interval.

The analysis of a cross in which a *Q* marker, located between the *O,P* region and *R*, is introduced provides support for the suggestion that the interval joining a pair of heterozygous alleles contains a substantial proportion of hybrid DNA. The progeny of a cross between heavy *cI857 Oam29 Pam80* and light *cI60 Pam3 Qam73 Ram5* were distributed on an equilibrium cesium formate gradient and the two-strand genotypes examined by nonpermissive single-burst analysis (WHITE 1971). Among the bursts showing heterozygosity of the *Ram5* allele, 90% (29/32) are also heterozygous for the *Qam73* allele. However, only 50% (29/54) of the bursts heterozygous for the *Qam73* allele are also heterozygous for the *Ram5* allele, suggesting that the heterozygosity is indeed polar from the region of selected recombination and further suggesting that coincident heterozygosity of a pair of genetic markers implies heterozygosity throughout the interval separating them. In sum, the finding of a high frequency of *Qam73*

heterozygotes supports the notion that the *Ram5* heterozygotes found associated with the *O,P* recombinants represent heterozygosity extending from *cl* to *R*.

The density distributions of the various classes of recombinant heterozygotes provide further support for the conclusion drawn previously that the phage heterozygous for a selected marker are asymmetrically distributed across the density gradient. In this case, among heavy recombinants 66% are heterozygous for *O*, with 20% giving only progeny that are wild type for the *O* and *P* alleles, while among light recombinants 41% are heterozygous for *Pam3* and 48% homozygous wild type for the *O* and *P* alleles. A reservation must be noted, however, since the *Q* allele, unlike the *R* allele, is a selected marker under these conditions. *Qam73* homozygotes are restricted in the *Su⁻* cell; however, since DNA replication occurs in *Q*-defective phage all *Q/Q+* heterozygotes as well as *Q+* homozygotes should grow on *Su⁻*.

DISCUSSION

Making use of density-labeled phage and conditions of blocked DNA synthesis, we have been able to examine the allele composition of recombinant bacteriophage λ particles as a function of their atom contributions from each of their parents. Among the recombinant progeny of a heavy-by-light cross, the phage heterozygous for a particular selected marker are asymmetrically distributed with regard to the relative material contribution of the two parents. This asymmetry is observed under conditions in which both the bacterial (*Rec+*) and phage (*Red+*) recombination systems are operative. STAHL and STAHL (1974) have shown that the same asymmetry is observed when the *Red* system is operative alone.

Heterozygosity often extends over a substantial fraction of the λ genome. The frequency of heterozygosity at *R* among the *Su⁻* single bursts is about the same as that found among the *Su⁺* single bursts, about 20%, and the frequency of heterozygosity at the *cl60* locus, about 40%, is also similar to that found among *Su⁺* single bursts.

Phage particles containing equal atom contributions from both parents (hybrid in density) often contain alleles derived from both parents in a portion of the genome that is less than 20% removed from the *R* end of the phage DNA molecule. As shown in Table 1, at least 15% of the hybrid progeny have demonstrable allele contributions from both parents in the *cl-P* region, and as shown in Table 2 at least 12% of the hybrid progeny fall into this category. Examination of the products of transfection with artificially constructed heteroduplex heterozygotes demonstrates the frequent loss of all of the alleles present on one strand of an infecting molecule (WHITE and FOX, manuscript in preparation), and suggests that substantially more than 12% to 15% of the molecules, hybrid in density, are in reality heterozygous in the *O,P* region of the genome. If we assume that these recombinants are the product of a single phage-phage interaction, then their atom and allele compositions are consistent with heteroduplex structures extending over a region including most of the phage genome.

Phage particles of hybrid density give rise to about the same number of wild-type recombinants whether the initial infection is in an *Su⁻* or an *Su⁺* host. Com-

parison of the allele composition of bursts containing a high proportion of wild-type phage reveals differences between Su^+ and Su^- in the kinds and numbers of heterozygotes observed. The overall frequency of heterozygosity observed is substantially lower after Su^- infection than after Su^+ infection. Furthermore, some classes evident after Su^+ infection are absent or nearly absent after Su^- infection and *vice versa*. Presumably some or perhaps all of these differences can be accounted for by the replication requirement of wild-type recombinant messenger-producing strands after Su^- infection. The absence of λ DNA replication in Su^- bacteria infected with a nonrecombinant heterozygote might be expected to result in a substantial *in vivo* persistence time, thus increasing the probability of rectification events.

We believe that the experiments presented here provide more detailed support for the proposal that recombination between closely linked markers, including both single and multiple genetic events, is primarily the result of reduction to homozygosity of base-pair mismatch heterozygotes. This process provides a molecular basis for understanding the phenomenon of high negative interference observed in bacteriophage λ crosses involving closely linked markers (AMATI and MESELSON 1965), and, possibly, for understanding the nature of "gene conversion" observed in genetic crosses with many higher organisms.

High negative interference would be expected if the limiting step in genetic recombination between closely linked markers were the inclusion of the markers within the heteroduplex region joining the parental contributions of the recombinant DNA molecules. Recombinants would result from the reduction to homozygosity of base-pair mismatches present in the heterozygous region. The observed classes of heterozygotes and their high frequency among recombinants further suggests that the events resulting in reduction to homozygosity are often confined to short regions of the heteroduplex DNA. Under these circumstances multiple genetic events in short intervals would be the rule rather than the exception. The details of "gene conversion" by base-pair mismatch repair have already been suggested in the early models proposed by MESELSON (1965), by WHITEHOUSE (1963), and by HOLLIDAY (1964). Our experiments, as well as unpublished experiments by JUDY WILDENBERG, provide evidence that at least in one biological system such events actually occur. It should be stressed, however, that these experiments do not provide a proper test for the occurrence, or significance in recombination, of single-strand insertion heterozygotes of the type that occur in bacterial transformation. The matured recombinants examined here may be limited to those that derive from DNA molecules that are recombinant for outside markers and therefore satisfy the maturation requirement of longer-than-unit-length molecules (STAHL *et al.* 1973).

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Corresponding editor: F. W. STAHL