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Properties of the Merozygotes Associated with a Sulfonamide-Resistant Mutation in Pneumococcus

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PROPERTIES OF THE MEROZYGOTES ASSOCIATED WITH A SULFONAMIDE-RESISTANT MUTATION IN PNEUMOCOCCUS

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PREFACE

I wish to take this opportunity to express my appreciation to all those who made this work, and the graduate education which it represents, possible.

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SUMMARY

Unstable prokaryotic strains arise occasionally under a variety of circumstances and with a variety of properties which make them amenable to genetic and physiological analysis at different levels of precision. A review of a number of such unstable systems, initially identified on the basis of a regular segregation to phenotypically non-parental types, has led to the recognition of a series of criteria for the systematic analysis of such strains. These criteria are applied to a sulfanilamide-resistant mutant of pneumococcus which regularly segregates to strains sensitive to sulfanilamide and resistant to p-nitrobenzoic acid, another analogue of paraminobenzoic acid.

From a combination of information gathered by previous workers and new data, it is confirmed that this sulfanilamide-resistant mutant, sul-c, is a genetically determined variant whose segregants are stable and indistinguishable from the strain wild type at the same locus (c^+) by criteria of drug-resistant phenotype, behavior as recipient and donor in DNA-mediated genetic crosses (transformation), and inability under a variety of conditions to give rise to the original sul-c type. By analysis of genetic crosses mediated by DNA from the c strain and from complex resistant strains designated cd, it is established that the segregants arise by separation of the mutant and wild type determinants pre-existing in the parental strain, rather than by some rapid reverse mutation. Thus strains containing sul-c are merozygotes, partial diploids for a region of genetic material encompassing the c locus and closely linked sites. Linkage of the c marker to the marker governing streptomycin resistance can be demonstrated although the latter marker is never seen in an unstable form. Consequently, the basis for the instability must lie within the pneumococcal chromosome, rather than on an extrachromosomal genome fragment. The complex cd types may assume a variety of properties with respect to frequency of wild type segregation and frequency of c, d, and d^+ markers in the DNA of the strains. These properties provide a basis for designating three classes of cd types, which have the same properties whether generated from a transformation of a d recipient

by c donor DNA or of a c recipient by d donor DNA. The various marker frequencies in the three classes probably result from a combination of an increased dosage of the genetic loci involved and an altered efficiency of integration of these loci in transformation.

The segregation process itself was investigated. It was established that a given c-containing strain will always give segregants at the same frequency of the total population, accumulation of these segregants with time being impossible to demonstrate. However the segregation frequency of a given strain is characteristic of the strain, and may vary among independent isolates. Perturbations of general cellular metabolism such as nutritional supplementation, changes in growth temperature, or repeated freezing and thawing had no consistent, progressive, and readily interpretable effect on segragation frequency. However, treatment with ultraviolet light or mitomycin C at doses permitting 80 to 90% survival of the total cell population resulted in a pronounced stimulation in the number of segregants. This stimulation failed to persist as cell growth resumed, suggesting that the process stimulated was readily reversible. Treatment with acridine orange gave the opposite effect, a depression in the frequency of segregants. From the known effects of these three agents on DNA and on processes known to be mediated by recombination, it is proposed that the mechanism of segregation involves a recombination step.

Two models are presented which account for the known properties of the c and cd strains. One involves a region of triple-strandedness around the c locus; the other involves a duplication in tandem of the c region. An attempt was made to distinguish the models on the basis of physical properties of the DNA of the strain: buoyant density, affinity for methylated albumin-kieselguhr, and ability to become inactivated by binding formaldehyde. In none of these tests was the c marker affected differently from markers known to be stable, although a triple-stranded structure might be expected to behave abnormally. However the presumed small size of the c region makes it possible that the inability to differentiate c was owing to insufficient sensitivity of the methods rather than to a strictly double-stranded structure. Data of my colleague, Dr. S.V.S. Kashmiri, is cited in which heteroduplex DNA molecules

bearing the c marker is one strand and its wild type allele in the other showed a markedly depressed ability to transform c and markers linked to it. Heteroduplexes in which both strands contained c or both strands contained c^+ did not show this depression. This evidence was interpreted as favoring the tandem duplication model, which is proposed as an hypothesis on which to base further experimentation.

The specific nature of the c mutation and the mechanism by which the mutation generates merozygosis are speculated upon, as is the basis for the mutant phenotype, the detailed mechanism of segregation, and the relationship of c to other unstable strains of prokaryotes.

TABLE OF CONTENTS

I
INTRODUCTION

Much of what is known of the genetics of higher organisms relies on studies of the assortment and segregation of parental traits in their offspring. When two strains differing in certain unit traits are crossed, the hybrid offspring exhibits some of those traits and not others. When its gametes form, the contributions of the two parents which have coexisted in the hybrid will become reassorted in chromosomal groups, each gamete receiving only half of the available information.

This phenomenon of segregation, so integral a part of the reproductive cycle of eukaryotic organisms, has been practically unknown in prokaryotes. Bacteria and the blue-green algae reproduce by binary fission, resulting in daughter cells which are exact copies of the mother. This life style has certain advantages for the microbial geneticist: pure strains are easily isolated and maintained free of genetic contamination; biochemical properties of large populations of cells may be taken as representing those of the cell giving rise to the population; variants arising in a line are usually the result of mutation.

Under some circumstances a sexual cycle may be imposed upon this asexual mode of growth. Exchange of genetic material may take place by means of elaborate cellular surface adaptations permitting conjugation, that is, contact between cells of opposite mating type, and subsequent transfer of DNA from one to the other. Facultatively lysogenic viruses may mediate exchange (transduction) by acting as vectors for host DNA. Perhaps the most straightforward of all processes of exchange is transformation, in which pure DNA isolated from one strain can introduce its characteristics into a second strain when that strain is in a state competent to pick up the exogenous DNA. These three phenomena differ from sexual cycles of higher organisms in a number of important respects. Specifically the two parental strains make unequal contributions to the

zygote, one (recipient) contributing its entire genome, the other (donor) only a fragment of its gene complement. Also the merozygote (a term denoting a cell which is diploid for only part of its DNA) usually persists only for a short time, the donor DNA rapidly becoming either incorporated into the recipient genome or eliminated. Thus segregation in the classical sense usually occurs immediately by a process analogous to haploidization.

There are certain circumstances in which the contributions of both parents governing the same genetic determinants can continue to exist in a single cell. This coexistence may be relatively transient, as in abortive transduction in Salmonella, where the donor material fails to become integrated and does not replicate, yet can express some of its genetic functions (flagellar synthesis, ability to use a certain sugar as sole source of carbon) in one of the two daughter cells per generation (Ozeki, 1956; Stocker, et al., 1953). A more permanent merozygosis results when episomes or lysogenic phages carry a segment of host DNA. If sufficient episomal or phage functions remain, this modified material can replicate and express the function determined by that DNA segment whether remaining free in the cytoplasm or integrated into the continuity of the host genome. In this latter state it is effectively a duplication of the corresponding host gene, two doses instead of the usual single dose. Only when the episomal particle is lost does such a strain lose its merozygosis.

A third--and until recently largely hypothetical--sort of merozygosis can occur by duplication independent of any episomal factor. Duplications in higher organisms have been known for many years. In prokaryotes such a duplication might arise through errors in replication or recombination. Its persistence is limited only by the frequency with which replication or recombination acts to reverse the original event.

For any merozygotic strain to be recognized and studied, it must meet two basic requirements: the extra determinant, whether brought in by phage or episome or generated within the host, must bear information for a function that may be selected in the presence of a large number of cells lacking the function; and the extra determinant must segregate at detectable frequency, losing simultaneously the merozygosis and the

extra function.

Despite the methodological difficulties of handling organisms which are unstable and too small to examine individually, a number of merozygotic systems in prokaryotes and viruses have been identified and characterized. Perhaps the most familiar of these are the heterozygotes of the T-even bacteriophages.

The T-even phage heterozygotes: An illustration of the nature of the problem

In 1951 Hershey and Chase reported the results of crosses of phage T2 in which mutant phages gave rise to clear plaques and wild type phages to turbid plaques. Among the offspring of each cross, they observed, at a frequency of about 2%, plaques that were neither clear nor turbid but mottled. Analysis of phages in the mottled plaques showed them to be predominantly either mutant or wild type, with again about 2% able to give rise to mottled plaques. After establishing that a single particle was responsible for this mottling and thus must carry both mutant and wild type alleles for the locus, Hershey and Chase analyzed the segregants genetically. The mottled plaques, inferred to arise from phage heterozygotes, so-called "hets," segregated for allelic pairs, double hets segregating only into parental marker combinations, unless the two sites were closely linked. Levinthal extended these studies by noting (Levinthal, 1954) that in three-factor crosses, particles selected as het for the middle marker were usually recombinant for the outside markers. He proposed a model for the structure of the het region: a partial diploid containing a small heteroduplex piece of biparental origin. Such internal hets would be continuously generated as intermediates in recombination, and continuously dissociated by semi-conservative replication. This explains their detection early in the course of phage infection and their failure to accumulate with time. Similar heteroduplexes result from mutagenesis of phages (Green and Krieg, 1961). By making certain assumptions about the regularity of segregation, one can calculate that

heterozygotes can account for all the recombinants eventually emerging from a cross.

Further studies by Edgar (1961) led to the description of a second sort of het which arose in selections for very unlikely recombinations. This class of het was not recombinant for outside markers and thus seemed more likely to be a true diploid. Its extent correlated well with the map distance over which high negative interference operates (Chase and Doermann, 1958). Doermann and Boehner later correlated the density of phage particles, as measured in a cesium chloride gradient, with the number of markers associated with the heterozygosity, and found that longer het regions were associated with denser phage particles, presumably containing more DNA (Doermann, 1963; Doermann and Boehner, 1963, 1964). Genetic mapping of the T-even phages at this time suggested that linear phage DNA could give rise to a circular linkage group by having the chromosome ends circularly permuted. When evidence for terminal redundancy of these circularly permuted chromosomes was found, it was easy to explain the second class of hets as terminal redundancy hets, in which the repeated segment carried two different alleles in double-stranded DNA. Stahl and co-workers used elegant techniques to generate preferentially one or the other het type (Shalitin and Stahl, 1965; Stahl, Modersohn, Terzaghi, and Crasemann, 1965).

Both terminal redundancy and internal hets are structures inferred from segregation patterns of progeny at least one growth cycle removed from the original particle containing the heterozygosis. Weil, Terzaghi, and Craseman (1965) found a more persistent heterozygote of phage T4. Two overlapping deletion mutants of the rII region were crossed, one in the rIIA and one in the rIIB cistron. Since the deletions overlap, no recombinants for these mutants arise, and internal hets are suppressed because of the inability of a non-deletion segment of one to base pair with a deletion segment of the other. Nevertheless these particular mutants complement each other. One, 1589, has a deletion extending into the left end of the B region, which is not essential for the expression of B function. Thus the only hets which can be generated are of the terminal redundancy type. Among these hets, about one in

10^6 can not only infect the non-permissive host for one cycle, but also forms plaques on that host. This requires that the heterozygosity persist for at least as many generations as are needed to infect and lyse a group of K12 cells. Parma and Ingraham in Hershey's laboratory (Hershey, 1969) have further analyzed these strains. Tandem duplications arising by illegitimate crossing over and segregating by unequal crossing over would have the properties adequate to explain the observed phenomena of the persistent hets. An additional assumption must be made: that the duplication of the rII segment be accompanied by a deletion in another segment to satisfy the restriction on the amount of DNA the phage head can accomodate. In testing this hypothesis Parma and Ingraham found that populations of hets segregate to both parental types and inviable particles. The frequency of segregants was 15 to 45% of the total particles and was characteristic for each line of hets, with parental segregants and inviable particles being of about equal frequency. The interpretation is that each segregant is formed by an unequal recombination which simultaneously generates an inviable triplication. That the original duplications are accompanied by deletions is suggested from the observation that segregants of the two parental phenotypes generate persistent hets more readily than the original parents do. The implication is that they already contain the deletion necessary to allow the persistent het to fit in the phage head.

In this brief summary of the process by which the structure of the T-even virus hets became understood, I have attempted to illustrate the kinds of difficulties which surround the problem of prokaryotic instability. Imaginative genetic methods, sophisticated physicochemical measurements, and empirical manipulations of physiological conditions have all played a part in leading to the conclusions. But even in viral systems there are certain advantages for the investigator which bacterial systems do not afford. The small and uniform size of the viral genome makes physical and genetic deviations much more accessible to the investigator. Bacterial chromosomes are many times as large, and unless the most exquisite delicacy is used in their isolation, they are broken into pieces of variable length and genetic content. Also physiological

heterogeneity of a cloned population of bacterial cells may be greater than that of a similar phage population, owing to the greater functional complexity of bacteria and a less homogeneous environment for growth.

The purpose of the thesis

The work to be described in this thesis is an attempt to clarify the genetic and physiological properties of an unstable pneumococcal mutant strain resistant to sulfanilamide. I have extended the body of work on the genetic behavior of this mutant. Its map position has been established. Its ability to serve as both a donor and recipient in transformation and the nature of the transformation products are investigated. Arguments are made, based on both old and new evidence, leading to the conclusions that the mutant strain owes its instability to a merozygosity inseparable from the mutation itself, that segregation to stable wild type occurs from a separation of the alternative alleles in the redundant region, and that the merozygosity occupies a unique position in the chromosomal continuum of the strain (Chapter III). Physiological studies on how various perturbations of the growth environment affect the characteristic segregation frequency lead to the hypothesis that segregation is mediated by an intrachromosomal recombination event (Chapter IV). Alternative models for the physical alteration of the DNA, derived from the observed properties of the mutant strain are proposed and tested using centrifugation of the DNA in cesium chloride density gradients, elution from methylated albumin-kieselguhr columns, and inactivation with formaldehyde. Additional evidence obtained by Dr. S.V.S. Kashmiri is cited, which together with my data strongly tends to favor the model of a tandem duplication as the structural basis of the mutation (Chapter V).

Before I begin to describe the experimental results, I would like to summarize briefly what is known about other unstable bacterial mutant strains.

An historical perspective

In the early studies of the conjugation system of Escherichia coli, Lederberg noticed exconjugant clones not only of the two parental types, but also mosaic clones which showed sectors of one parent type in a colony of the other type. Markers able to display such variegation included fermentation of maltose (Lederberg, 1949), xylose, mannitol (Lederberg, 1951), lactose (Lederberg, 1949), galactose (Lederberg, 1950), and resistance to streptomycin (Lederberg, 1951) and T1 phage (Lederberg, 1949). By developing some media in which the merozygote could be maintained, and others permitting growth of segregants, Lederberg established that segregation was highly aberrant and seemed to result from preferential elimination of one of the parental contributions. Half of the segregants appeared to be lethals (Lederberg, 1950). Segregants were pure and thus were usually considered as haploid, rather than homozygous diploid, although some of the latter were identified on the basis of their rapid regeneration of variegated clones. Micromanipulation studies on individual variegated cells and their progeny (Zelle and Lederberg, 1951) showed a high degree of inviability among the offspring. Segregation was stimulated by doses of ultraviolet light low enough to cause only 5 to 10% cell death (Lederberg, 1951; Lederberg, Lederberg, Zinder and Lively, 1951). Since this work was reported before the sexual polarity of E. coli was known, there is some question as to what the proper interpretation ought to be. Nevertheless the observation is reproducible and may result from a prolonged stabilization of the donor DNA in the recipient cytoplasm without its being integrated.

In 1954 Nelson and Lederberg took account of the new-found sexual differentiation of E. coli. In application to segregation studies they reported that the F^+ contribution to the merozygote is preferentially lost. But the partial diploid strains segregate independent of the presence or absence of F itself (Campbell, 1969). Anderson in 1957 and 1958 described micromanipulation studies in which single zygotes yielded

recombinant progeny for up to nine divisions after the conjugation event, and rarely bred true before three divisions. Lederberg (1957) in similar experiments found pure clones after only two divisions in 75% of the zygotes but his donor strain was of the opposite donor polarity from the one used by Anderson (Curtiss, 1969). In any event the partial diploids were unstable over much longer periods, and thus cannot be considered normal intermediates in conjugation.

The interpretation that the merozygosis is a stabilization of the donor DNA fragment in the recipient cytoplasm in a non-integrated form is supported by Low's observations (Low, 1968) that in conjugations where the recipient is a recombination-deficient strain of the recA, or "reckless", type, prototrophs for a selected marker are prototrophic for all nearby unselected markers, whether they originated in the donor or recipient. Since in rec^+ crosses the frequency of co-inheritance of two markers is determined by their frequency of recombination and is dependent on the degree of linkage of markers, Low took his results to imply a failure of the donor fragment to recombine with the recipient, as well as a maintenance of its capacity for independent replication. Thus a merozygosity of the sort similar to F' strains can be established.

In the transduction system using phage P1 Lennox (1958) observed prolonged instability of transductants with respect to both selected and unselected markers. Such instability can persist through five passages, equivalent to 150 cell generations. It seems to be specific for fermentation (catabolic) markers. When selection was based on amino acid requirement (anabolic), selected markers were rapidly stabilized, although unselected markers continued to segregate. Luria, Adams, and Ting (1960) found that P1 transduced the lac^+ marker from E. coli donor to E. coli recipient giving stable transductants, but that if the recipient were Shigella dysenteria, the transductants were predominantly variegated and unstable. The latter are thought to result from integration of the defective transducing particle at its locus for lysogeny leaving the recipient lac locus (of uncertain homology between the two species) intact, and effectively producing a duplicated and heterologous lac region.

"Mutational heterozygote" is the term used to describe the phage structure in which one DNA strand has been affected at a particular locus by mutagenic treatment and the other remains unaltered. Such a structure was inferred by Pratt and Stent (1959) as the basis for their observation that T2 or T4 phages treated with 5-bromodeoxyuridine give rise to mixed clones of mutant and wild type phages. They obtained the maximum heterozygote frequency if the minimum amount of replication were allowed to occur after mutagenesis, evidence supporting their interpretation that the heterozygote segregates on replication. However not all mutagens induce mutational heterozygotes of such classical behavior. Green and Krieg (1961) exposed T4 phages to ethylmethanesulfonate, a divalent alkylating agent, and found mutants in mixed clones. But the proportion of the clone that was mutant was variable, and did not correspond to a simple model of segregation at the first replication. Assuming no delayed reproduction of phages carrying the mutant locus, one must postulate a delayed mutagenesis, in which the initial action of the mutagen is required for the subsequent generation of a mutant phage several cycles of replication removed from the primary event (Green and Krieg, 1961).

Revertibility by base analog as opposed to acridine dyes or ultraviolet light is an accepted criterion of the original mutation being a base substitution rather than an insertion or deletion. Demerec using Salmonella (Demerec, 1960) and Magni using Saccharomyces (Magni, 1969) demonstrated that the majority of spontaneous mutations are of the insertion-deletion type. Magni proposed that unequal crossing over during meiosis is the source of these spontaneous mutations, noting that their frequency is greatly increased in a meiotic division over a mitotic division where crossing over is much rarer (Magni, 1969). Demerec also invoked unequal crossing over to account for the phenomenon of "selfers," bacterial mutants which give rise to wild type recombinants in transductional crosses to themselves (Demerec, 1962).

In Salmonella typhimurium a mutant strain carrying a deletion for a small part of the histidine operon was found to lack all eight of the closely linked, metabolically related enzyme activities (Ames, Hartman,

and Jacob, 1963). Revertants, selected for their ability to grow on histidinol, a biosynthetic intermediate, were active for seven undamaged enzymes. However enzyme sythesis was no longer subject to regulation by histidine, and the genes were no longer transferrable by conjugation. In addition the linkage between the damaged operator region and the seven his markers measured by transduction was destroyed. The majority of these revertants could be demonstrated to carry a duplicated his region on an extrachromosomal DNA segment. These segregated to cells unable to grow on histidinol and identical to the original mutant strain in their complementation, reversion, phenotype, and map position. Transductants for this revertant type also give rise to segregants identical in all respects to the recipient (Ames, Hartman, and Jacob, 1963).

Another unstable system in Salmonella was investigated by Dawson and Smith-Keary (1963). The leu-151 strain, unable to grow without added leucine, gave rise to unstable as well as stable revertants. The unstable reversions were associated with a second suppressor mutation, which mapped at the su-leuA locus, although stable reversions at the same locus were also found. Different unstable reversions segregated to the original leu-151 type (as diagnosed by reversion and transduction) at different frequencies. Similar unstable strains also arose among the stable revertants caused by the su-leuA mutation. The site of instability was restricted to a limited region of the su-leuA locus. The authors suggested as a mechanism for this behavior a "controlling episome," an extrachromosomal regulator whose attachment to a specific chromosomal locus may activate or repress the normal functioning of that locus. Evidence supporting this interpretation was that 1) certain cell populations originally found to be unstable progressively lost their instability; 2) the instability of a su-leuA allele was transducible; 3) in strains mutant not only at the leu-151 and su-leuA loci, but also at pro-401, the proline requirement could also show unstable reversion, but only under conditions in which the su-leuA was stable; 4) when pro-401 became unstably revertible, the instability could be transduced along with the pro locus. The authors carefully eliminated a mutator gene, an inherent instability at the su-leuA locus, and partial diploidy

for a portion of the chromosome as bases for their observations. Their idea of a controlling episome, derived in part from analogy to McClintock's controlling elements in maize (1956), is an attractive explanation for the variety of their system.

Controlling episomes have also been invoked to explain problems of instability and mutation in E. coli. Hill (1963) found spontaneous and ultraviolet-induced revertants from auxotrophy which tended to regenerate the original auxotrophic type. Also Taylor (1963) described a mutation in E. coli K12 associated with integration of the temperate phage Mu-1 at that locus.

Several reports have been published concerning species of Streptomyces, a morphologically complex prokaryote with a mycelial growth habit. Newcombe found that changes in colonial morphology and color induced by ultraviolet or gamma irradiation of spores were heritable and usually associated with instabilities which became manifest over a period of numerous generations or during spore formation. Each unstable strain retains its instability indefinitely, and the variants it throws off arise in characteristic proportions, each subsequently having a pattern of instability of its own. These unstable mutations arose in 30 to 60% of the mutagenized cells. Newcombe proposed that the mutation being induced might have more the nature of a chromosomal rearrangement than a single gene alteration, especially because of its high frequency of induction and the superior effectiveness of x-rays to ultraviolet light (Newcombe, 1953).

In 1967 Hopwood reviewed genetic analysis in Streptomyces coelicolor and what can be learned of its genome structure. Although the genome is a single chromosome, some spores behave as if partially diploid. By the next round of sporulation, the segregation of these heteroclones is essentially complete. In a cross between two mutant strains in which recombinants are selected, the proportion of heteroclones increases as the tightness of linkage of the markers, and the heterozygosity most often occurs for the selected marker, though the total region of diploidy may be quite short. Among the segregants from a heteroclone, there is a gradient of allele frequency. The segregants may carry parental or recombinant marker combinations. This array of properties is remini-

scent of the terminal redundancy phage hets discussed earlier, and Hopwood proposes a similar model for heteroclones, suggesting that they arise from an odd number of exchanges between the recombining parent chromosomes. If an even number of exchanges occurred, stable recombinants would be generated (Hopwood, 1967).

In almost every system in which merozygosity cannot be demonstrated to result from extrachromosomal material, the model proposed is essentially a tandem duplication. The mechanism of segregation from a tandem duplication has a ready-made model in Campbell's looping-out hypothesis for episomal detachment (Campbell, 1969). The initial basis for this hypothesis was observations on E. coli cells doubly lysogenic for two genetically distinct lambda prophages. By studying the marker arrays on segregant phages obtained after prophage induction, Campbell realized that they could only have arisen by one of the following two mechanisms: 1) an internal recombination involving looping-out of one of the prophage genomes, or 2) a duplication of the bacterial chromosome in the region containing the prophages, followed by unequal crossing over to eliminate one of them (Campbell, 1963). Since it was initially proposed, support for the first explanation has come in abundance from experiments showing that the map of the vegetative lambda is a circular permutation of the prophage map (Campbell, 1969), and that loss of a prophage brings flanking bacterial markers into tighter linkage (Rothman, 1965).

That the duplication could occur in the absence of an episomal particle was shown when Campbell isolated a gal^+/gal^- partially diploid E. coli strain. The behavior of this strain was unaffected by F factors, and it could be lysogenized by two lambda prophages, one linked to gal^+, the other to gal^- (Campbell, 1965). Thus the original diploid region must extend to the prophage attachment site for lambda. This sort of strain bears remarkable resemblance to the strains described by Lederberg, cited earlier (p. 7).

Other unstable E. coli strains have been discovered with a variety of sources in instability attributed to them. Mutator genes have been described, which can affect mutation rates of the strains in which they

arise (Treffers, Spinelli, and Belser, 1954; Goldstein and Smoot, 1955). In these strains drug-resistant or auxotrophic mutants arise at much higher frequencies than normal: 100 times normal (Treffers, et al.), or several percent of the total population (Goldstein and Smoot). In the former instance although mutations resistant to streptomycin arose in a random manner, indicating the independence of each separate mutational event, they often seemed to be associated with development of an amino acid requirement. However, the presence or absence of the requirement in no way influenced the maintenance of the high mutation rate (Treffers, Spinelli, and Belser, 1954). In the strain used by Goldstein and Smoot, the newly formed auxotrophs persisted through single colony isolations and retained their nutritional requirement through many passages in fully supplemented medium. Here too a fluctuation test demonstrated that the auxotrophs arose de novo as do normal spontaneous mutants. A specific period in the history of the original strain could be identified, before which it behaved normally, implicating a mutator gene in the phenomenon. The mutations induced are functionally diverse, although an amino acid requirement is particularly common. Thus single genes, not linkage groups, are involved. Although ultraviolet light increased the frequency of auxotrophs generated, the frequency of stable auxotrophs was unchanged (Goldstein and Smoot, 1955).

Zamenhof, DeGiovanni, and Greer (1958) extended the study of this unusual strain. Three of its loci were identified, a mutation in any one of which is accompanied by dramatic diminution in the high mutation rate of many others. An auxotrophic mutation was as likely to revert to prototrophy as a similar marker in a normal strain; but having reverted, its likelihood of regenerating the auxotrophy was again dramatically enhanced. The authors tested the DNA of the strain and found it to be normal in its heat stability and in its ability to serve as a substrate for pancreatic DNase. Cell-free extracts were not mutagenic (Zamenhof, DeGiovanni, and Greer, 1958).

Although both groups accounted for their results in terms of a tendency of the strain to mutate, it is not clear to me that other possibilities, such as partial diploidy, have been rigorously excluded.

However, it must be remembered that certain essential prerequisites of establishing partial diploidy, such as a more complete genetic map, and ways to select segregants in the presence of large numbers of non-segregant types, were not yet in widespread use.

The first example of apparently spontaneous generation of partial diploidy was reported for an E. coli strain capable of producing β-galactosidase at rates up to four times normal (Horiuchi, Horiuchi, and Novick, 1963). The hypersynthesizing strains arose during growth in a chemostat in which the limiting nutrient was lactose. The basis for the hypersynthesis was discovered to be extra copies of the lac gene which are very closely associated with the lac locus on the bacterial chromosome and with which they are co-transducible. These extra determinants are not eliminated by acridines (a test frequently diagnostic for episomes), but they do segregate at frequencies of 2 to 5% to strains synthesizing normal levels of enzyme (Horiuchi, Horiuchi, and Novick, 1963).

A duplication of the gene coding for glycyl transfer RNA synthetase in E. coli has been reported by Folk and Berg (1971). A mutant for this enzyme had a gly^- phenotype, owing to a reduced affinity of the enzyme for glycine. However at a frequency of more than 10^{-5}, gly^+ revertants could be selected. The enzyme produced by these revertants was identical in its properties to the original enzyme, but two to four times the normal activity could be recovered. Only the original mutant allele could be transduced by phage P1. The gly^+ revertants segregated gly^- cells at frequencies varying from 0.1 to 10% of the population, depending on the particular revertant being examined, but segregation was prevented by rendering the cells recombination-deficient. That the revertant cells were partially diploid was shown by making them heterozygous at the enzyme locus and observing the segregation of types producing the two parental enzymes (Folk and Berg, 1971).

Among the partial diploids some have been amenable to definite assignment as episomal or chromosomal, while other cases are more speculative. The F' factors are perhaps the clearest example of diploidy for chromosomal genes in which the extra determinants are episomally borne. A rather more complicated strain, described by Curtiss (1964),

was diploid over 9% of the *E. coli* genome, from *ara* to pro_2, and segregated at a rate of 0.4% per bacterium per generation. Segregation required a preliminary recombination, but recombination could occur with retention of the partial diploidy at a rate of 0.04%. The endogenote carried a deletion of a fairly sizable region at one end of the duplicated segment which was a significant factor in stabilizing the exogenote, since the same exogenote in a strain whose endogenote carried only a point mutation in the same region segregated at a rate of 5 to 10% per bacterium per generation. The exo- or endogenote could be transferred to recipient strains if an F factor were introduced, but the two sets of markers were transferred independently. Acridine orange cured the strain of its F, but had no effect on the exogenote (Curtiss, 1964).

Schwartz induced *lac*$^-$ mutations in *E. coli* by treating cells with ultraviolet light or ethylmethanesulfonate. These mutant strains reverted to two classes of *lac*$^+$ phenotype. One class was identical to wild type, while the other contained revertants all of which were unstable, produced reduced amounts of β-galactosidase, and behaved in transduction in a allele-specific manner. On this evidence they were considered to be suppressor-mediated reversions. Their instability is reminiscent of the similar suppressor-associated instability of the *leu*-151 in *Salmonella* (Dawson and Smith-Keary, 1963). A particular suppressed strain, carrying *su*-lac_o-5, segregated colonies of *lac*$^-$ phenotype at a frequency of 0.5% even after single colony isolation. These segregant colonies were identical to the original lac_o mutant (Schwartz, 1964). The frequency of segregation was enhanced by treatment with acridine orange. An occasional *lac*$^+$ derivative may be obtained which is stably suppressed. The unstably suppressed strain transfers its suppressor soon after initiation of mating at a low frequency, but the suppressor in the stably suppressed strain is transferred at high frequency and maps at a different location on the chromosome (Schwartz, 1965). All this evidence led Schwartz to the conclusion that in the unstable strains, the suppressor is borne on an extrachromosomal genome segment, which may be lost, leading to *lac*$^-$ segregants, or recombined with the chromosome to yield a stably integrated suppressor (Schwartz, 1965).

By this time enough suppressor mutations had been described that it became possible to classify them on the basis of similar allele specificity (Eggertson and Adelberg, 1965). There continued to be reports of instability of suppressor genes, but frequently the unstable mutants were abandoned in favor of the more readily studied stable mutations (Gallucci and Garen, 1966, for example).

A most elegant study of suppressors and their role in correcting missense or nonsense mutations has been the detailed investigation of the tryptophan synthetase of E. coli by Yanofsky and coworkers. By correlating specific alterations in the amino acid sequence of the enzyme with the presence of specific suppressor genes, Brody and Yanofsky (1963) established the basis for allele-specificity of suppression. Further work in which the ratio of active protein to total cross-reacting material (a measure of the efficiency of suppression) was compared under a variety of metabolic conditions led to the conclusion that the suppressor gene acts to permit formation of active protein before or during the formation of the polypeptide chain, but not after (Brody and Yanofsky, 1965). This discovery directly implicated mutant transfer RNA species as the suppressor gene products, acting to correct structural defects in proteins by mistranslating the mutant condon in the messenger RNA (Carbon, Berg, and Yanofsky, 1966a,b).

In the same paper the authors reported an observation as important to the present study as the mechanism of suppressor action. For each suppressor gene the efficiency of suppression was characteristic of that particular gene. When the suppressor gene was transduced into a different host strain of E. coli, this efficiency was markedly enhanced, and the enhancement was correlated with an instability of the suppressor gene. The newly suppressed strains could yield stable tryptophan-requiring segregants which retained the high efficiency (Brody and Yanofsky, 1965).

In 1969 new suppressor mutants able to correct the same tryptophan synthetase mutation were isolated (Hill, Foulds, Soll, and Berg, 1969). These new mutations grew more slowly in minimal medium, were very sensitive to a shift from minimal to enriched medium, and did not segregate. Either by forcing growth on enriched medium, or by transduction to a strain lacking the suppressor mutation, Hill, et al., were able to recover

strains still capable of suppressing the tryptophan synthetase mutation, but now possessing the full spectrum of properties characteristic of the Brody-Yanofsky suppressor. Considerable evidence was presented that the new suppressor mutants themselves were haploid, containing only the mutant gene for the suppressor, while the Brody-Yanofsky type of suppressor strains were diploid, possessing both mutant and wild type genes for the suppressor transfer RNA. Presumably the presence of the wild type gene is essential for the strain to grow rapidly in minimal medium, survive a shift-up to enriched medium, and segregate unsuppressed tryptophan-requiring clones. Evidence supporting this interpretation was as follows:

Analysis of segregation patterns from the occasional fast-growing unstable variants showed them to be partial heterozygotes for the region containing the suppressor locus. One such partial diploid included the linkage group containing thi, argH, and metB. Both wild type and mutant alleles of these loci could be recovered among transductants or segregants. However no segregation could be observed if a recA mutation were introduced into the strain. Also time of entry studies using the unstable strains as donors in conjugation showed distinct biphasic curves for entry of those markers which segregate. The initial slope was similar to that of stable markers, and the subsequent slope was about twice as great. This is clear evidence for a tandem duplication of the markers in the diploid region (Hill, Foulds, Soll, and Berg, 1969).

The authors also provide an intriguing explanation for the frequent observation of instability at a suppressor locus in general. Some transfer RNA species are more dispensable for translation than others, but might be important to the cell in other roles, as regulators or modifiers of metabolic activity. A mutation in one of these might allow a strain to suppress a defect preventing its growth, but might at the same time destroy essential modulating functions. However, if the strain had duplicate copies of the gene, one copy could mutate to the form needed for suppression, while the other in its wild type form could function in the other roles (Hill, Foulds, Soll and Berg, 1969). That certain suppressor mutations can be lethal to the cell was demonstrated by Soll and Berg (1969), who were able to isolate new nonsense suppressor mutants by selecting them in strains bearing an F' episome. The suppressors were

invariably found in an unstable form and were localized on the episome (Soll and Berg, 1969).

Hill, Schiffer, and Berg (1969) reported that when the partially diploid suppressor strain was used as a donor in P1-mediated transduction, the entire duplication could be regenerated in the recipient strain, although this represents a much larger DNA fragment than the transducing phage can accomodate. Furthermore some of the duplicated markers can be demonstrated to be duplicates of those from the recipient cell. The authors propose that only a fragment of duplicated material containing an essential "repeat point" is necessary to regenerate the entire duplication in the recipient. This hypothesis finds some support in some of our findings about the unstable sulfonamide-resistant strain to be described.

Perhaps the most direct demonstration of a tandem duplication has been achieved for the gene controlling synthesis of tyrosyl transfer RNA (Russell, Abelson, Landy, Gefter, Brenner, and Smith, 1970). The gene for a minor tyrosine transfer RNA maps near the attachment site of transducing phage ϕ80. This gene can mutate to a form active in suppression of amber mutations (Carbon, Squires, and Hill, 1969). In strains containing the suppressor mutation, not all of the minor transfer RNA is altered, suggesting that perhaps more than one gene copy specifies the transfer RNA normally. Russell, et al., confirmed that suppressor strains and defective ϕ80 phages derived from suppressor strains both segregated the triplicated and single derivatives, presumably by unequal recombination. The genotypes of the phage segregants was confirmed by hybridization experiments in which the purified transfer RNA gene product was hybridized to the DNA coding for it. The haploid segregant can bind only half as much of this specific transfer RNA as the parent strain (Russell, et al, 1970). It is clear that such elegant analysis requires the isolation of the primary gene product, the corresponding RNA species.

In transformable bacteria, persistent instabilities have also been described and characterized. Tryptophan-synthetase mutants in Bacillus subtilis can revert to partial tryptophan independence. The partial revertants could not recombine with the original mutation, and could be generated at high frequency in a cross of wild type DNA by mutant recipient

cells. Occasional segregating forms were seen, but they resolved to stability upon sporulation (Anagnastopoulos and Crawford, 1961). More recently the same laboratory has been able to generate partial diploids for many loci for which variants may be selected. These have proven useful in analyses of complementation and dominance relationships (C. Anagnastopoulos, personal communication).

In 1965 Iyer reported an auxotrophic mutant induced by ultraviolet irradiation of B. subtilis which yielded unstable transformants (Iyer, 1965). However this apparent case of abortive transformation was shown to be an artifact of a second mutation and cross-feeding (Jensen, 1968). Thus there are many possible sources for an unstable phenotype.

Using pneumococcus Bernheimer and Wermundsen have described an instability of the locus controlling binary capsulation. Usually pneumococci synthesize only one of a variety of possible capsular polysaccharides, if any at all. A rare transformant type can simultaneously synthesize polysaccharides of two antigenic types, type I and type III, apparently owing to the integration of the type I determinant at an unusual location so that it coexists with that of type III, rather than replacing it (Bernheimer, Wermundsen and Austrian, 1967). However some of the binary strains that can be obtained are unstable, losing their type I genome at high frequency, so that after several subcultures 30% of the cells were segregant. The instability has been traced to a mutation in the type III gene, permitting covalent integration of the type I genome at a closely linked site. That the source of instability lies in the type III genome is demonstrated by the loss of instability when the type III determinant is replaced in transformation by an alternative capsular polysaccharide genome. The authors propose that the mutation occurs in that part of the capsular genome which determines the type-specific uridine diphosphoglucose dehydrogenase, the only enzyme common to the pathways of synthesis of both type I and type III polysaccharides. If the mutation permitted homology in the base sequences of the two enzyme determinants which previously was lacking, tandem integration of the two capsular determinants could ensue (Bernheimer and Wermundsen, 1969).

Ravin and Takahashi have isolated streptomycin- and erythromycin-resistant mutants of pneumococcus which are merodiploid and one of which segregates to the more sensitive form. In both cases, as in the capsular polysaccharide mutants, the merodiploidy is associated with particular mutations. During transformation the mutant loci in donor DNA add to, rather than replace, their homologous sites in sensitive recipients. Nearby markers do not become heterozygous; thus the unstable region must be of limited size. A single molecule of DNA is sufficient to introduce the diploidy, suggesting that its structural basis is a duplication of the determinant within the continuity of the bacterial genome. From the kinds of segregants obtained, the authors propose a looping-out of the duplicated segment as a mechanism for its segregation (Ravin and Takahashi, (1970).

Unfortunately both capsular antigen synthesis and drug sensitivity are difficult segregant phenotypes to quantitate, particularly if there is a large excess of cells of the alternative form. Hotchkiss, Abe, and Lane (1967) have published preliminary descriptions of a sulfonamide-resistant pneumococcal mutant which segregates at a moderate frequency to wild type. Selection both for mutant and wild type is possible, so that segregant frequencies and variations of transformation frequencies can be readily quantitated. The instability and drug resistance are transformable to stable sensitive strains, and may involve markers at closely linked loci as well (Hotchkiss and Abe, 1968). It is an extensive discussion of the behavior of this strain which constitutes the remainder of this thesis.

The purpose of this rather detailed introduction has been to provide a background in the realm of segregating genetic systems. Strains of a variety of origins with instabilities of different extent and probably different structures have been reviewed. Certain common features may have been discernible, but each individual system has unique features which permit the investigator to emphasize a particular aspect of the organism's behavior. The availability of selection, of a mating system, of a readily isolated enzyme or RNA species which may be related to the segregating properties themselves--all these determine the certainty with

which the structure can be elucidated.

Given the indirect nature of most of the analytical tools we have discussed, it is rather amusing to follow the chronological progression of proposed sources of instability. In the early 1950's pure genetic description was sufficient, and non-disjunction, diploid, and haploid were satisfying terms to explain the observations. By the middle to late fifties, mutator genes (popularized from Drosophila) were more likely to be invoked. With McClintock's examinations of controlling elements in maize (1955), a good analogy could be drawn to many instabilities. And as the episome concept grew and became refined, it seemed to serve as an admirable model for instabilities described in the same period.

Today the tandem duplication may appear to be a popular model to propose. Many of the systems I have cited seem to have strong supporting evidence for its existence. However it is inescapable that a duplication is difficult to demonstrate at the molecular level, and one must rule out other reasonable structural alternatives before settling on it as a final answer.

II
MATERIALS AND METHODS

Media

1. Casein hydrolysate medium (Fox, 1959) modified so as to be suitable for transformation (CH-T)

Per liter of glass-distilled water:

2 g sodium acetate
5 g casamino acids (Difco)
6.25 mg L-tryptophan
62.5 mg L-cysteine-HCl
8.5 g dipotassium phosphate

The pH is adjusted to 7.6 and aliquots are autoclaved. This constitutes pre-CH. The final mixture is made by completing a 400 ml aliquot of pre-CH with sterile additions as follows:

2 ml combined salts (0.02M $MnSO_4$; 1% $MgCl_2$; 0.05% $CaCl_2$)
0.3 ml 1% $CaCl_2$
4 ml 20% glucose
10 ml Adams III (Adams and Roe, 1945) (a solution of vitamins, metal ions, asparagine, and choline)
5 ml 4% albumin
12 ml fresh yeast extract

The pH is again adjusted, and any precipitate (usually $Ca_3(PO_4)_2$) is filtered out using a Millipore membrane filter, 0.22 mμ pore size.

This CH-T medium is used for passing and storing stock cultures, growing cells to the competent state, transforming them with DNA, or rejuvenating them after a selection step.

2. Basal scoring medium (BX)

Per 4 liters of glass-distilled water:

37 g brain heart infusion (Difco)
42 g NaCl

20 g neopeptone

Aliquots are sterilized by autoclaving. This is basal medium, BM.

Before use, complete with the following sterile additions:

To 400 ml BM:

8 ml 20% glucose

12 ml 4% albumin

13 to 26 ml FSB supplement (for contents, see the recipe for FSB)

10 ml 0.5M K_2HPO_4

This medium is used for assaying pneumococcus cells on agar plates. The medium is warmed and mixed with an equal volume of 2% agar held at 45 to 50° C., just before it is pipetted over samples of cells in petri dishes.

3. Pneumococcus liquid scoring medium (FSB) (Hotchkiss, 1957)

Per liter of distilled water:

1/2 lb. beef heart per liter to give a meat infusion

1% Difco neopeptone

0.3% NaCl

0.2% K_2HPO_4

Adjust pH to 7.8. Boil fifteen minutes. Filter through filter paper. Autoclave in 1.5 liter aliquots. This is scoring broth, FB basal. Dilute 1 volume FB basal with 2 volumes 0.85% NaCl. Distribute and autoclave. This is FB x/3.

To 400 ml FB x/3 add:

10 ml 0.5M K_2HPO_4

5 to 10 ml FSB supplement (0.28 mg adenosine, 4.0 mg casein hydrolysate, 0.28 mg shikimic acid, 0.64 mg thymine, 14 mg sucrose per ml)

10 ml 4% albumin

0.05 ml 20% glucose

Sufficient antibody against the pneumococcal R antigen to yield good agglutination of clones after overnight incubation. FSB is used for scoring particularly sulfonamide-resistant cells in liquid medium.

4. Preparative broth for growing cells to make DNA

Per liter of distilled water:

1 lb. beef heart to give a meat infusion

1% neopeptone

Boil ten minutes, filter through filter paper, autoclave. Complete a 1.5 l aliquot with 15 ml 0.5M K_2HPO_4 and 3 ml 20% glucose at the time culture is inoculated.

5. D medium (Fox and Hotchkiss, 1957) for dilutions and holding cells in a viable state but unable to grow

200 ml 0.85% NaCl

12 ml 0.5M K_2HPO_4

2.8 ml 20% glucose

10 ml 4% albumin

1.0 ml 1% $CaCl_2$

Components of all media were reagent grade. Amino acids and nucleosides were supplied by Calbiochem, vitamins by Nutritional Biochemical Company, Merck, and Eastman Kodak.

Strains

All strains used were derivatives of R36A, a rough, unencapsulated strain of Diplococcus pneumoniae originally described by Avery, et al. (1944). Drug-resistant mutants of this strain have been isolated, then purified from other mutations by genetic transformation of the wild type by DNA extracted from the mutant. In this way multiply-marked stock strains have been developed. These are designated by the drug-resistance phenotype of their mutations:

sul denotes resistance to sulfanilamide (SA). Subclasses of this group are sul-a, sul-b, sul-c, and sul-d, each resistant to characteristic level of drug. These subclasses are referred to as a, b, c, and d for convenience.

mic denotes resistance to micrococcin, 0.3 μg/ml.

str denotes resistance to streptomycin at high levels; it is usually selected between 50 and 150 μg/ml.

Q denotes resistance to optochin (ethylhydrocupreine hydrochloride), 1 μg/ml.

ery denotes resistance to erythromycin, 0.25 μg/ml.

Our present standard wild type strain is R1-26.

Table I is a comparison of the resistance levels of the various *sul* mutants to sulfanilamide (SA), and to two other analogs of paramino-benzoic acid, p-aminosalicylic acid (PAS), and p-nitrobenzoic acid (NOB). These mutants may be combined in various ways to yield double mutants whose resistances are shown in Table II.

Table I: Maximal drug level yielding optimal growth in FSB (μg/ml)

Strain	SA	PAS	NOB
sul-a	20	20	20
sul-b	15	400	20
sul-c	150	400	2*
sul-d	80	0.5	less than 0.5

* Also yields segregants resistant to 20 μg/ml

Table II: Complex sulfonamide-resistant strains; maximal drug level yielding optimal growth (μg/ml)

Strain	SA	PAS	NOB
sul-ab	70	400	50
sul-ac	150	200	2*
sul-ad	400	4	1
sul-bc	200	----	5*
sul-bd	300	400	1
sul-cd	800	400	1*
sul-abd	1200		
wild type (= $a^+b^+c^+d^+$)	5	2	20

* Also yields segregants resistant to 20 μg/ml

It is evident that combinations of two or more unit mutations generate a strain whose drug resistance is markedly enhanced over that of either component strain. It was this evidence which prompted Hotchkiss and Evans (1958) (see also Wolf and Hotchkiss, 1963) to conclude that the mutations *a*, *b*, *c*, and *d* all affect the same enzyme in the synthesis of folic acid,

the one which couples paraminobenzoic acid to the pteridine moiety. The sulfonamides act to inhibit competitively this coupling because of their structural similarity to the true substrate, paraminobenzoic acid. Ortiz has demonstrated, at least for the enzyme of the sul-d mutant, that it is the relative binding affinity for the inhibitor that is reduced, thus permitting normal growth in the presence of enhanced drug levels (Ortiz and Hotchkiss, 1966; Ortiz, 1970). This same mechanism was proposed by Davis and Maas (1952) in a more general sense as the basis for sulfonamide resistance in a wide variety of bacteria. It is perhaps reasonable then to project this mechanism to the other three sul mutants, a, c, and b, especially since a, c, and d are very tightly linked in transformation. However, the originally reported linkage of b to the other markers is still at present in question (Hotchkiss and Evans, 1958).

Other strains used in this study include R1-26str27A-2, a transformant of R1-26 for a mutation conferring recombination deficiency. Dr. Gerald Vovis supplied me with competent cells of this mutant stock. He isolated and characterized the mutant in C13str-41, another subline of R36A, as a γ-radiation sensitive variant in a population mutagenized with nitrosoguanidine. The strain gives about 10% of the normal yield of transformants per unit of DNA taken up from the medium. It is also more than usually sensitive to ultraviolet irradiation, is "cautious" in its breakdown of DNA following irradiation, and is demonstrated to lack an ATP-dependent exonuclease present in wild type strains (Vovis and Buttin, 1970).

Hex-1, supplied by Dr. Sanford Lacks, is also an R36A derivative, mutant in its ability to differentiate donor markers according to efficiency of integration. In the wild type strain different mutant markers, even within the same locus, may show dramatically different yields of transformants when compared to a standard marker in the same DNA. In this mutant strain, all markers tested integrate with an identical efficiency, that is, the ratio of their yield to the yield of the standard marker is always about 1.0 (Lacks, 1970).

A second strain with similar properties to hex-1 is Rx-1, supplied

by Dr. Minna Rotheim. This is a pneumococcal strain of different origin from R36A and is used as wild type in the laboratories of Dr. Walter Guild and Dr. Arnold Ravin. In 1959 Green reported that it gave no differences in transformation efficiency for a pair of markers, while a variant, Rz, showed a consistent depression of efficiency of one of the pair. He attributed this phenomenon to a depressor factor present in Rz but absent in Rx (Green, 1959). In studying his hex-1 strain, Lacks compared it with Rx-1 and found the behavior of the two to be similar for a wide variety of markers (Lacks, 1970).

Methods

1. Growth of a culture

My standard growth medium has been CH-T, although for certain purposes I have used FSB or a modified medium as indicated at the appropriate place in the text. The inoculum can be anything from a single cell to a population of several hundred thousand. The source of the inoculum can be a freshly thawed culture stored in 10% glycerol at -20°C., a suspension of cells from a colony picked from an agar plate or from a liquid scoring tube, or a dilution passage of a culture in the process of growing. Any stage in the growth cycle may be used as a source of inoculum although stationary phase cells (or single colony suspensions) usually display a lag before resuming growth. A lag may also precede exponential growth if the growth medium is quite different from the medium of the inoculum. The optimal growth temperature is 37°C. at which temperature actively growing wild type cells have a doubling time of 25 to 30 minutes.

2. Passage and storage of cultures

Stock cultures must be passed regularly to maintain their viability, yet too frequent passage will lead to accumulation of variability in the stock. In order to preserve stocks in a state of maximum viability for the maximum time, an old stock is thawed, diluted twenty-fold into fresh medium, and grown at 37°C. until turbidity is barely visible to the eye (about 5×10^6 colony-forming units/ml). This culture is then diluted about threefold into medium containing glycerol of final concentration 10% and returned to the deep freeze (about -20°C.). Cells frozen at this

density will remain viable for over a year.

3. Growth of a culture to competence

Competence is a physiological state of pneumococcal cells in which they are capable of binding and incorporating DNA present in the medium and integrating it into their own genome. It is characterized by a wave of production of a labile substance, probably protein (Tomasz and Mosser, 1966), capable of inducing competence in incompetent cells, and thus called "competence factor" or "activator" (Tomasz and Hotchkiss, 1964). The attainment of competence usually occurs at a particular cell density, 1 to 4 x 10^7 colony-forming units/ml, when the pH is in the range 7.6 to 8.0, and is accompanied by an increased agglutinability of the cells. The cells become competent in an autocatalytic manner, reach a competence peak, then experience an equally rapid decay of competence, probably owing to release of an inhibitory factor (Tomasz and Hotchkiss, 1964). Fox and Hotchkiss found that cells grown to competence, then frozen, could be preserved in the competent state for up to two months (Fox and Hotchkiss, 1957).

The standard procedure is to inoculate CH-T medium with about 10^4 cfu/ml of a culture that has been competent recently. This culture is grown until lightly turbid; then at half-hourly intervals, aliquots are placed at 25°C. for fifteen minutes (a procedure which seems to synchronize the cells in their division cycle), (Hotchkiss, 1966). Glycerol is added to a final concentration of 10% and the cells are frozen. Usually three or four aliquots are taken to be sure that one will include cells in the competence peak. Often it is possible to select the most competent aliquot by observing the clumping and increased agglutinability of the cells.

If there is difficulty in obtaining a competent culture, owing to age of the cells, or a progressive diminution of the competence level, it may be necessary to subclone the culture and pick a colony which can achieve high competence as the progenitor of the competent line. One must be extremely careful to insure that the clone selected is typical of the strain by all available criteria. A less drastic stratagem is to pass the culture several times in rapid succession with either a single overnight freezing step or none whatsoever. This has frequently given highly competent cultures.

Competence of frozen cultures is tested in a transformation assay (see below) using as donor a DNA whose activity is known.

4. Scoring methods

It is frequently necessary to enumerate the number of cells in a culture either for their total number or for the fraction capable of growth in the presence of selective pressure, such as a drug. There are three means routinely employed in our laboratory. The first is a microscopic cell count, in which a Petroff-Hauser chamber is filled with a cell suspension and the number of cocci or chains in a given area determined using phase contrast optics. From the known dimensions of the chamber, the concentration of cells can be calculated. This number bears no relation to viability of the cells, since living and dead cells are visually indistinguishable. It is useful however if there is a question about chain length. As may be guessed from its name, Diplococcus pneumoniae, the customary mode of growth is as two-coccus chain. However the culture conditions in the laboratory seem to favor chains of about four cocci in length. An occasional check is made to be sure the cultures are behaving normally, but rarely is there reason to suspect deviations from the norm. All data are reported in terms of viable count or colony-forming units (cfu). Our practice of reporting the ratio of transformants for a given marker to those for a standard marker make it unnecessary to correct for chain length.

A second scoring method is plating in scoring medium, BX, solidified with 1% agar. Since pneumococcus is facultatively anaerobic, it grows best deep in the agar. After overnight growth in this medium, colonies may be seen as ovoid white forms of uniform size in the clear agar. They may be counted using a dissecting microscope or a magnifying glass.

The third method, and the one on which most of my results are based, is a liquid scoring method in medium containing the anti-pneumococcal antibody, anti-R (Hotchkiss, 1957). The advantages of this method are its economy of time and materials and the many internal controls it is possible to perform. Cells growing in this medium remain agglutinated to their progeny as they divide. As the colony of sister cells increases in

size, it falls to the bottom of the scoring tube. After an overnight incubation, the colonies may be counted using an indirect light source and a dental mirror. Using a serial dilution technique developed by Hotchkiss, one can usually find at least two out of six tubes with an appropriate number of colonies (20 to 100) to give an accurate estimate of the viable count. For increased accuracy it is necessary only to make replicate dilution series; once the range of expected count is known, more tubes can be prepared containing dilutions in that range. Supplementary data obtainable through this method, and particularly valuable in scoring with drugs such as sulfanilamide, which starve rather than kill, is information on the effect of cell density on colony formation, the growth in drug of normally sensitive cells in the presence of sufficient numbers of resistant cells (cross-feeding) or in those tubes lacking resistant cells (breakthrough).

5. Preparation of DNA

The main elements of this procedure are to grow cells to a maximum density while still maintaining exponential growth, to lyse cells and deproteinize rapidly to minimize the effects of cellular nucleases, and thoroughly to deproteinize under conditions designed to minimize shear breakage of the DNA. The procedure has been described in Hotchkiss (1966). Preparative broth is the growth medium; its enrichment allows the attainment of high cell concentrations. Growth is followed by measurements of turbidity using a Coleman Nepho-colorimeter with an 8-212 filter (red), and the pH is maintained about 7.2 to 7.5. At the desired cell density (about 400 to 500 nephelos units) a sample is taken to confirm the phenotypic properties of the cells and the viable count (usually about 1 to 2 x 10^8 cfu/ml). The cells are rapidly harvested in a refrigerated centrifuge, resuspended in ice cold 0.10 M citrate-0.15 M NaCl, pH 7.4, and lysed with 0.05% deoxycholate between 25 and 37°C. As soon as lysis is complete, chloroform and a few drops of isoamyl alcohol are added to denature and gel the protein. This mixture is shaken about 15 minutes on an alternating shaker at room temperature, then centrifuged to separate the layers. The protein is found in a fluffy cream-colored layer at the interface of the aqueous and organic layers. The aqueous layer containing

nucleic acids is carefully pipetted off, and the nucleic acids precipitated with 50% ethanol. The fibers are collected on a glass rod and redissolved in citrate-saline (modified from Sevag, Lackmann, and Smolens, 1938). This procedure is repeated until there is almost no protein layer. Then RNA is digested with 20 μg/ml pancreatic RNase A, the solution deproteinized twice more, and the DNA fibers finally dissolved in 0.15 M NaCl. The DNA content of the resulting solution is determined by its absorption at 260 nm and its hyperchromicity upon alkaline denaturation. Its activity is determined relative to a DNA of known activity in a transformation assay (Hotchkiss, 1966). DNA prepared in this way is active for a number of years. It is suitably pure for physical studies.

Occasionally an assay for qualitative transforming activity (content of markers) is performed on the DNA in a crude lysate, or one deproteinized only once.

6. Transformation assay

The standard assay method is designed to maximize the yield of transformants. Certain variations may be introduced to facilitate various sorts of experiments. The important point is to use the same method in all experiments whose results are to be compared.

A tube of frozen competent cells is thawed in ice water, then placed at 30°C. (preincubation) for an amount of time previously determined to give the optimal transformability of the culture. Usually as the culture ages, this time increases from as little as 5 minutes to as much as 25. At the end of preincubation an aliquot of the culture is diluted with cold CH-T containing the donor DNA at the required concentration. The maximal transformation response is usually obtained by 0.01 to 0.02 OD units/ml of DNA, and can affect as many as 10% of the total colony-forming units in the culture. The mixture is returned to 30°C. (determined by Fox and Hotchkiss (1957) to permit maximum transformation with minimum cell growth) for a precisely determined interval, usually five to fifteen minutes, and specified in each experiment (exposure). At the end of that interval 1.5 μg/ml pancreatic DNase is added to terminate the reaction and the tubes are placed at 37°C. Immediately a sample is taken to assay

for viable counts. Samples are also taken at appropriate intervals to score for genetic markers whose phenotypic expression is known to be complete in that interval (Hotchkiss, 1966). Usually the minimum interval is twenty minutes, within which time any irreversible recombination which will take place will have had a chance to occur. The sul markers can be scored immediately, partly because of the dominance of the phenotype, partly because of the period of grace allowed by the starving action of the drug (3 to 4 divisions). Mic is fully expressed at 30 minutes, Q at 60 minutes, and str, a recessive marker with a killing selection, requires 90 minutes. I chose to score each marker as it completed expression in order to avoid any error arising from division of transformants. Except for str, this is unlikely, as the influence of the chain length and probably single-stranded integration, make it improbable that viable counts of transformants will begin to increase in fewer than four cell generations.

Occasionally I have made use of a qualitative transformation when I wanted to see whether a particular isolate could be transformed to a particular drug resistance. For this, a passage of the culture of interest is made in CH-T, the cells grown for several hours at 37°C. until lightly turbid, a drop of the required DNA added, and the incubation continued for several more hours. At the end of the experiment, the tube is chilled and scored for the marker of interest and some other standard markers as a control on the competence of the culture. The DNA concentration is high enough for activity to persist over the period during which the cells become competent. This method is useful for introducing a marker into a strain or for verifying genotypes of isolates.

In some experiments where it has been difficult to develop intrinsic competence in a recipient strain, I have used "competence factor" generously supplied by Mrs. Eva Zanati and Dr. Richard Ziegler, to induce competence. I have described the procedure at the appropriate point in the text.

7. Single-colony isolation and rejuvenation

In a number of instances I have isolated single colonies and tested

their genetic properties. In this work it is desirable to have all colonies to be compared in as similar a physiological state as possible. Colonies as they are picked are not optimally healthy. Cells toward the center of the colony may be starved for nutrients and poisoned by accumulated waste products, and total cell growth may have become limited by exhaustion of the medium. Moreover, if the cells have been selected in drug medium there may be some residual effects of the drug not conducive to immediate and optimal growth. Consequently whenever colonies are picked, it is into 1 ml of CH-T; then a two-hour period of "rejuvenation" of this culture at 37°C. is allowed before testing or freezing for subsequent testing. From agar plates, sterile toothpicks are used to remove as much of the colony as possible. From a liquid scoring, tubes containing only one colony are chosen, resuspended by agitating the tube, and diluted into the CH-T sufficiently to reduce the final drug level below that toxic to the most sensitive known type.

8. Other methods

For the details of determining segregation frequency, treating cells with mitomycin C, ultraviolet light, or acridine orange, treating DNA with formaldehyde, or fractionating DNA on methylated albumin-kieselguhr or by cesium chloride equilibrium density gradients, the reader is referred to the appropriate section of experimental results.

III

GENETIC PROPERTIES OF THE SUL-C STRAIN

Introduction:

Sul-c is a mutant strain which segregates to wild type

The particular sulfonamide-resistant mutation whose properties occupy the rest of this thesis is known as sul-c. It was the first sulfonamide-resistant mutant of pneumococcus, isolated in this laboratory in 1952 (Hotchkiss and Evans, 1957) and first described in 1954 (Hotchkiss and Marmur, 1954) as a mutant which could be selected from wild type by growth in 15 μg/ml sulfanilamide (SA).

With the isolation in 1955 of the complex sulfonamide-resistant strain (Hotchkiss and Evans, 1958) in a single selection step, work on the sul-c strain flagged. The new strain was eventually determined to be subdivisible into the unit markers we now refer to as a, b, and d. The possibilities of this group of markers for investigating the effects of various sulfonamide analogs as substrates and relating these to theories of enzyme structure stimulated efforts in that direction (Hotchkiss and Evans, 1960).

Meanwhile sul-c was being passed periodically along with the rest of the stock cultures. In 1960 it was discovered (Hotchkiss and Evans, personal communication) that only about half the total number of cells in the stock culture retained their resistance to SA. Hotchkiss and Abe (Hotchkiss, personal communication) established that those cells which were sensitive to SA were now resistant to NOB, much like wild type. Thus two distinct populations were represented in the stock culture. The NOB-resistant portion never again showed the high SA-resistance characteristic of the parent strain. The SA-resistant portion, on the other hand, continued to give rise to SA-sensitive, NOB-resistant progeny even through successive single colony isolations. Thus the instability is an intrinsic property of the strain, not an artifact of cell clumping or something similar. The c strain as we know it now is resistant to up

to 400 μg/ml PAS and 150 μg/ml SA, and at any given time, 5 to 10% of the total population can be shown to be resistant to NOB.

The c property is probably not a long-lived physiological modification of the strain for the following reasons:

1. I have looked for a similar phenotype in wild type strains without success. It could not be generated in transformations of wild type cells by wild type DNA, even when recombination was stimulated by low doses of ultraviolet irradiation (Guerrini and Fox, 1968). Thus the effect is more than a transient destabilization of the normal physiological processes.

2. If DNA is isolated from cells of the c strain and used to transform wild type cells, the c SA-resistance, the associated high-level resistance to PAS, and the instability are always found to be associated in the transformants, no matter what the basis of selection (for more details, see Appendix A). Either PAS or SA may be the selective agent, and its concentration may vary from just above the threshold of sensitivity of the recipient cells to close to the maximum resistance level of the transformants without affecting this outcome. Thus the c phenotype is a genetically determined property transferrable through the purified DNA of the strain.

3. If the above transformation is performed at different DNA concentrations while holding the cell concentration constant, the yield of transformants follows a linear relation to DNA concentration, and reaches plateau, or saturation of receptor sites on recipient cells, at the customary concentration, indicating that the information for all the c-associated properties is borne on a single piece of DNA.

4. If the transformation mixture is sampled at various intervals after addition of the DNA, c transformants accumulate linearly with time, as do transformants to all other single mutations (Kent and Hotchkiss, 1964).

From these arguments it is apparent that the basis for all of the c phenotypic properties is genetic and that c itself is a mutation which is responsible directly or indirectly for each of them.

Let us direct our attention for a moment to the nature of the

"segregants" or "revertants." They are similar, at least superficially, to wild type, in that they are sensitive to SA and resistant to NOB at the same levels of drug. Abe studied thirteen segregant colonies, and none of the 10^9 cells in these colonies had any demonstrable SA resistance. She also tested a number of other analogs of sulfanilamide on the segregants. These analogs often are useful in distinguishing sul mutants similar by other criteria. But the segregants showed similarity to wild type in their response to the entire spectrum of drugs tested. No reversion of segregants to the c phenotype has ever been seen. Segregants used as recipients in transformation are transformed by all donor DNA's, including c, as efficiently as wild type (Abe and Hotchkiss, personal communication).

I attempted to confirm and extend these observations, in order to ascertain whether the c property might be present but suppressed by some other factor in the segregants. I selected segregants by two methods from agar plates. (For a discussion of some segregation anomalies in liquid selection, see Appendix B). In the first I plated a culture of c in agar containing 20 μg/ml NOB. The colonies that formed represented 3.3% of the population plated. Nine of these were selected, rejuvenated, and tested for residual SA or PAS resistance. None of them showed any resistance to either drug under conditions where 0.2% could be detected. One of these nine colonies, designated N12, was chosen as a prototype, streptomycin resistance was introduced by transformation, and the resulting strain, N12-str, was used to prepare DNA in order to test donor activity. In the second method, a c culture was plated on medium lacking any drug and colonies were picked, rejuvenated, and tested in NOB. All but one of the 47 colonies gave frequencies of NOB resistance in the range expected for the c strain, 5 to 10%. That one showed 100% NOB resistance. Subsequent testing showed it to have completely lost its SA and PAS resistance. Thus it is a segregant which arose without a drug selection. It was treated similarly to N12, streptomycin resistance introduced, and DNA prepared from the resulting strain, designated 42-str.

The presence of the streptomycin marker in the two donor DNA preparations allowed a control on the level of transformation, determined by

the competence of the recipient culture and the activity of the DNA. The results are summarized as follows:

Table III: Inability of DNA from segregant strains to donate SA or PAS resistance to wild type or d recipients.

Donor strain	Concentration of DNA	Recipient strain	Percent transformation to str (150 μg/ml)	SA* (50 μg/ml)	PAS** (50 μg/ml)
N12-str	plateau	R1-26	0.50	0.002	0.002
	linear		0.21	0.0003	0
42-str	plateau		0.49	0.001	0
	linear		0.27	0.001	0
			str (150 μg/ml)	NOB (20 μg/ml)	SA* (600 μg/ml)
N12-str	plateau	sul-d	2.8	2.4	0.001
	linear		0.88	1.1	0.0002
42-str	plateau		2.4	3.0	0.001
	linear		1.1	1.5	0.007

* Selected colonies, when purified and tested, showed complete resistance to SA, PAS, and NOB. This is unexpected behavior. Such colonies cannot be demonstrated when the untransformed competent cells are scored. However, they were also seen in a transformation of the same cells by two other DNA preparations. Possible explanations are mutation induced by the transformation process (Yoshikawa, 1966) or a contamination of the DNA preparation cultures with cells bearing the anomalous marker. The former explanation is unlikely, as I have tried to demonstrate mutagenesis resulting from transformation in pneumococcus, without success. The possibility remains however that in this particular experiment, conditions favored such an event. In any case the effect is of low enough frequency that the primary result is not in doubt.

** This is the PAS-resistant colony count corrected for the background PAS resistance in the untransformed recipient population. It is doubtful that any excess of PAS resistance at the level indicated is significant. For further discussion, see Appendix A.

Thus it is clear that the segregants carry in their DNA no determinants of their former SA resistance or PAS resistance. Their complement of the determinant of NOB resistance, the wild type phenotype, is normally active, and they are unable to enhance the SA resistance of a d recipient.

A second experiment performed to see whether the segregants had lost

all of their instability was the following. N12 and 42 were used as recipients in a qualitative transformation by DNA from a d strain. Transformant clones resistant to 50 μg/ml of SA (a characteristic selective level for d) were tested for any possible ability to give NOB-resistant colonies. Such colonies are never found in d cultures, or d transformants of wild type recipients, but might be anticipated if the recipient were not truly wild type, but retained some instability. Eight of nine clones selected in liquid culture showed very low but measurable frequencies of NOB-resistant cells. Two of the eight were chosen for further testing and showed no other property associated with the presence of c, that is, they were sensitive to PAS and to high levels of SA. In fact twenty independent transformant clones selected from agar plates showed no NOB resistance in about 10^6 cells per clone, nor did subclones of two of the transformant clones originally selected in liquid medium. Further discussion of possible sources of discrepancy in colonies selected in liquid medium and from agar plates is given in Appendix B.

Thus it is clear that by all available criteria, segregants are indistinguishable from wild type. They have identical phenotypes, they carry no memory of their drug-resistance when used as donors, and as recipients there is no residual instability. Henceforth I shall refer to them as true wild type, cells carrying only the c^+ allele.

Segregants result from separation of coexisting alleles in the c strain, not from a high frequency of reversion.

The only direct way to prove that the segregants are not generated by a reversion event is to demonstrate the simultaneous presence in a pure c culture of DNA bearing both mutant and wild type determinants. Unfortunately this is impossible for c itself, because we have no means of assaying c^+ activity in transformation. A c culture used as recipient will give rise to wild type, but it is impossible to distinguish wild type segregants from wild type transformants.

Nevertheless a slightly less direct means is available resulting

from an association of c with a second SA-resistant mutation, d. I have mentioned that c DNA can transfer all the c properties, including the instability, to wild type recipients. Thus wild type cells freshly transformed to c will segregate to wild type just as the original c strain does. Recipient strains bearing mutations in genes other than sul can also be transformed to the c SA resistance. These transformants segregate to cells identical to the original recipient.

When other sul mutants are used as recipients, c transforms them to yield doubly mutant strains of markedly enhanced SA resistance (see Chapter II, Materials and Methods, p. 25). Thus c DNA transforms a recipients to give ac transformants; b recipients to give bc transformants; and d recipients to give cd transformants. Each of these is readily selected on the basis of its enhanced SA resistance (Hotchkiss, Abe, and Lane, 1971).

Each of these transformant types can be demonstrated to segregate to the original recipient type. Thus ac colonies give a segregants; bc give b; and cd give d. However, some of the cd transformants can also segregate to wild type (Hotchkiss, Abe, and Lane, 1971). These can be seen as NOB-resistant colonies, while the transformants which segregate to d only are entirely NOB-sensitive. Since a wild type segregant must possess the wild type allele of d, that is d^+, and since it is clear that this allele is not present in the d recipient strain, the two possibilities are that a d^+ allele is brought in along with the c in the original transformation or that c can induce reversion of the d locus to d^+ in some cases. These two possibilities are distinguished by preparing DNA from two cd strains, one of which gives rise to both d and wild type segregants, the other of which segregates only to d (Hotchkiss, Abe, and Lane, 1971). The cells from which the DNA is prepared are grown in the presence of SA sufficient to suppress the growth of segregants, so that the DNA represents as closely as possible the genome of the parent cd. When DNA prepared in this way is assayed for its transforming activity on wild type recipients, both cd types can be shown to have the full amount of c and d transforming activity. Moreover, when assayed on a d recipient, the strain segregating to wild type can transform the d to

$\underline{d}^+$ (NOB-resistance) at frequencies similar to wild type DNA. Thus the $\underline{d}^+$ allele coexists in the genome of these $\underline{cd}$ strains, and the wild type segregants arise from a true segregation of this $\underline{d}^+$ from the resident $\underline{d}$, not from a conversion comparable to reverse mutation. As anticipated, those $\underline{cd}$ types which do not segregate to wild type have no $\underline{d}^+$ transforming activity in their DNA.

Although this demonstration does not prove that the segregation of $\underline{c}$ itself occurs by the same mechanism, it seems to be valid inference, particularly when it is remembered that the $\underline{d}$ region does not segregate at all without $\underline{c}$ being in association with it, and that when $\underline{d}$ segregates, $\underline{c}$ does too.

Thus we must consider $\underline{c}$ to be a merozygote, a partial diploid of pneumococcus, in which the diploidy can extend at least to the $\underline{d}$ region of the genome.

The c mutation occupies a unique position in the chromosomal continuity of pneumococcus.

Merozygotes can be of two kinds: those in which the extra genetic determinant(s) are borne on an extrachromosomal piece of DNA, such as an F factor, and those in which the extra material has a unique position in the continuous structure of the chromosome, such as an integrated episome, or a duplication of a portion of the chromosome. In the former case an assay of the DNA for genetic linkage will show the unstable marker to be linked only to other markers whose instability can be demonstrated. In the latter case it should be possible to demonstrate linkage to markers outside the region of instability.

Linkage in pneumococcus is measured most readily as "cotransfer frequency" (Hotchkiss and Marmur, 1954), the ratio of the number of cells simultaneously transformed by doubly-marked DNA for both markers, x and y, to the total number of cells transformed to the less efficient of the two, x.

$$\text{Cotransfer frequency} = \frac{N_{xy}}{N_x}$$

Thus it is a measure of the proportion of the times a molecule is integrated bearing marker x that marker y also enters. When cotransfer frequency is high, and linkage is tight, this can be an unambiguous measure not only of the presence of linkage, but also of its degree: the higher the cotransfer frequency, the more closely linked the two markers. However, below a frequency of about 5% it becomes a less conclusive test. At the high DNA concentrations needed to obtain adequate yields of transformants, there is an increased probability that a single cell will be doubly transformed by successive interaction with two different DNA molecules. When this is the case, it is necessary to resort to more analytical methods, such as a kinetic analysis of the rate of accumulation of transformants (Kent and Hotchkiss, 1964) or the dependence of transformant yield on DNA concentration (Hotchkiss, 1966).

We have seen that the c marker can influence the d locus to develop instability. Our deduction that in the original transformation c brought the d^+ locus along with it into those cd transformants capable of segregating to wild type suggests that the c and d regions are probably linked. About 50% of the cd transformants I examined were of the type able to segregate to wild type, suggesting this as the range of linkage. DNA isolated from cd strains was used to transform wild type. DNA from c strains was used to transform d strains. The results are in Table IV.

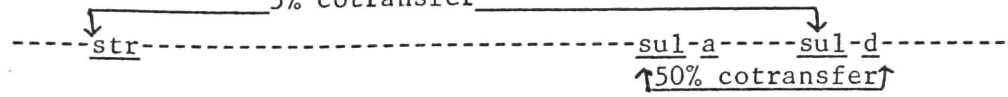
Table IV: Linkage of c to d and d^+

Donor	Recipient	Ratio measured	Value of the ratio
cd	wild type	cd / total c	0.66 ± 0.32 (n=14)
c	d	(c - cd / total c	0.66 ± 0.15 (n=14)

Therefore c is very closely linked to the locus of d. Abe, Lane, and Hotchkiss (personal communication) have obtained similar, and slightly higher cotransfer ratios in the past.

The current map of the stable markers in the sul region is as

follows:



The next point to establish is whether other markers besides d can be unstabilized in the presence of c. I chose to examine in particular str and a. Segregation of the former marker is especially difficult to follow, since there is no way to select the str-sensitive segregants. Only in the case of an unstable clone segregating so rapidly that a large proportion of the cells lose their resistance could such a segregation be detected by differential scoring. If segregation occurred less readily, it would be necessary to select resistant colonies and subclone them in the hope of finding a few sensitive segregants among the subclones.

I decided at the outset that to examine c transformants of str-resistant recipients by DNA carrying c and to look among them for the (hypothetical) rare colony in which c had carried with it a linked str^+ (sensitive) allele would be unrewarding. Assuming a linear genetic map for pneumococcus, c would be only weakly linked to str, if at all, judging by its close relationship to d. Thus it would be a rare transformant indeed with even the potential for segregating at the str locus. It might be more reasonable to select str-resistant SA-resistant double transformants of wild type recipients, and to look among these for a str-sensitive segregation. However, in that case it would be necessary to impose a selection using streptomycin, which might tend to favor stably integrated transformants. Perhaps the greatest chance of getting str transformants in an unstable form would be to use DNA from a str-resistant strain to transform str-sensitive c recipients. In this case if it were possible for the str marker to integrate into the merozygotic segment, it would probably do so; and it would be highly unlikely for both the chromosomal and duplicated str^+ alleles to be substituted by transformation to the resistant allele.

Table V summarizes the results of five independent experiments.

Table V: Streptomycin resistance in transformants of c recipient cells by str donor DNA

Experiment no.	No. colonies tested	Str-resistant cfu / total colony-forming units × 100
1	6	77.5; 124**; 115; 66**; 128; 76.5
2	4	74*; 89; 100; 111
3	6	109; 66*; 98; 109; 61*; 122
4 (subclones of 98% str-resis. colony in expt. 3)	8	83; 104; 53*; 72*; 99; 89; 154*; 97
5	37	Similar to the above; five looked potentially like segregators, but proved to be normal.

* signifies that the percentage is beyond the limits of one standard deviation of the determination in its difference from 100%.
** signifies colonies which gave at least one subclone which appeared to be entirely sensitive to streptomycin. In no case were these subclones segregant for c; further testing showed them to be SA-resistant contaminants.

Two clones from experiment 3 and two from experiment 4 were used to prepare DNA in order to see whether both streptomycin-sensitive and -resistant alleles were present. However a scoring of the cells in the culture for the DNA preparation showed that only one of the four clones still gave a value significantly lower than 100%, that is 83%. The DNA of this strain was as active in transferring str-resistance to sensitive recipients as was a very active non-c DNA preparation:

Table VI: Ability of Scd-2 DNA to transform various recipients to streptomycin resistance

Source of DNA	Percent transformation of wild type recipient	c recipient	d recipient
str-mic-sul-d	4.8	2.1	0.33
str-sul-c-sul-d	5.5	2.0	0.34

Thus out of a total of 53 independent transformants of c, of which 37 were chosen with particular care with regard to their independence, limited exposure to the selective agent, and minimum number of divisions before testing (experiment 5, Table V), none showed an unstable integration of the str marker which persisted through the duration of a passage.

It is clear that either the instability detected by differential scoring is very short-lived, or the scoring error is greater than estimated. In either case no persistent instability was detected.

It is conceivable that the generation of a $\underline{str}^+/\ \underline{str}$ merozygote would show a streptomycin-sensitive phenotype (Lederberg, 1951; Matney, McDonald, and Goldschmidt, 1962). Then only when all possible str loci carried the resistant allele would the cell show a resistant phenotype. In that case no segregation of str could be demonstrated insofar as no sensitive alleles would remain. However the partial heterozygotes formed as intermediates in the normal transformation process are capable of surviving at least a brief challenge with streptomycin as long as two divisions after transformation (Hotchkiss, 1956; see also Breckenridge and Gorini, 1969). Thus it is unlikely for a $\underline{str}^+/\ \underline{str}$ merozygote to be sensitive to streptomycin, or for all $\underline{str}^+$ loci to be str before resistance is expressed. The yield of str-resistant transformants in the c recipient is as high as in any other strain, and increases linearly with DNA concentration, demonstrating a need for interaction with only one DNA molecule to transform a given cell.

Hence by all available criteria we must conclude that the presence of c does not confer instability on the str locus, which is thus beyond the region of merozygosis.

The determination of the involvement of the a locus in the segregating region has been slightly more straightforward. The experiment could not be done by introducing an a marker into a c recipient by transformation because the SA resistance of ac cells is so close to that of c that the transformant type could not be selected against the vast background of untransformed c cells. Thus it was necessary to transform an a recipient with DNA from a c strain and pick ac transformants. From a study of three such ac transformant clones, Abe (Abe and Hotchkiss, unpublished results) had found only a segregants. However I repeated the experiment on an expanded scale, since it seemed likely to me that the probability of c DNA transforming a recipient cell for c and at the same time bringing in the wild type allele of the resident marker might be related to the degree of linkage between c and the marker in question. Since c is much

less tightly linked to a than to d, the map order must be ---a----d--c-- (Hotchkiss and Abe, personal communication). Only about half of cd transformants are able to segregate to wild type, correlating with the 65% cotransfer frequency of c and d^+. Since a is on the other side of d from c and linked to d (50% cotransfer), it might be expected that a still lower proportion of ac transformants would be able to segregate wild type. Accordingly I isolated ac transformant colonies from agar plates. After a period of rejuvenation, an aliquot of each colony was plated in agar medium containing 20 μg/ml of NOB, to which the transformant ac is sensitive but a segregants and wild type are resistant. These segregant colonies in their turn were picked from the plates, four per transformant, rejuvenated, and tested for the presence or absence of PAS resistance. Wild type cells are sensitive to PAS while a cells are resistant. In the first 12 colonies tested I found ten, each of which gave four out of four segregants completely resistant to 20 μg/ml PAS. One of the others produced three resistant segregants and one sensitive. The twelfth yielded four sensitive segregants. The PAS-sensitive segregants are true wild type by the criteria of drug resistance spectra. Thus two sorts of segregants have clearly been demonstrated. There is the possibility that owing to the similarity of SA resistance between ac and c, some original transformants might be ones in which the c DNA brought an a^+ allele along with it which replaced the resident a marker rather than coexisting with it, giving rise to a pure c transformant. This may well be the case for the transformant whose segregants are all PAS-sensitive, or wild type. However, the one clone which seems able to give segregants of both types might be an ac transformant in which the a region has been unstabilized in a manner analogous to the d region in cd transformants.

To test this possibility, I attempted to determine the nature of segregants from subclones of the original transformant which gave rise to the two kinds of segregants. Four segregants were tested from each of 8 subclones. Seven out of the 8 yielded all four segregants of the a type. One out of 8 gave all four segregants of the wild type. Thus it appears that the original transformant rapidly segregated to produce a mixture of ac and c, the former giving rise only to a segregants, and the latter only to a^+. An alternative possibility, that two transformant clones

grew together during selection, is most unlikely under these experimental conditions.

Thus 13 (3 of the earlier work and 10 reported here) ac transformants gave fewer than 25% wild type segregants. The one transformant which may have had an unstable a region lost the instability too readily to study it more closely. Thus the chances are less than 1 in 14 (0.07) that ac transformants can be stabilized in a form which readily segregates wild type. In other words it seems very unlikely that the segregating region can be extended toward the a region at a frequency at all similar to its linkage of a to the unstable marker c.

It follows from this conclusion that the possibility of the str locus being in the segregating region is still more unlikely. For all practical purposes it may be considered a stable marker even in strains carrying the unstable c marker.

Having defined the limits of the extent of the c region in the direction of a and str, we can proceed to the question whether c occupies a site in the chromosomal continuity of pneumococcus, or whether it behaves as an extrachromosomal element. To answer this question, I chose to measure the linkage between c and str.

Str and d are cotransferred about 5%, the lower limit of accurate measurement in our transformation system. Str and c seemed to previous workers to show regular cotransfer frequencies in the range of 2 to 4%; but as I mentioned above, at this level of co-transformation accurate determination of double transformants requires that the concentration of DNA be relatively high leading to a sizable danger of consecutive single transformations. At various times and under various conditions I measured the c-str cotransfer frequency with the results indicated in Table VII. It is clear that overall these values are rather variable. However if they are grouped on the basis of the source of the donor DNA, the variability diminishes dramatically.

Table VII: Cotransfer frequency of c and str in three DNA preparations

DNA preparation	c-str / c
PN 479	0.044; 0.024; 0.033; 0.033; 0.032; 0.032; 0.030
	0.026; 0.027; 0.020
	Mean = 0.030 ± 0.006
PN 488	0.012; 0.014; 0.007; 0.006; 0.006; 0.003
	Mean = 0.008 ± 0.004
PN 490	0.010; 0.016; 0.007; 0.012; 0.011; 0.012; 0.012
	Mean = 0.011 ± 0.002
Overall mean = 0.019 ± 0.011	

Thus although these data cannot be said to demonstrate linkage between c and str, there is certainly some property of the DNA, which can vary from preparation to preparation to which the cotransfer frequency provides an index. If this property be physical linkage, then it is reasonable to attribute the source of the variability among the different preparations to variable amounts of shear and consequent breakage of the linkage to a greater (PN 488) or a lesser (PN 479) degree. That linkage of this weakness is susceptible to controlled shear forces has been amply demonstrated by a number of workers (Mindich and Hotchkiss, 1964a; Berns and Thomas, 1965; Kelly and Pritchard, 1965). Nevertheless a more conclusive demonstration of linkage is needed.

Figure 1 is a plot of the transformation yield for single markers or simultaneously scored marker pairs, as a function of DNA concentration. The curves for the single markers, c, str, and mic display the well-established properties of linearity (slope = 1 on a log-log plot) at low DNA concentrations and a plateau region at higher DNA concentrations (Hotchkiss, 1966). The former reflects the interaction of one marked DNA molecule with a cell as a sufficient condition to yield a transformant, and the latter results from the progressive saturation of cellular receptor sites at higher concentrations of DNA. That this saturation occurs at the same concentration of DNA for all markers attests to the validity of this interpretation.

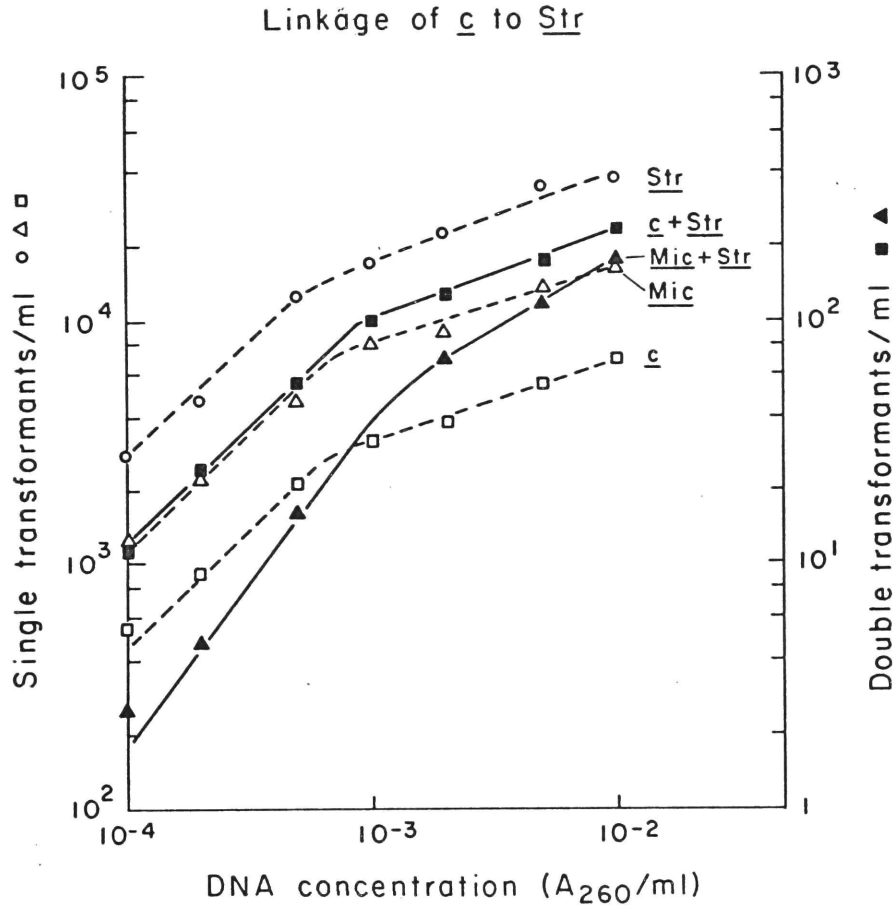


Figure 1: Transformation frequency as a function of DNA concentration for single markers and marker pairs in DNA from a strain bearing c, str, and mic. Exposure to DNA: 15 minutes; scoring performed in agar plates.

When two markers known to be borne on separate pieces of DNA are assayed, such as str and mic (Kent and Hotchkiss, 1964), the slope of the curve at the lower concentrations is two, that is, two pieces of DNA must interact with a cell to doubly transform it. However when c and str are the two markers assayed, it is clear that the curve reflects those of the single markers, with a slope of one at the lower DNA concentrations. Interaction of a cell with a single DNA molecule can transform it for both markers. Thus the two markers must be borne on the same molecule, that is, linked. The degree of linkage is probably best reflected by the figure for PN 479 in Table VII above. This is the maximum linkage I obtained, 3%.

Since c is linked to str, and str is shown to be well outside the region of instability, c must have a location in the continuity of the chromosome. Thus it is probably not exclusively extrachromosomal in nature, although the possibility that it exists in an episomal mode as well as an integrated mode is not ruled out.

The d marker may associate with c in a variety of configurations.

In the preceding discussion (p. 39) I used some properties of cd strains to demonstrate the merozygotic nature of strains containing the c marker. At that time I mentioned that cd strains could be distinguished from one another by two criteria: 1) by their ability to segregate to wild type as well as to d, and 2) by the presence or absence in their DNA of the d^+ allele, as assayed by transformation. Both of these properties may be more finely quantitated than indicated in that discussion.

With respect to the ability to segregate wild type, of those cd strains which segregate any wild type at all, at least two categories can be distinguished on the basis of the frequency of wild type segregation. One group gives rise to 5 to 10% NOB-resistant wild type segregants; the other yields only about 0.1 to 1% wild type. Similarly the frequencies of the d and d^+ alleles in the DNA may be quantitated, and they too seem to fall into categories of more d correlated with less d^+, and vice versa.

Prototypes of the various segregation classes of cd were designated for a more intensive investigation. The type segregating frequently to wild type is cd-10, the less frequently segregating type is cd-13, and the type which apparently never segregates to wild type is cd-11. Table VIII is a summary of their properties reproduced from Hotchkiss, Abe, and Lane (1971).

Table VIII: Segregation frequencies and marker frequencies of various strains.

Strain	Phenotype	Segregants type	Segregants frequency	Marker frequencies d^+/str	Marker frequencies d/str
wild type	c^+d^+	-	-	1.8	0
sul-d	c^+d	-	-	0	1.8
sul-c	cd^+	wild type	3%	2.7	0
cd-10	cd	wild type	6%	1.6	0.7
		d	low		
cd-13	cd	wild type	0.4%	0.8	1.7
		d	medium		
cd-11	cd	d	1%	0	2.8

It is apparent that the c strain has a great deal of d^+ activity and no d; cd-10 has less d^+ and some d; cd-13 has some d^+ and much more d; while cd-11 is very active as a donor of d while completely lacking in d^+ activity. Furthermore if one considers the sum of d and d^+ activities for the four c-containing strains, it is remarkably constant (2.3 to 2.8) and exceeds significantly the dosage of d or d^+ marker in the non-c strains (1.8). A striking feature of these data is the almost integral increments by which the various strains differ in their activities for one or the other marker. Thus despite the fact that the three cd strains originated from the same cross, c donor by d recipient, it is clear that each has a characteristic activity for the markers d and d^+.

In pneumococcal transformation each marker has an intrinsic transforming activity which is independent of the quality of the DNA preparation or the competence of the recipient cells. This activity is generally expressed as the transformation frequency for that marker as a fraction

of the transformation frequency for a standard marker in the same DNA. In our system the standard marker is str-r7. If all markers had the same intrinsic activity, this fraction would always be 1.0. However, two factors, dosage and efficiency, act to make the fraction different from 1.0 for a number of markers.

Dosage is a term reflecting the number of alleles of a locus that are present in the total genome at a given time. A clear example of a dosage-related change in transforming activity is the situation in heteroduplex DNA. From denatured DNA's the strands may be separated and annealed either to their original complementary partners (homoduplex) or to the complementary strand of a heterologous DNA (heteroduplex). If the two original DNA's both contain one marker, for example str, but only one contains a second marker, such as d, then the reconstituted DNA's will have very different activities of d relative to str, with the heteroduplex being about half as active for d as the homoduplex. This effect is presumably a reflection of the dosage of d, it being present in both strands of the homoduplex but in only one strand of the heteroduplex. A similar reduction in the apparent marker activity will occur if the cells from which DNA was originally isolated are contaminated with unmarked cells. Enhancement in the dosage of a marker has been seen in Bacillus subtilis where a marker which replicates early in the replication cycle is found to be more abundant in exponentially growing cultures relative to stationary phase cultures or spores than is a marker which replicates late. While the replicating fork lies between the two markers, the former will be replicated and the latter not, thus making the relative abundance of the early marker twice as great as usual (Yoshikawa and Sueoka, 1963; Yoshikawa, O'Sullivan, and Sueoka, 1964).

Efficiency is a phenomenon restricted to transformation systems (Hotchkiss and Gabor, 1970; Yoshikawa and Sueoka, 1963; Day and Ruppert, quoted in Hotchkiss and Gabor, 1970). It is as much a characteristic of a particular genetic marker as is phenotype or map position, and is measured as the yield of transformants for the marker of interest relative to the yield for a standard marker in the same donor DNA. Marker efficiencies of transformation vary from as little as 0.1

relative to str (the classic low efficiency types, Ephrussi-Taylor, Sicard, and Kamen, 1965; Lacks, 1966) to several times str (Lacks, 1966). The efficiency of c is in the vicinity of 0.2, while d is about 1.6 to 1.8. A great deal of effort (Sicard and Ephrussi-Taylor, 1965; Gray and Ephrussi-Taylor, 1967; Lacks, 1970; Tiraby, 1971) has been devoted to understanding the physiological basis of efficiency. In effect certain mutations which transform at low efficiency may regularly be eliminated from or repaired out of the donor-recipient complex during the recombination event leading to the production of transformants, while mutations of high efficiency are much more often tolerated. Since extremely closely linked sites within the same genetic locus, such as c and d, can display vastly different efficiency in independent transformations, it is thought that certain specific base-pair mismatches between donor and recipient DNA molecules, monitored by a specific enzyme system are preferentially eliminated (Gray and Ephrussi-Taylor, 1967). In support of this hypothesis is the existence of a number of mutant strains which all tested markers transform with equal efficiency (Green, 1959; Lacks, 1970; Tiraby, 1971).

Returning then to our cd strains and their unusually variable transforming activities (see Table VIII above) as donors of d and d^+, the question arises whether these are effects of dosage or of efficiency or of some combination of the two. If they could be viewed entirely as a phenomenon of dosage, some fascinating possibilities arise. Since the total activity at the d locus in c-associated strains is almost exactly 3/2 that in non-c strains, models can be constructed in which the standard strains have two doses of a region and the c strains three doses. From here it is a short step to the hypothesis that the two standard doses are represented by the two strands of DNA, and therefore the three doses in c strains might represent a triple-strandedness.

Alternatively, if the explanation were to be based entirely on efficiency, it would be necessary to hypothesize a c region with two parts, one of higher efficiency than the other. Overall the unstabilized d region is integrated only 1.5 times as efficiently as the d region of non-c strains, presumably owing to some structural difference between the two portions. The cd strains then would differ among themselves in the

way in which d and d^+ alleles are assorted on the higher and lower efficiency portions.

An attempt was made to differentiate these two factors by testing the efficiency of transformation of c in two of the strains described above which tend to normalize marker efficiencies. Each single site marker transformed into these strains has an efficiency close to unity (Green, 1959; Lacks, 1970). Clearly markers which normally transform at the same frequency as str cannot be distinguished in such a strain. Markers which transform with higher efficiency than str have not been tested (Tiraby, 1971). Since c is normally of low efficiency, about 0.2, we wondered whether this low efficiency were of the sort caused by mismatch at a single site, the sort that could be normalized in the recipient strains Rx-1 and hex-1 (see pp. 26-27).

The first step was to grow the cells to the competent state. Both strains grew poorly probably because of subtle and undefined differences in requirements of the cells. At first the Rx-1 culture grew with a doubling time of about 45 minutes, 50% longer than our wild type, R1-26, and it went into the stationary phase of growth at a cell concentration too low to be compatible with competence. By increasing the yeast extract content of the CH-T medium, I was able to promote exponential growth up to a titer of 2×10^7 cfu/ml, but there was still no appreciable transformation. Subcloning the culture still gave a maximum competence of about 0.2%, too low for any meaningful comparisons of relative transforming efficiencies. Similarly the hex-1 strain was recalcitrant to our customary procedures of development of competence.

Therefore I turned to members of the laboratory of Dr. Alexander Tomasz, Dr. Richard Ziegler and Mrs. Eva Zanati, who very kindly supplied me with preparations of the pneumococcal competence factor (Tomasz and Mosser, 1966). Generally I preferred to use cells whose competence was internally developed to avoid unnecessary sources of possible variation, but that did not seem feasible for this experiment.

Frozen cultures at a concentration of about 3×10^6 cfu/ml were thawed at 4°C., diluted twenty-fold into fresh CH-T and exposed to freshly thawed competence factor, 0.1 ml/ml of recipient culture, at 30°C. for

20 minutes. Then the appropriate DNA was added and exposed to the now competent cells for another 20 minutes at 30°C. The transformation was terminated by DNase and the cultures sampled to score the various drug resistances at the appropriate times as detailed on p. 32. Untransformed control cells were included in all scorings for drug resistance, but the background levels were negligible.

The results are given in Table IX.

Table IX. Transformation of various strains which normalize marker efficiencies

Recipient strain	Donor DNA	d/str	c/str	cd/str	mic/str	opt/str
Rx-1	c-str-mic		0.31		1.0	
	cd-13-str-mic	1.7	0.28	0.15	0.71	
	opt-str					0.80
hex-1	c-str-mic		0.26		0.95	
	cd-13-str-mic	---	0.07	0.06	1.1	
	opt-str					1.3
	d-str	1.1				
R1-26	c-str-mic*		0.22		0.44	
	cd-13-str-mic*	1.9	0.28	0.16	0.56	
	opt-str**					0.42
	d-str	1.5				

* from unpublished data of Mrs. Esmeralda Party
** from unpublished data of Dr. Gerald F. Vovis

Donor DNA's are identified by the drug resistance markers they bear. R1-26 is the wild type recipient, in which efficiency differences are manifest; Rx-1 and hex-1 are the recipient strains in which they are not. For micrococcin and optochin resistance it is clear that the strains are behaving as anticipated. Efficiencies of about 0.4 to 0.5 in R1-26 become about 1.0 for both markers in either normalizing strain. The d marker in a non-c

strain also appears to be normalized; these data may be the first reported for a marker of efficiency greater than str normally. However, for c the transforming efficiency appears to be unaltered. Even more striking is the observation that d and the cd pair in cd-13 DNA are similarly unaltered. This suggests that c strains have a basic structural abnormality which makes it hard for them to be integrated into the recipient genome at transformation, and which is different in kind from the abnormality that confers low efficiency of integration on other single site markers. It is unlikely to be entirely a dosage abnormality: even if the dosage of c were the minimum imaginable, one allele on one strand of DNA, one would anticipate c/str = 0.5 in Rx-1 or hex-1. Thus clearly some other factor is involved. We know that the c merozygosis extends over more than a single site, since the neighboring d region can become involved. Perhaps integration of an extended region altered in an unknown fashion involves difficulties on a different scale from that determining classical low efficiency.

In any event we have made little progress toward distinguishing whether the unusual marker activities for the d and d^+ locus are related to the gene dosages of the markers, or might be rather a reflection of transforming efficiency modified in a different way. We can only say that dosage is not the sole determinant of the marker activities; and that if dosage does play a role, perhaps it is modified by an intrinsic efficiency of the c region.

Perhaps the problem could be approached from a different direction. The prototypic cd strains, cd-10, cd-13, and cd-11 were all generated from a cross in which the c component was the donor and the d component the recipient. Perhaps more could be learned about the possible variety of associations of c and d if transformants from the reciprocal cross were investigated.

I first set out to develop a competent c strain. Since the stock culture had never been grown to competence, there was some difficulty. However isolation of a subclone provided a culture which yielded 2% transformation. It was this culture which was used in the study of str-c transformants and of the possibility of the str marker segregating (see pp. 42-44 above).

At first, hoping to study the possibility of the resident c marker being removed by transformation, I grew the culture in the presence of PAS or SA at concentrations high enough to just inhibit the growth of

wild type segregants. The reasoning was that under those conditions wild type transformants might be distinguishable from segregants. However, it became obvious that although growth of segregants might have been inhibited in the growing culture, it was rapidly restored to its former frequency when the culture was diluted into non-selective medium.

Table X: Segregant frequency and transformability of the c recipient grown to competence in the presence and absence of drug.

Conditions of growth	% NOB resistance	% str transformants
No drug	5.2; 3.6	0.93
SA, 20 μg/ml	5.4; 2.8	1.1
PAS, 20 μg/ml	3.3; 4.4	1.5

The transformation yield for other markers, here exemplified by str, was not affected in any consistent way in the presence or absence of drug. Although for the most competent preparations, the yield of NOB-resistant cells was greater in cultures containing c^+ DNA than in untransformed controls, there was no way to separate or distinguish segregants from transformants, so this approach was not pursued.

The linkage of d to str is maintained when a donor DNA bearing these two markers is introduced into a c recipient (cd-str/str = 0.03 ± 0.006, n = 4). However the efficiency of d is about half that in a wild type recipient (d/str = 0.9 ± 0.3, n=5).

In the body of work done with the competent c the procedure was as follows: a competent culture was thawed and preincubated at 30°C. for a time previously determined to give the optimal transformation yield, usually 10 to 15 minutes. Then the cells were diluted twenty-fold into CH-T containing DNA marked with str and mic in addition to d. The DNA concentration was in the low plateau range, 0.01 to 0.02 OD units/ml. After a 10-minute exposure at 30°C., DNase was added to stop the reaction, and the tube was incubated at 37°C. for phenotypic expression. In experiments in which selection was imposed immediately after transformation, cd transformants were scored by diluting the transformation mixture into medium containing 600 μg/ml of SA after 20 minutes at 37°C. This medium was either liquid containing anti-R, or solidified with 1% agar (see

Materials and Methods). In experiments in which selection was delayed, the following procedure was used: An aliquot of the transformation mixture was assayed for its content of transformants after the appropriate expression periods. The rest of the mixture was frozen after 20 minutes at 37°C., an arbitrary period to allow a minimal stabilization of newly integrated DNA, but well below the time at which transformants begin to replicate. From the results of scoring of the aliquot, the number of cd transformants per ml of the frozen culture was calculated. The contents of this tube were thawed, diluted, and aliquots distributed so as to contain an average of 0.2 transformant per tube.

According to the Poisson distribution this procedure ensures that most tubes which upon subsequent incubation and scoring are found to contain transformants, will contain the progeny of a single transformation event. Thus about 100 aliquots are distributed, and among these about a quarter will have one transformant each. In addition, of course, all tubes have a 20- to 100-fold excess of untransformed cells. The aliquots are grown 8 to 10 hours to permit multiplication of the transformants to a point where they may be recognized in a sample of the aliquot. Then samples are scored in medium with 600 μg/ml of SA to identify those tubes containing the progeny of a cd transformant.

This elaborate process was designed to ensure the independence of the transformants whose properties would subsequently be tested. The selection step used to identify the transformant-containing tubes can also be used as an isolation of the transformants themselves. However, the original tubes have never been exposed to drug, so they may always be incubated for longer periods and more transformants selected at any time.

The results of this series of experiments are summarized in Table XI. I have classified the results according to whether selection was immediate or delayed (see above), and whether the transformant colonies eventually tested were picked from liquid medium in tubes or from agar plates. My working hypothesis was that the generation of types of cd transformants when c DNA and d recipient cells were crossed was related to some feature of the merozygosity itself, not to the polarity of the

cross. Thus reversing this polarity, using a d donor and a c recipient, I expected to find two distinct classes of transformant based on frequency of segregation to wild type. The third class corresponding to cd-11, which never segregates to wild type, was not expected to arise, since the recipient cell always contained the d^+ allele.

Table XI: Segregant frequencies of transformants from the cross of a c recipient culture by d donor DNA.

	Immediate selection		Delayed selection	
	tubes	plates	tubes	plates
Expt. 1	1.9 ± 1.1% (n = 6)			
Expt. 2			type I 4.9 ± 1.0% (n = 5)	
			type II 0.53 ± 0.12% (n = 15)	
Expt. 3	type I 3.3 ± 0.7% (n = 11)		type I 3.7 ± 1.3% (n = 6)	
	type II 1.0 ± 0.5% (n = 6)		type II 1.1 ± 0.23% (n = 4)	
Expt. 4		type I 6.1 ± 3.7% (n = 16)		type I 3.2 ± 1.7% (n = 5)
		type II 0.63 ± 0.39% (n = 27)		type II 0.51 ± 0.26% (n = 25)

In a preliminary experiment, Experiment 1, six transformants were tested for their segregation frequency in 20 μg/ml NOB. The numbers reported are the means of the indicated numbers of transformants plus or minus the standard deviation, as a rough measure of the variability of the mean. There was no evidence of any division of the first six colonies into sub-classes of any sort, although the segregation frequency was by no means uniform and the sample size was small.

In this first experiment the SA challenge may have come before the newly-transformed cells were able to stabilize into some preferred configuration. I therefore repeated the transformation, diluting early and

selecting only after about 12 hours of growth in the absence of drug. These transformants (Experiment 2) showed two very distinct categories of segregant frequency, one about ten times the other. Within either of the two categories the type of transformant is very similar, in that the variation among descendants selected from the same tube (i.e., a single original transformation event) is about the same as among transformants of the same category selected from different tubes.

Experiment 3 was an attempt to establish whether it was early, as opposed to late, selection that determined whether or not the two categories could be detected. The result here was that in both conditions two distinct classes were obtained, with the mean of one about three times the mean of the other. Similarly when the selection was performed in agar plates (Experiment 4), the two categories were identifiable both early and late. The conclusion from Experiments 2 to 4 is evident: cd transformants generated from the cross of a d donor by a c recipient fall into two categories (types I and II) with respect to their wild type segregation frequency. These two categories are superficially similar to the cd-10 and cd-13 types generated in the reciprocal cross (Hotchkiss, Abe, and Lane, 1971). Out of 126 transformants tested, none was unable to segregate to wild type. Thus the prediction of the hypothesis is fulfilled.

DNA was prepared from subclones of two of the transformants in Experiment 2, one from each category. Table XII shows the results of duplicate assays of these DNA's for their frequencies of d and d^+. The resemblance of type I to cd-10 and type II to cd-13 is striking.

Table XII: Marker frequencies in DNA from type I and type II strains.

Source of DNA	Segregant frequency of cells	d^+/str	d/str
Type I	5.8%	1.6	0.7
Type II	0.47%	0.8	1.5
cd-10*	6%	1.6	0.7
cd-13*	0.4%	0.8	1.7

* From Hotchkiss, Abe, and Lane, 1971.

Thus it appears that no matter in which direction the transformation cross is performed, cd transformants can fall into categories typified by the cd-10, cd-13, and (using a c donor and a d recipient) cd-11 of Hotchkiss, Abe, and Lane. These must therefore represent favored, if not unique, associations of c with d, although the structural basis for these associations remains unclear.

IV

CHARACTERISTICS OF THE SEGREGATION PHENOMENON

Segregation frequency is a characteristic parameter of a c culture

That the original sul-c strain was unstable became evident from the accumulation of segregants in the stock strain. If c stocks were passed through SA or PAS to remove all pre-existing segregants and then repeatedly passed in non-selective medium, the proportion of the culture that was NOB-resistant rose from 2% to 5% to 10% to as high as 30% (Hotchkiss, personal communication). And the first notice of the instability of c was taken when a culture was observed to contain 50% segregants.

However in recent years it has become more and more difficult to demonstrate this accumulation. Although the frequency of segregants in a population may vary even in repeated scorings of the same culture, it has not been possible for us to show a systematic accumulation over the course of many generations.

In Experiment 1 described in Table XIII (treated in less detail in Table X, p. 56), a culture of c was diluted and distributed for single colonies in FSB with 20 μg/ml of SA, 20 μg/ml of PAS, or no drug at all. Ten colonies from each group were then tested for their segregation frequency on two separate occasions. The total cell population in each colony was about 5×10^5 to 3×10^6 cfu/ml. Among the colonies grown without drug, four were pure segregant, that is, 100% resistant to NOB and sensitive to SA. The means and standard deviations for segregant frequencies among the other six as well as the ten from SA and the ten from PAS are given in the table.

In Experiment 2, 47 colonies were isolated from an agar plate in the absence of drug. The number of cells per colony ranged from 1×10^5 to 3×10^7. One of the 47 was a pure segregant. The mean of the other 46 is given in the table.

Table XIII: Segregant frequencies of subclones of c

	Drug in the growth medium	% NOB-resistant colonies	
1	No drug	5.2 ± 2.9	3.4 ± 1.4 *
	SA, 20 μg/ml	5.4 ± 2.2	2.8 ± 1.0
	PAS, 20 μg/ml	3.3 ± 1.8	4.4 ± 1.5
2	No drug	8.6 ± 4.5	

* These are duplicate results obtained on two separate occasions.

It is clear that even under conditions in which segregants are unable to accumulate in a colony, their proportion of the total after scoring is indistinguishable from the case where there is no overt selection against segregants. The theory developed and demonstrated by Luria and Delbrueck (1943) shows that mutation is a process occurring randomly in time and without regard to the imposition of selection. Accordingly in any such process the variance of the distribution of frequency of mutations in cultures grown from small initial inocula should be much greater than the mean. This reflects the clonal distribution of mutations. Clearly this pattern does not hold for the segregating cultures. Segregants will arise in the absence of NOB, so there is no suggestion that NOB induces segregation in a constant subpopulation of the culture. And the division time for segregants is indistinguishable from that for the parent c culture, so it is unlikely that differential growth could account for the result.

The Luria-Delbrueck fluctuation test carries with it the assumption that the process involved is a single step and reversible only rarely. Let us suppose that the process of segregation involves more than one step and that each step except the final one is reversible. If for example, NOB could permit growth of all cells which had passed the first step, but the cells retained their SA resistance until the last step had been passed, this might explain the apparent paradox of stable segregants that fail to accumulate. Moreover, NOB-resistant colonies selected in liquid culture and then tested for residual SA resistance often have as many as 30% of the cells still able to grow in SA. This is far more than

can be accounted for from mere survival of incompletely starved non-segregant types (see Appendix B).

Although measured segregant frequencies do vary in consecutive determinations, it is nevertheless possible to distinguish strains on the basis of their segregant frequency. We have already seen this in the 3 to 10 fold difference between cd-10 and cd-13 (Table XI and the data quoted there; other examples appear below, Table XXI). Another example is the following:

Scd 3 was a single colony transformant of a c recipient by str-d donor DNA. It was subcloned in drug-free medium, and two of the subclones were Scd 3D and Scd 3H. The segregation frequencies of these three cultures in the course of three serial non-selective passages is given in Table XIV.

Table XIV: Change in segregation frequency with serial passage.

Strain	Passage no.	No. of generations/passage	% NOB resistant
Scd 3	0	0	4.9
	1	6.5	3.6
	2	9.5	3.2
	3	8	3.3
Scd 3D	0	0	18.6; 14
	1	6.5	8.4
	2	9	8.8
	3	9	7.0
Scd 3H	0	0	0.88; 6.0
	1	5.5	1.5
	2	8	1.7
	3	9.5	1.6

This is a representative demonstration that for a given strain the segregation frequency tends to be a dependable parameter of the strain.

Attempts to influence the segregation frequency of the c strain

1. General intervention into cellular metabolism

By perturbing some of the conditions of cell growth, I hoped to alter

the segregation frequency in a measurable and interpretable way. There was a report that in B. subtilis, lysine in the medium increased the segregation frequency of an unstable strain (C. Anagnastopoulos, personal communication). I therefore tried to grow c, cd-10, and cd-13 in three media: 1) FSB without anti-R, relatively unenriched medium; 2) the FSB without anti-R, but supplemented with 600 μg/ml of L-lysine; 3) SB, three-fold more concentrated than FSB, with all the normal additives except anti-R. The results of two experiments are presented in Table XV. The initial inocula were adjusted so that the first experiment encompassed a 7-hour incubation in late log phase and early plateau, while the second experiment fell entirely in the log phase over the course of a 6-hour incubation.

Table XV: Segregation frequencies in a variety of growth media

			% NOB- resistant after growth in:		
Strain	Initial % NOB		FSB	FSB+lys	SB
c	4.5	Experiment 1	4.4	4.2	0.3
	6.8	" 2	6.9	3.3	0.8
cd-10	14.5	" 1	23	26	30
	13.5	" 2	30	11	16
cd-13	1.4	" 1	2.2	2.5	1.4
	6.5	" 2	1.8	1.6	1.4

For c and cd-13 it might seem that segregation frequency decreases as the richness of the medium increases. However cd-10 does not follow the same trend. The extremely low values of segregation for c in SB medium could be the result of the extremely rapid growth of c in this medium, about 50% more than in the other media, rather than an effect on the generation of segregants per se. Perhaps there is a lag in production of segregants per cell division which increases as the rate of cell division increases. However, the effect did not seem to offer convenient possibilities for further analysis, so I discontinued my investigation of it.

I next tried growing c cells at various temperatures to see how this might affect the segregant frequency. The medium was CH-T. A freshly thawed culture of c was diluted into CH-T and incubated one hour at

37°C. to allow the cells to come out of lag and begin growing actively. Then aliquots were diluted again, placed in water baths at various temperatures, and assayed for segregant frequency at various intervals at 37°C. The results are summarized in Table XVI. Here too, although fluctuations occur, no consistent pattern emerges.

Table XVI: Effect of incubation temperature on segregant frequency of c

Temperature	Division time	% NOB-resistant segregants (0 hrs.)	2 1/4 hrs.	4 hrs.	6 hrs.	16 hrs.
44-47°C.	All dead by 2 hrs.	2.9				
37°	32 min.	"	3.5	4.1	4.3	
30°	42 min.	"	3.0	4.0	3.8	
22°	252 min.	"	4.8	9.0	3.8	7.5
0°	half-life = 14 hrs.	"	1.7	2.5	10.9	4.1

Occasionally it seemed as if cultures scored several times over a period of days and frozen between scorings gave rather more variable frequencies of segregants than otherwise. I set out to test whether multiple freezing and thawing of cells in CH-T with 10% glycerol had any consistent effect on segregation frequency, with the results shown in Table XVII.

Table XVII: Effect of multiple freezing and thawing on segregant frequency

Strain	Yield of NOB-resistant segregants after freezing and thawing the following number of times: 1	2	3
c	4.5%	6.8%	5.1%
cd-10	14.5%	13.5%	7.6%
cd-13	1.4%	6.5%	1.5%

Once more there is considerable variability with little perceptible pattern. The main lesson gleaned from this experiment was that the variability tends to increase with the need for repeated thawing. Accordingly it was decided to freeze two aliquots of any culture that might need to be tested more than once. I found that the reproducibility of scorings made on freshly grown cells and those cells after one freezing was quite

good. This procedure thus gave three chances for an accurate scoring: one on the cells on the day of preparation and one on each of the two frozen aliquots.

2. Specific intervention in DNA metabolism

a. Background

Next I tested the effects of ultraviolet irradiation on the segregation frequency of c. As mentioned in the introduction, Lederberg's group noticed complex changes in the behavior of heterozygous diploids--generated in conjugation--after a dose of ultraviolet irradiation permitting 90 to 95% survival of the viable cells (Lederberg, 1951). At this dose more than 50% of the survivors now behaved as haploids. The maximum degree of haploidization occurred at about 80% survival. If after irradiation, the cells were grown on broth agar, they remained genetically haploid. However when incubated for a long time on minimal medium, variegated sectors reappeared in the center of the segregant colony (Lederberg, Lederberg, Zinder, and Lively, 1951). Apparently the basis of the diploidy was disrupted by ultraviolet irradiation and permanently lost during growth on broth agar, but was able to regenerate the diploidy if grown on minimal medium.

In the following years Hayes reported that a dose of ultraviolet light allowing 25% survival of one of his E. coli K12 strains stimulated the yield of recombinants 5 to 20 fold when this strain was then mixed with unirradiated cells of another strain. This stimulation occurred despite the decrease in cell viability. Again for the effect to be manifest, incubation of the mating mixture in complex medium was needed; and supplemented minimal medium was an ineffective substitute. In his discussion Hayes postulated that the action of the ultraviolet light was to "increase the proportion of the cells able to extrude potent gametes," and noted that the optimal dose for this effect was similar to that for the induction of lysogenic phages (Hayes, 1952). A more recent series of experiments (Evenchik, Stacey, and Hayes, 1969) confirmed that the fertility of F^+ populations was enhanced and that of Hfr population was diminished by ultraviolet doses permitting 40 to 60% cell survival. The induced donor state was not heritable, and was not shown in uvr mutants or in rec

mutants. A similar effect was noted for mitomycin C, and the effect was inhibited by acriflavine. The authors propose that in the course of repairing an ultraviolet lesion, a single-stranded region of DNA is exposed which may be homologous to a part of the autonomous F factor. A partial recombination ensues in which the F is integrated sufficiently to mediate transfer but not enough to replicate.

Ultraviolet irradiation of a transducing phage lysate at a low dose has been found to increase the yield of transductants (Zinder and Lederberg, 1952). The authors suggested that the ultraviolet stimulates an increased frequency of integration of the lysogenic phage carrying the donor markers.

Jacob and Wollman studied the effects of ultraviolet irradiation on the yields of recombinants in crosses of phage lambda. They found enhanced yield of phage recombinants under the following conditions:

1) A lambda-lysogenic strain is induced with ultraviolet, then superinfected with a genetically different phage;

2) Bacteria are mixedly infected with irradiated phage; or

3) The mixedly infected cells are irradiated. In all three cases, doses of ultraviolet light allowing 40 to 60% survival were most effective in stimulating recombination. It is worth mention that this is also the range of dose that gives optimal induction of lysogenic phage. The kinetics of generation of recombinant phage is also different in irradiated from control crosses. In the latter there is a two-fold increase in recombinant yield over the course of the latent period, reflecting the increased size of the intracellular phage pool. In irradiated crosses, however, the maximum recombination frequency is seen from the beginning. Their interpretation was that the presence of ultraviolet lesions increased the likelihood of recombination switches (Jacob and Wollman, 1955, 1961). They also tested the effect of irradiating an Hfr donor on its ability to form recombinants with F^- recipients. Their finding was that as dose increased, the number of recombinants decreased exponentially. Apparently at the doses used, conjugation and transfer of the donor chromosome were unaffected (Jacob and Wollman, 1961).

Similar results to those of Lederberg, et al. were obtained by Hill (1957), who found that ultraviolet-induced revertants from auxotrophy to prototrophy were unstable and likely to revert back to auxotrophy. His hypothesis of "controlling episomes" proposed that the auxotrophy resulted from attachment of an episome which effectively blocked the activity of the site it occupied. The ultraviolet light served to induce the episome, which upon its release restored the locus to activity. But the episome remained in the cell, and at some later time could spontaneously reattach, restoring the auxotrophy (Hill, 1963).

In a review of mutagenic mechanisms, Drake concluded that ultraviolet irradiation stimulates recombination and can produce deletions in bacteria, presumably via a mechanism such as unequal crossing over (Drake, 1969).

Perhaps of greater relevance to this work is the report by Guerrini and Fox (1968) that in pneumococcal transformation, the heteroduplex structure of the primary recombinant may be reverted to homoduplex prior to replication by administering mitomycin C or ultraviolet light. This conversion was demonstrated by showing that the frequency of pure clones increased with the dose of mitomycin or UV. The doses were such that there were many repairable hits per lethal hit.

In summary, then, several segregating systems had been shown to be affected by ultraviolet light, and the similar optimal dosages and need for nutritional supplementation were sufficiently suggestive that it seemed quite likely that a process mediating recombination were somehow involved. It was postulated that ultraviolet light might influence the segregation frequency of the c strain.

b. Experimental results

The ultraviolet light source used was a General Electric 15-watt germicidal lamp (low pressure, mercury) at a distance of 80 cm from the suspension to be irradiated. This corresponded to a dose rate of 1200 ergs/cm^2/sec as measured on a YSI-Kettring Model 65 Radiometer. The intensity of irradiation of the lamp followed the inverse square law to distances as close as 20 cm, but deviated markedly at closer distances, owing to the asymmetry of the source, a tubular lamp. The suspension to

be irradiated was contained in a sterile glass Petri dish. A frozen stock of c cells was thawed and diluted into CH-T at a concentration of 1×10^5 cfu/ml. This preculture was incubated 3 hours at 37°C. until it reached a concentration of 1×10^6 cfu/ml, then diluted tenfold into D medium (see Materials and Methods, p. 24) in the glass Petri dish containing a sterile magnetic flea. The solution was stirred with a magnetic stirring apparatus, and irradiation was controlled by removing and replacing the glass lid of the Petri dish. After each dose, a sample was diluted tenfold in CH-T in a foil-wrapped test tube on ice. Pneumococcus has been found to be relatively insensitive to photoreactivation (G.F. Vovis, personal communication), so no unusual precautions were taken. The doses reported were cumulative, with no more than 30 seconds required for sampling between each ultraviolet pulse.

In one experiment controls were performed in which D medium alone was irradiated and then used as diluent for the cells, or D medium containing cells was irradiated for twice the maximum dose with the cover on. In neither of these cases was there any difference in survival or segregation frequency between the control and the untreated sample included in every experiment. The tubes containing the diluted irradiated cell samples were scored immediately after termination of the experiment and after one and two hours of incubation at 37°C. for the total number of colony-forming units and the colony-forming units resistant to 20 μg/ml NOB. The results of the scoring before incubation in two experiments are given in Figure 2. The total cell population declines exponentially with dose of ultraviolet, as anticipated, with a possible shoulder in the survival curve at very low doses. The number of segregants, however, is stimulated two- to threefold at an ultraviolet dose at which 70 to 90% of the cells are still viable. If the curves were in terms of percent segregants, the stimulation would appear to be even more dramatic.

Another effect of ultraviolet light is the inhibition of DNA synthesis and consequently cell division. In testing segregation frequencies after various intervals of post-irradiation incubation, I was interested to learn whether the stimulation was maintained as growth resumed. Table XVIII shows that as the incubation progresses, the peak stimulation

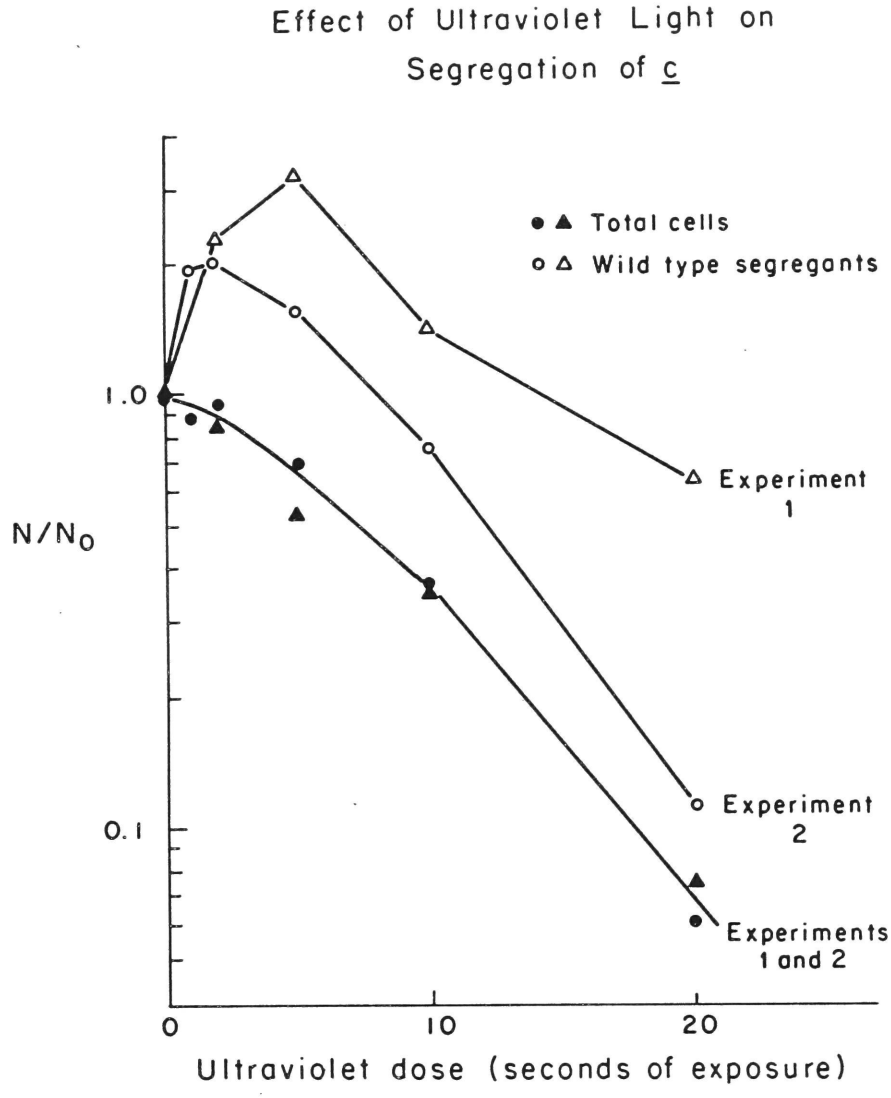


Figure 2: Survival curve of total cells and NOB-resistant segregants in a c culture exposed to 1200 ergs/cm^2/sec ultraviolet light and assayed immediately. N = number of cells at the indicated times, N_o = number of cells at 0 time.

occurs in samples which received higher doses. This peak need not be the maximal stimulation shown by that sample, but rather seems to reflect the fact that at the higher ultraviolet doses, resumption of growth is slower.

Table XVIII: Correlation of decline of enhanced segregation frequency with resumption of cell division.

Incubation after treatment:	0		1 hour		2 hours	
Dose of ultraviolet	% in NOB	Relative growth	% in NOB	Relative growth	% in NOB	Relative growth
0 seconds	3.4; 4.3	1.0	7.2; 5.0	3.0; 2.8	6.4; 4.9	16.6; 15.8
1 second	9.7	"	7.2	3.2	6.8	13
2 seconds	7.5; 9.7	"	8.1; 6.4	2.8; 3.1	3.8; 5.6	17.6; 11
5 "	17; 10.2	"	12; 9.6	2.1; 2.1	6.2; 6.4	9.4; 6.2
10 "	11; 9.3	"	16; 18	0.6; 1.0	13; 12	2.0; 2.3
20 "	23; 8.5	"	9.4; 9.2	0.5; 0.9	12; 15.1	0.9; 1.2

The data for two independent experiments are shown.

Apparently then the modification of segregation frequency induced by ultraviolet does not persist, or is not heritable by the division products of the irradiated cells. This is reminiscent of the temporary modifications of Hfr and F^+ donors (reported by Evenchik, et al., 1969) in which the alteration to lower or higher conjugation activity is not retained as the cells divide.

Mitomycin C is a radiomimetic cross-linking agent which was first described as an inhibitor of DNA synthesis. It is one of a group of agents to which certain resistant mutations exhibit cross-resistance (see for example Terawaki and Greenberg, 1965). The damage induced in the DNA by mitomycin C is usually alkylation of a base in one of the DNA strands, and this damage may be repaired with a cellular system at least partly involved in repairing ultraviolet lesions (Evenchik, Stacey, and Hayes,

1969; Otsuji and Murayama, 1972). It mimics ultraviolet in other ways too: transformants in the heteroduplex state are converted to homoduplexes after exposure to mitomycin C but prior to replication of the DNA (Guerrini and Fox, 1968); Hfr donors lose ability to transfer their chromosome, while F^+ donors increase that ability under the influence of mitomycin C (Evenchik, et al., 1969). In both cases the dose yielding the maximal effect is very similar to the optimal dose for induction of this effect by ultraviolet light in terms of cell survival.

The stimulation of segregation caused by ultraviolet light may be a direct effect on the DNA and segregation mechanism, or it could be a relatively non-specific effect involving alterations in metabolism at critical points leading more indirectly to the observed effect on segregation. It would help to distinguish these two possibilities if the effects of mitomycin C and ultraviolet light could be compared. It is hard to imagine that two agents so different in intrinsic physical and chemical properties both could affect a population of cells so similarly as to indirectly influence segregation frequency. However, if the ultraviolet effect is mimicked by mitomycin C, it strengthens the argument that the segregation mechanism is intimately connected with the state of the DNA, a main common site of action of the two agents.

The critical dose of mitomycin C was found by preliminary experiment to be 20 μg/ml. This gives a survival of 40% in 4 minutes. It is a considerably higher concentration than the 0.5 μg/ml used by Guerrini and Fox or the 10 μg/ml found optimal by Evenchik, et al. for E. coli. I attribute this to the rather high albumin content of our growth medium. Albumin is very efficient in binding toxic agents of various sorts, and in fact that is one reason it is included in the medium.

The experiment was designed to imitate as nearly as possible the one using ultraviolet light. Naturally though, the effect of mitomycin C might be expected to lag in its onset, and require high dilution or washing to terminate its action, unlike ultraviolet, which can so easily be pulsed. A culture of stock c was thawed, diluted in fresh CH-T to a concentration of 1.3×10^5 cfu/ml, then incubated 3 1/2 hours at 37°C. to reach a concentration of 4×10^6 cfu/ml. This preculture was chilled

and mitomycin C was added to a final concentration of 20 μg/ml. Immediately a sample was taken and diluted 500-fold into ice cold CH-T in a foil-wrapped tube on ice. The reaction mixture was incubated at 30°C., and further samples were taken after various intervals had elapsed. At the end of the sampling period, the foil-wrapped tubes were scored for total colony-forming units per ml and for NOB-resistant colony-forming units per ml. Then they were placed at 37°C. and scored again after one and two hours incubation.

Two experiments were performed, one of which is illustrated in Figure 3. The other differed only in minor detail. Again there was an exponential decay of the survival of the total cell population, and a pronounced enhancement in the segregant frequency at a dose at which more than 90% of the population still survived. The enhancement was not as great as with ultraviolet light. Once again the samples treated for longer times resumed growth more slowly than those treated for shorter times, but in this case it was harder to decide whether there was a corresponding delay in the decline of segregation frequency to normal range. In part this is owing to the fact that the samples treated longer with mitomycin show practically normal segregation frequencies to begin with. Also the stimulation is not as strong, so the return to normal is harder to differentiate from customary variability. Table XLX summarizes these data.

Thus at least in gross aspect the changes in cultures of c induced by mitomycin C appear to be similar to those induced by ultraviolet light. The direction of the shift in frequency is the same and it occurs at the same dose of agent, considered in terms of cell survival.

Preliminary experiments performed in this laboratory by Mrs. Isobel Mackenzie suggested that the dye acridine orange might have the effect of diminishing the segregation frequency of a culture of c. Acridines have been known for over a decade to cure certain bacteria of their plasmids. Hirota and Iijima were able to cure F^+ cells of the F factor by overnight treatment with sublethal doses of acriflavine. Hfr strains could also be rendered F^- by three passages through acriflavine (Hirota and Iijima, 1957; Hirota, 1960). The mechanism for this curing is thought to be a

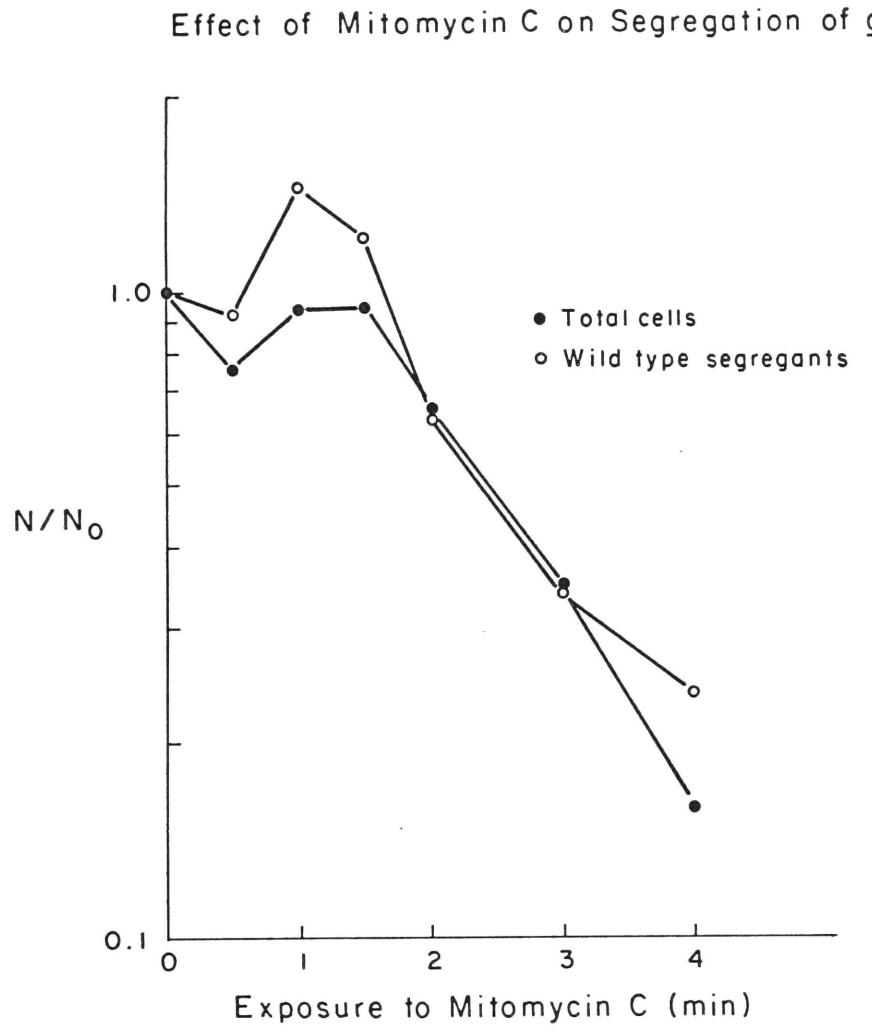


Figure 3: Survival curve of total cells and NOB-resistant segregants in a c culture exposed to 20 μg/ml mitomycin C and assayed immediately after dilution. N = number of cells at the indicated time; N_o = number of cells at 0 time.

Table XIX: Correlation of decline of enhanced segregation frequency with resumption of cell division

Incubation after treatment:	0		1 hour		2 hours	
Exposure to mitomycin C	% in NOB	Relative growth	% in NOB	Relative growth	% in NOB	Relative growth
Experiment 1:						
0 seconds	7.8	1.0	6.3	6.6	7.5	17
30 seconds	9.4	"	6.7	5.3	7.6	15
1 minute	12.7	"	7.1	4.0	6.5	14
1 3/4 minutes	9.6	"	11.2	1.3	6.6	10
2 1/3 "	13.5	"	10.7	1.9	8.3	12.5
3 "	5.5	"	4.7	1.0	8.6	1.9
4 "	3.4	"	7.2	0.81	5.5	2.9
Experiment 2:						
0 seconds	6.6	1.0	8.7	2.3	10.8	10.8
30 seconds	8.0	"	10.0	2.5	8.9	8.9
1 minute	10.3	"	24	2.5	8.3	12.2
1 1/2 minutes	8.4	"	10.7	1.7	8.4	10.2
2 "	6.5	"	9.2	1.6	7.5	7.5
3 "	6.5	"			8.7	3.3
4 "	10.0	"	9.7	1.3	14.1	3.3

differential inhibition of replication of the plasmid, leading to its eventual loss by dilution (Hohn and Korn, 1969). Acridines also lead to the elimination of resistance transfer factors with a low frequency. The efficiency of curing increases if the cells have previously been exposed to ultraviolet irradiation (Watanabe, 1963). By 1963 the effect of acri-

dines on plasmids was so widely accepted that when Mitsuhashi, et al., found a multiply drug-resistant strain of Staphylococcus aureus, three of whose resistances were coordinately lost during acriflavine treatment, they concluded that the three resistances were determined by a cytoplasmic element (Mitsuhashi, Morimura, Kono, and Oshima, 1963). Investigators of segregating strains of various sorts have tested response of their systems to acridines as a clue to whether they might be of episomal structure (Hill, 1963; Curtiss, 1964). However, some known episomes are unaffected by acridines, for example the FP sex factor of Pseudomonas aeruginosa (Holloway and Fargie, 1960).

As early as 1961 Witkin was observing that basic dyes such as acriflavine added to cells after irradiation with ultraviolet light were able to delay certain of the normal effects of irradiation, such as reversion of auxotrophic mutations, and fixation of mutations. In combination with ultraviolet light, acridines caused marked enhancement of the mutagenic potency of low doses of irradiation. Her interpretation was that mutagenesis by ultraviolet light initiates lesions in the DNA which require a repair process. The presence of acridines delays this process and hence the eventual production of mutants (Witkin, 1961). In a discussion of acridine mutagenesis, Streisinger, et al., cite a personal communication of L. Lerman stating that high levels of acridines decrease the frequency of recombination (Streisinger, Okada, Emrich, Newton, Tsugita, Terzaghi, and Inouye, 1966). E. coli strains carrying mutations conferring extra sensitivity to ultraviolet irradiation are found to be more than normally inhibited by acriflavine in their repair of the ultraviolet lesions (Harm, 1967). Strains of E. coli bearing mutations in the rec locus have been tested for acridine sensitivity and for the ability of acridine to eliminate resident episomes. The rec^- strains of type A, B, or C could be cured of an F factor as readily as a rec^+ strain (Bastarrachea and Willetts, 1968).

In a lucid review of this complicated area, Witkin (1969) proposed

that DNA damage produced by mitomycin C or ultraviolet light (among other agents) is usually repaired by excision-repair. A second process, post-replication repair, acts on the single-stranded gaps left when regions containing unrepaired thymine dimers--the product of ultraviolet damage--are replicated. This latter kind of repair may involve a recombination, because ultraviolet-sensitive mutants are so often recombination deficient. Acriflavine inhibits the excision of thymine dimers, thus shifting the burden of repair to the post-replication repair process and bringing recombination into play (Witkin, 1969). However she acknowledges that particularly regarding the acridine work, the results are controversial.

Thus in testing the effects of acridines on the segregation of c an effect was predicted which would be opposite in direction to that of ultraviolet and mitomycin C, that is, a depression in segregation frequency.

The acridine chosen was acridine orange (2,8-bis-dimethylaminoacridine). This compound is said to be less toxic to E. coli cells than acriflavine and other analogs (Hirota, 1960; Silver, Levine and Spielman, 1968). A thawed stock of the c strain was diluted to a concentration of 1×10^5 cfu/ml in CH-T. After a three-hour incubation period, the culture was diluted 100 fold into fresh CH-T or CH-T containing various amounts of acridine orange. These dilutions were scored in FSB with and without 20 μg/ml NOB, immediately and after various intervals of incubation at 37°C. The results are indicated in Figure 4. Since the acridine concentrations affecting segregation frequency were toxic not only to the cells but to the generation of segregants as well, the results are given as per cent segregants among survivors, rather than survival of segregants. Thus a differential survival can be discerned, although there is considerable variability in the actual ratios. Nevertheless, at acridine orange concentrations greater than that sufficient to arrest cell division, there is a clearly visible trend toward progressive diminution of segregation frequencies. In a control experiment I found the growth of segregants to be no more sensitive to acridine orange than is wild type. Hence the dye must be acting to diminish the generation of segregants, that is, to inhibit segregation.

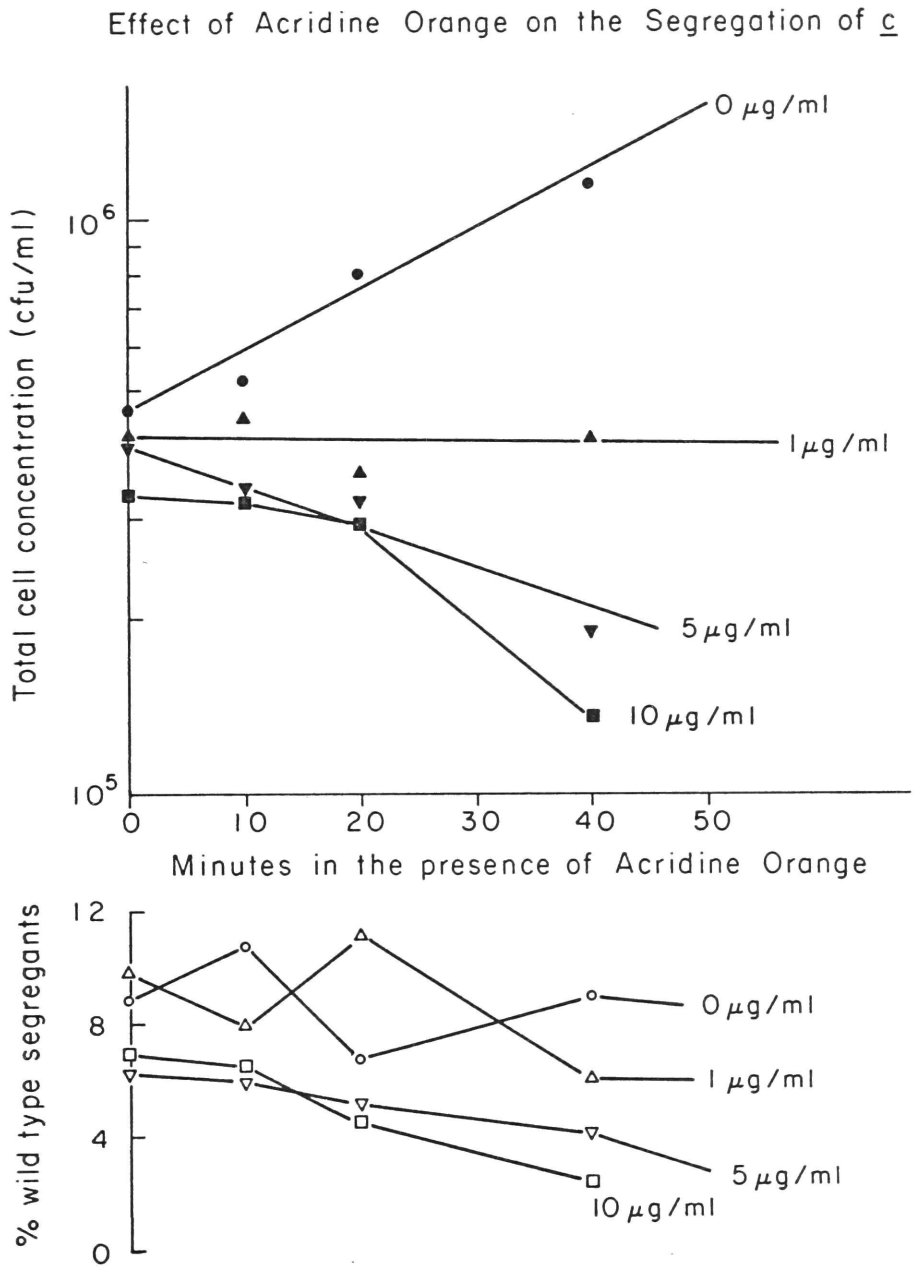


Figure 4: Changes in total cell concentration and in segregant frequency as a function of duration of exposure to various concentrations of acridine orange.

The foregoing observations may be summarized as follows: cells carrying the c mutation have been treated with the agents ultraviolet light, mitomycin C, and acridine orange under conditions for which each agent is progressively toxic to continued cell growth. The first two agents stimulate the generation of segregants, while the last inhibits it. The three agents are of such different physical natures that their tendency progressively to influence segregation can readily be interpreted only in terms of their action on the DNA of the strain, the only known site of action common to all three.

Can the nature of the involvement of DNA in the segregation mechanism be elucidated? The thymine dimers caused by ultraviolet light and the nucleotide bases alkylated by mitomycin C are both subject to excision-repair by the same enzyme system in E. coli. Acridines seem to inhibit the activity of this enzyme system. Assuming excision-repair in pneumococcus is analogous in its major features to excision-repair in E. coli, it is a reasonable hypothesis that this enzyme system plays some role in mediating segregation. It has become increasingly clear that DNA repair and recombination share common features. In other stable partial diploids, haploidization requires recombination (Curtiss, 1964; Hill, Foulds, Soll, and Berg, 1969). Since the merozygote of c has a specific location in the chromosomal continuity of the cell, perhaps a recombination event is required to remove it and lead to the haploid segregant.

Ability of the c marker to transform and segregate in a recombination-deficient recipient

In E. coli there are a variety of mutant strains in which recombination ability is deficient. The recombination ability usually tested is the ability to yield recombinants after conjugation or generalized (P1-mediated) transduction (Franklin, 1967). The strains may be classified in addition according to their sensitivity to ultraviolet irradiation and their tendency to break down their DNA following exposure to ultraviolet light. The mutations in the complementation group designated recA undergo extensive breakdown ("reckless"), while those in groups B

and C exhibit less breakdown even than wild type ("cautious") (Hayes, 1968). Although all rec mutants exhibit a depressed frequency of recombinant formation, those exconjugants which do appear to be recombinant differ distinctly depending on whether the mating is between recA or recB parents. In the latter, the recombinants are normal haploids with anticipated linkages of markers. In recA matings, however, there is unusually high coinheritance of selected and unselected markers. This coinheritance results from the generation of merodiploids in which the donor material was brought in on a defective F particle retaining just enough function to permit the exogenote to replicate (Low, 1968).

Rec mutants which are F^+ or F' are unable to mobilize their chromosomes for conjugation, consistent with the proposed mechanism that the F factor must be integrated to stimulate chromosome transfer (Evenchik, Stacey, and Hayes, 1969). There is evidence that other kinds of events thought to be recombination-mediated may occur even in rec^- strains. Wilkins observed that although recA F' donors were inhibited by a factor of more than 10^4 in their ability to transfer their chromosome, recB and recC F' donors could transfer their chromosome at more than 50% of the wild type frequency. And ϕ80 and lambda c, lysogenic phages with specific sites of integration, are inducible by heat in recB strains, although not by ultraviolet light nor in recA strains (Franklin, 1967). Thus apparently recombination between homologous DNA segments, as in conjugation, may be different from the recombination required to insert an episomal factor into a (largely) non-homologous bacterial chromosome. This latter sort of recombination seems to be less affected by the recB and recC gene functions.

From pneumococcus a recombination-deficient strain has been isolated on the basis of its sensitivity to γ-irradiation (Vovis and Buttin, 1970b). This strain is more than usually sensitive to ultraviolet light and is "cautious" in its breakdown of DNA following irradiation. The mutation is associated with loss of a specific ATP-dependent deoxyribonuclease whose biochemical properties are remarkably similar to those of enzymes in other bacterial species (Vovis and Buttin, 1970a). This association of properties is very similar to that of the recB and C mutants of E. coli.

After transforming his mutation into our R1-26 strain and confirming that its properties were unaltered, Dr. Gerald Vovis provided me with competent cells on which I could test the behavior of the c marker in this recipient, R1-26str27A-2. Since the str marker appears necessary for activity of the mutation, it is present in the recipient strain, and I used micrococcin resistance as my standard marker. The cells were diluted only four-fold into the transformation mixture instead of the customary twenty-fold, and were exposed to the DNA for 15 minutes rather than ten, owing to their low intrinsic level of transformability. Otherwise the usual procedures obtained. The results are indicated in Table XX.

Table XX: Ability of c to transform recombination deficient strain, R1-26str27A-2

Strain	c(SA)/mic	c(PAS)/mic
R1-26str27A-2	0.23 (n=2)*	0.39 (n=2)*
R1-26	0.32 (n=9)	0.35 (n=9)

* a single experiment using two different concentrations of DNA
n = the number of replicate determinations.

It is apparent that, whether scored as SA resistance or as PAS resistance, c is not affected in its efficiency of transformation into the recombination-deficient strain. In this experiment the maximum transformability of the culture for micrococcin resistance was 1.8%. According to Vovis and Buttin (1970b) one would expect less than 20% of the normal yield of transformants per molecule of DNA irreversibly bound by the cells.

The next question investigated was whether the c marker segregates normally in the strain lacking the ATP-dependent deoxyribonuclease. Table XXI shows the results of testing the segregant frequencies of transformants selected on the basis of their PAS resistance or their SA resistance in liquid scoring tubes or from agar plates. For purposes of comparison I include similar data for c transformants of R1-26 recipients and for subclones of sul-c itself. As might be expected, the variability of segregant frequency of the freshly selected transformants

tends to be greater than that of the subclones (or of established populations of c in general). Thus although the transformants of the rec⁻ strain seem to segregate with a lower frequency than those of the wild type--especially in the sample selected in liquid SA-containing medium--it is highly unlikely that this difference is significant. In any event a much greater depression in frequency would be anticipated if the enzyme system active on the segregation mechanism were the one whose activity in normal transformation is depressed more than five-fold.

Table XXI: Segregant frequencies of c transformants of R1-26str27A-2 compared with c transformants of R1-26 and subclones of sul-c

	SA-selected		PAS-selected	
Strain	tubes	plates	tubes	plates
R1-26str27A-2	2.5 ± 0.6 (n=9)	5.1 ± 2.4 (n=24)	4.0 ± 4.2 (n=6)	4.3 ± 1.5 (n=8)
R1-26	8.0 ± 4.0 (n=38)		5.5 ± 2.1 (n=25)	
Subclones				
sul-c	5.4 ± 2.2 2.8 ± 1.0 (n=10)		3.3 ± 1.8 4.4 ± 1.5 (n=10)	

The numbers reported are the mean % NOB-resistant regregants ± one standard deviation unit, where n is the number of clones tested.

At first glance it would seem that these data refute the hypothesis that recombination is necessary for segregation of the c strain. However it is apparent that certain kinds of recombination may occur in recombination-deficient strains, specifically those strains which are of the recB or recC type (Franklin, 1967; Wilkins, 1969). Since this is just the mutation carried by the pneumococcal recombination-deficient strain, we can only conclude that if segregation is a recombination event, it must be more similar to episomal integration and induction than to the sort of recombination occurring in conjugation or transformation.

Table XXII presents data similar to those in Table XXI, except that

the recipient strains whose transformants are being tested for segregation frequency are the Rx-1 and hex-1 strains. This is the transformation described in greater detail in Chapter III, pp. 53-55. Here it is evident that segregation frequency is similarly unaffected by absence of the function determining efficiency of integration.

Table XXII: Segregant frequencies of c transformants in strains normalizing efficiency of integration.

Strain	% segregants (NOB-resistant colonies) SA-selected	PAS-selected
Rx-1	4.1 ± 1.9 (n=7)	6.3 ± 4.5 (n=8)
hex-1	3.6 ± 0.7 (n=5)	
R1-26	8.0 ± 4.0 (n=38)	5.5 ± 2.1 (n=25)

Although the indirect nature of the available experimental approaches makes it difficult to obtain direct evidence concerning the events determining the segregation process, it is reasonable to consider them related to recombination. Segregation is discontinuous and regulated in overall frequency. It seems to occur in several steps, one of which is stimulated by ultraviolet light and mitomycin C and inhibited by acridine orange. The ability to segregate is unimpaired in a strain lacking one of the enzymes mediating recombination. Perhaps when mutant strains of the recA type become available, it will be possible to confirm more directly the hypothesis proposing that recombination is necessary for segregation of the c strain.

V

PHYSICAL PROPERTIES OF THE DNA FROM THE c MUTANT

In the preceding two chapters data have been presented which demonstrate that the merozygosis of the c strain is determined by a mutation having a fixed location in the bacterial genetic map. The merozygosis is associated with an enhanced level of transforming activity for the closely linked d markers. Some, if not all, of this extra activity may be accounted for by extra genetic determinants for the locus. It seems likely that the process by which merozygosis is lost (segregation) is the result of a recombination event which acts to restore the region to its normal complement of determinants for the d locus.

A structural abnormality of the DNA of the c strain determined by the presence of the c mutation must underlie this array of genetic and physiological properties. It is possible to imagine two mutually exclusive hypotheses as models for this structural abnormality. Each explains all the properties discussed, and each has features which it should be possible to test. Although other structural models may be formulated, these two are the simplest and most general.

One is the model proposed by Hotchkiss, Abe, and Lane (1967, 1971) and illustrated in Figure 5. In the region of merozygosis, including the loci for c and d, there is a stretch of the genome which is triple-stranded. A single continuous strand is in hydrogen-bonded equilibrium with two discontinuous complementary strands of the opposite polarity. It is hard to predict how physically stable such a structure would be. Synthetic polynucleotides can be constructed, all of whose bases are the same; and some combinations of these homopolynucleotides have been found able to associate in triple complexes, as determined from measurements of buoyant density, melting curves, and ultraviolet absorption spectra. However, only very specific combinations of homopolynucleotides have shown this three-fold association, and for deoxyribopolynucleotides the possibilities are even more limited than for ribopolynucleotides (Felsenfeld and Miles, 1967). From studies with structural models, the association seems to be a triple-stranded helix, at each level of which

Strain designation	Frequency of wild type segregants	Marker ratios as proportion of non-c strain	
		d^+	d
c	5-10%	1.5	0
cd-10	5-10%	1.0	0.5
cd-13	1%	0.5	1.0
cd-11	0	0	1.5

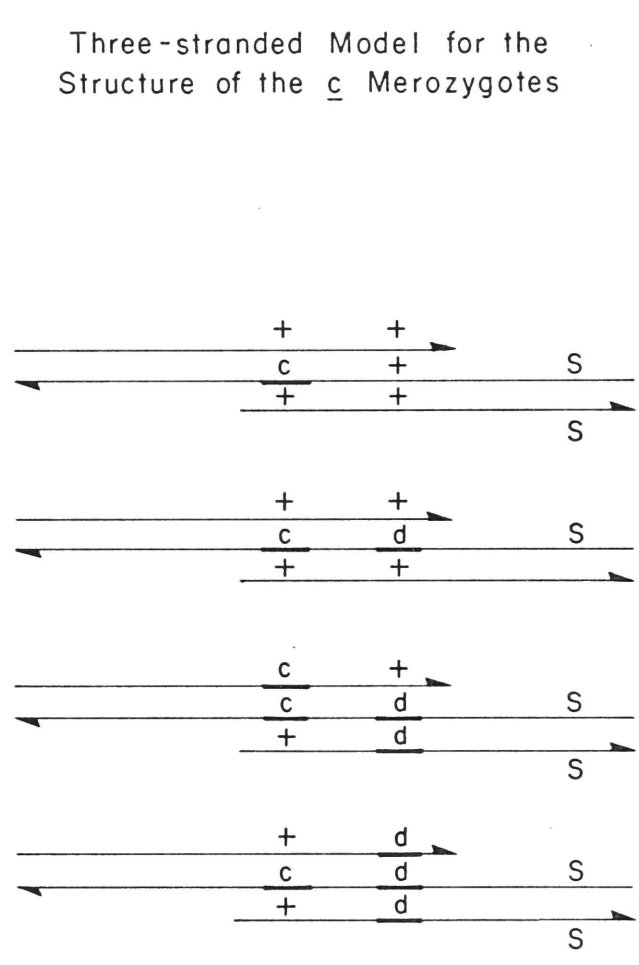


Figure 5: Triple-stranded structure of the c and cd merozygotes, and its correspondence to certain properties of the strains. See text for more detailed explanation.

a given base in one chain is hydrogen bonded to two different partners in the other two chains.

It might be questioned whether a triple-stranded structure could exist in DNA from a natural source. The exquisitely controlled conditions in which the structures arise *in vitro* and the many base combinations for which no three-fold association has been demonstrated make it hard to imagine that a single, albeit unusual, mutation could convert a normally double-stranded region into one bearing triple-strandedness, at least that kind which has been demonstrated *in vitro*. However, Hotchkiss, *et al.*, do not suggest that their merozygotic region is stabilized by such a three-fold sharing of a triple helix, but rather that the structure is in dynamic equilibrium, first one discontinuous strand, then the other being bonded to the continuous strand in a given region in the classical base-pairing combinations. In fact just such a locally discontinuous structure is a feature of most models as an intermediate in recombination (Hotchkiss, 1971) and is thought to play a role in transcription as well.

There is recent evidence that triple-stranded DNA's can occur in nature. Mitochondrial DNA of many vertebrates may be isolated in closed circular form. When such DNA from mouse L cells was banded in a gradient of cesium chloride containing ethidium bromide (an intercalating dye which enhances the density difference between circular and linear DNA molecules), two bands of circular DNA were obtained. Denaturation of the DNA from one of these bands or diminution of the ionic strength of the solution released a small single DNA strand having a sedimentation coefficient of 7S. Using electron microscopy, the authors could see in the native circular DNA a single region of local denaturation which contained a short discontinuous DNA segment. After denaturation and annealing, this "displacement loop" was no longer visible (Kasamatsu, Robberson, and Vinograd, 1971). A similar structure was inferred by TerSchegget and Borst (1971) for newly-synthesized mitochondrial DNA from chick livers. In this case the evidence is based primarily on centrifugal separation of DNA species allowed to replicate for a limited time in medium containing a nucleic acid precursor labelled both for density and radioactivity (TerSchegget and Borst, 1971). Thus, however it be stabilized *in vivo*,

the triple-stranded structure in the model of Hotchkiss, et al., may not be excluded a priori on theoretical physical grounds.

Another objection might be raised on the grounds that a structure such as the one in Figure 5 could never survive a round of replication without becoming normalized. However, the more that is learned about the enzymatic mechanism of DNA replication, the clearer it becomes that a great deal of flexibility may be tolerated. What appears on a large scale to be a continuous, unidirectional process, may prove to involve fragmentary syntheses followed by correctional processes (see Hotchkiss, 1968). As for transcription of a triple-stranded region, that process need not be regular, since we do see, in fact, the mutant phenotype for the strain.

If the basic structural features of the model are accepted, it provides an admirable account of the genetic properties of the c and cd strains. Alternative forms of either allele coexist by having their determinants on one or another of the DNA segments. Most specifically the marker frequencies for d and d^+ correspond to those measured in transformation, and represent a dosage half again as great as in non-c strains. Segregation of a strain containing such a structure could occur by a completion of the recombination process of which the structure resembles an intermediate (Figure 6). According to current ideas, one or both of the discontinuous strands could be trimmed the appropriate distance, then the homologous ends joined by a ligase. The resulting heteroduplex would resolve at the next cycle of replication to a normal wild type structure and a structure which might be inviable, indistinguishable from wild type, or able rapidly to regenerate the original structure.

The alternative model is that of the tandem duplication, as shown in Figure 7. Reading from left to right, the first "+" denotes the wild type allele of c; the second "+" or "d", the state of one copy of the d locus; the third symbol, the mutant c allele; and the fourth, the second copy of the d locus. Either copy of the d locus may have either the mutant or wild type configuration, but the positions of the two c alleles are invariant. This structure would pose no problem in physical stability

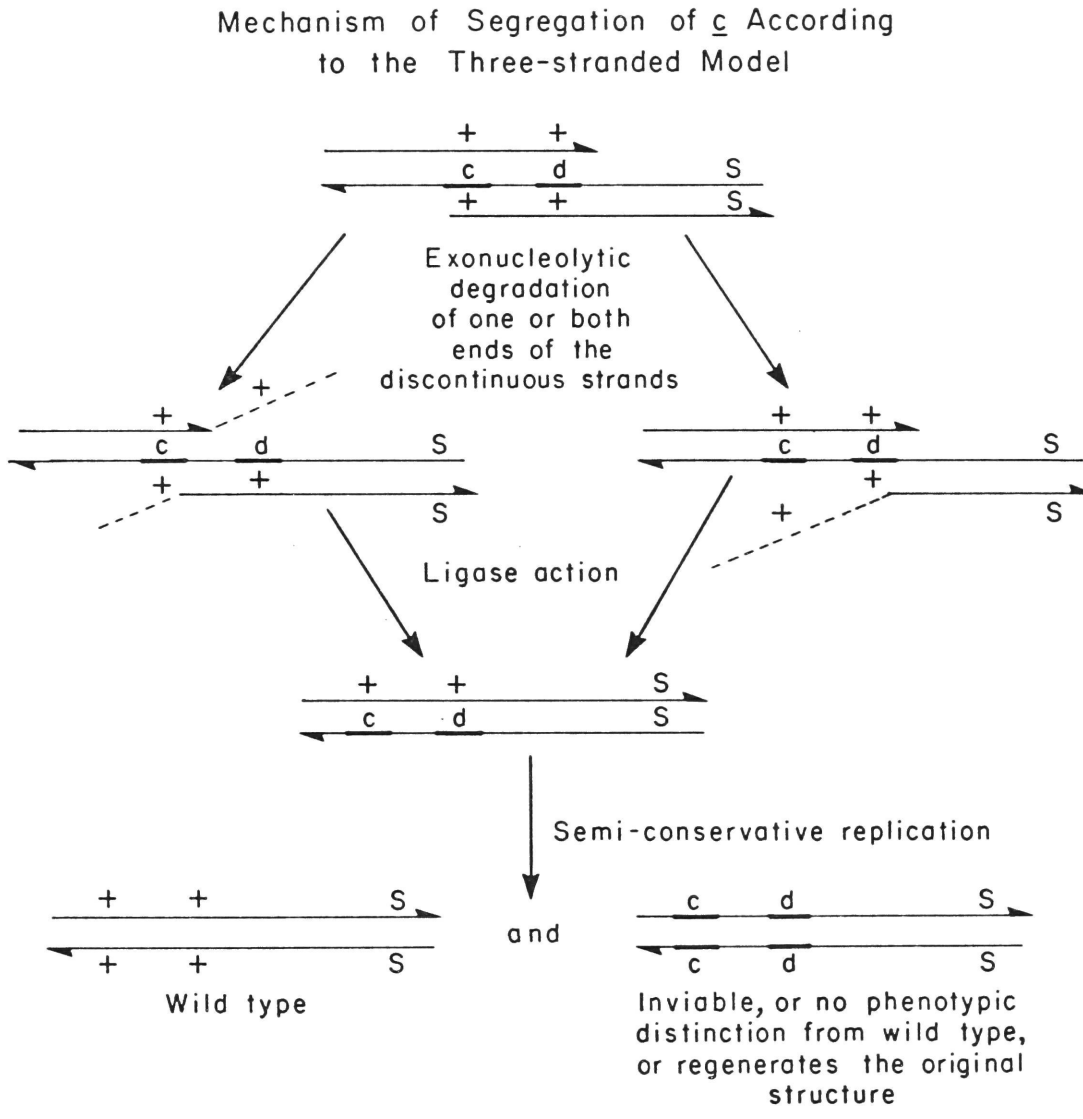


Figure 6: Scheme for the mechanism of segregation of c in the triple-stranded configuration.

Strain designation	Frequency of wild type segregants	Marker ratios as proportion of non-c strain	
		d^+	d
c	5-10%	1.5	0
cd-10	5-10%	1.0	0.5
cd-13	1%	0.5	1.0
cd-11	0	0	1.5

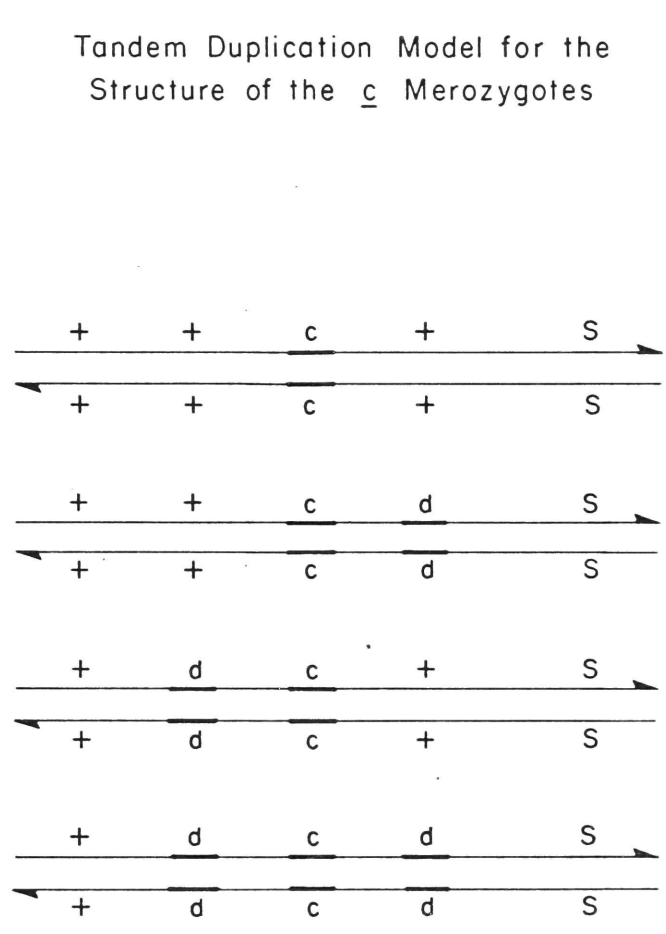


Figure 7: Tandem duplication structure of the c and cd merozygotes, and its correspondence to certain properties of the strains. See text for more detailed explanation.

replication, or transcription. In fact the extra sequence of nucleotides could in itself account for the production of a mutant enzyme, since extra amino acids in the enzyme primary structure would very likely perturb its function. The enhanced SA resistance of the cd strains could result from incorporation of a d allele at either or both available positions. According to most ideas of duplication, one would expect a doubling of the frequencies of those genes included in the extra segment in a c strain compared to a non-c strain. The observation of only a 50% increase may be accounted for by assuming markers in the duplicated segment containing the c allele to be depressed in efficiency over those in the segment containing c^+. Thus cd-10 would have normal efficiency for d^+, but reduced efficiency for d, while cd-13 would have normal d dosage and reduced d^+.

Segregation could occur by a looping-out process as in Figure 8, followed by a single reciprocal recombination, a process analogous to Campbell's model for release of integrated episomes or temperate phages (Campbell, 1969). The products of the recombination event would be a normal non-c chromosome and a circular piece of DNA, which might persist in the cell, unable to replicate or express a function and eventually lost. Applying this mechanism, it is further proposed that the region of the duplication containing the c allele and its right-hand d locus should preferentially be lost, although occasionally the recombination could eliminate the c together with the d allele on its left. Thus cd-10 would give rise to wild type segregants at a frequency greater than cd-13 and both would have the potential to yield d segregants.

These two alternative models represent two ways of considering an intrachromosomal merozygosity: either a longitudinal amplification of information, or an increased stratification at a given position. Variations and combinations of these models can be constructed, but their properties should at least resemble those of the simpler model from which they were derived.

The DNA of the merozygotic region in the triple-stranded model has several unusual features which could lead to abnormal behavior of the DNA. Specifically, a triple-stranded region should have an increased

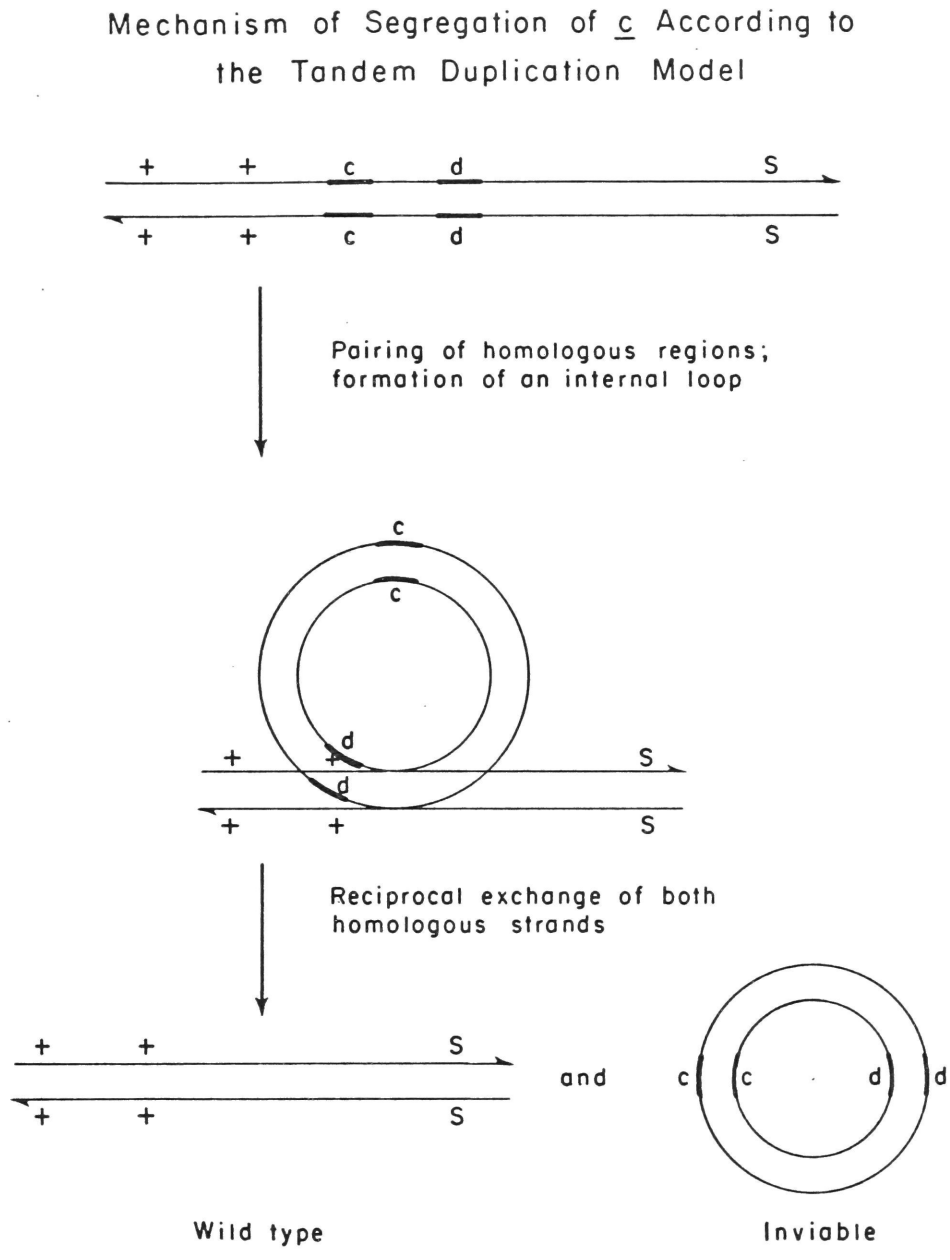


Figure 8: Scheme for the mechanism of segregation of c in the tandem duplication configuration.

buoyant density and an altered affinity for methylated albumin-kieselguhr (MAK). The presence of unbonded ends should render DNA of the c region more sensitive to treatment specific for single-stranded, or loosely double-stranded DNA.

However, there could be a problem detecting DNA of abnormal behavior in the presence of the overwhelming amount of normal DNA corresponding to the remainder of the bacterial genome. Using even the most gentle methods of isolation of DNA, usually about 50 fragments of 2×10^7 daltons molecular weight are obtained per genome. Since linkage can be detected only between markers lying in the same fragment to begin with, the str and the merozygous region must usually be borne on the same fragment, probably at its extremities. But the str marker is a stable marker, and the a marker, lying much closer to c, can be unstabilized only transiently, if at all. Thus the region of structural abnormality must occupy only a part of the fragment of DNA on which it is found. Based on this realization, that the abnormal piece occupies a small region of one of the fifty DNA fragments in the preparation, our expectation of detecting a physical separation between c-bearing DNA fragments and the rest of the genome must be slight. Nevertheless, a positive result would be useful not only for what it might tell us about the structure of the region, but also for providing a method for enriching the population of DNA molecules for those bearing the c structure.

In the following experiments, two highly purified DNA preparations were used. One, designated cSK, bore markers for sul-c, str, and mic (micrococcin resistance); the other, cK, had only c and mic.

Behavior of c-containing DNA in cesium chloride equilibrium centrifugation

The buoyant density of DNA from the R36-A strain of pneumococcus has been reported to be 1.701 g/cc, corresponding to a content of guanine + cytosine of 39 to 42% (Schildkraut, Marmur, and Doty, 1962). I confirmed a value close to this, and found the cK DNA to be indistinguishable from a similar DNA preparation made from wild type pneumococcus.

The centrifugation was performed in a Model E analytical ultracentrifuge using a two-cell rotor. One cell contained 0.1 OD unit of the

cK DNA and the other 0.1 OD unit of the wild type. Each cell also contained 0.1 OD unit of a standard DNA prepared from Micrococcus lysodeikticus, of known density 1.731 g/cc. The density of the cesium chloride solution was controlled by diluting weighed samples of crystalline optical grade cesium chloride with weighed amounts of water. This density was confirmed by measurement of the refractive index of the solution.

After 20 hours of centrifugation at 44,770 rpm at 25°C., photographs were made using ultraviolet absorption optics. After development, the images on the negatives were traced using a Joyce-Loebl recording densitometer. By measuring the radial distances of the two resulting DNA peaks and correcting them to the actual corresponding distances in the rotor, values could be obtained to use in the equation of Schildkraut, Marmur, and Doty (1962). Based on this calculation, the densities of both wild type and cK DNA proved to be 1.699 g/cc.

The cK peak appeared to be symmetrical and there was no evidence of absorbing material in any other position. However there was a possibility that a secondary peak might have been obscured by the control M. lysodeikticus DNA, or present in such small quantities that it was not visible above the baseline. Accordingly I repeated the experiment several times, omitting the control DNA and increasing the sample size to 1.0 OD unit. In each trial wild type DNA was compared with cK. In one run there appeared to be some absorbing material floating on the gradient. It failed to band, even after the density at the top of the gradient was reduced to 1.6500, so it could not be nucleic acid. It did not disappear when the DNA solution was digested with pronase, eliminating the possibility of a protein contaminant. It must therefore be an artifact of the centrifugation, perhaps a scattering of the ultraviolet light owing to drying of the solution. No peak other than the main one was ever seen. Thus, if DNA bearing the c region has different physical properties from the bulk DNA, those properties do not allow it to separate from the main peak sufficiently to detect by analytical density gradient centrifugation.

An increased opportunity of detecting resolved c-specific DNA might be possible to preparative equilibrium density gradient centrifugation. DNA present in quantities undetectable by ultraviolet absorption may still

possess transforming activity. Also a skewing of c-specific activity within the major peak might be detectable. However perturbations of the gradient during collection of fractions and the increased intrinsic variability of a biological assay might act in the opposite direction. Nevertheless the approach was tried.

Centrifugation was performed in a Model L ultracentrifuge using an SW39 rotor. The temperature during spinning was 55°F. and the speed was 35,000 rpm., leading to a shallower gradient of density than in the analytical experiments. The cesium chloride solution was adjusted so that the main DNA band would come to equilibrium at the center of the gradient. The duration of the centrifugation was about 70 hours. Fractions were collected from the bottom of the cellulose nitrate tube through a small bore needle which delivered an average of 0.011 ml per drop. The drop size of the cesium chloride solution varied as the solution density changed. Flow rate was controlled at about 1 drop per second using a syringe attached to the top of the tube containing the gradient.

In the first experiment 1.0 OD unit of cK DNA was banded and 220 two-drop fractions were collected. The fractions were all diluted with 0.5 ml of 0.15 M NaCl solution, and their ultraviolet absorption was measured. Since cesium chloride at high concentrations is toxic to pneumococcal transformation, selected fractions were assayed for transforming activity both at 0.1 and 0.01 the concentration of the diluted fraction. The transformation procedure was the standard one, with a 15 minute exposure of the competent cells to the fractions. Within the peak there was no fraction containing either c or mic which did not have strong activity for the other. However the ratio c/mic seemed higher in the more dense edge of the peak than in the less dense. Only two of the fractions selected from the areas of the gradient on either side of the peak had any detectable activity, but both of these had activity for mic as well as c.

There was the possibility that such low concentrations of DNA were below the detectable limit of activity. So a second experiment was performed, overloading the gradient with 3.0 OD units of cK DNA. All parameters of the centrifugation were the same as in the first experiment,

except that the run lasted 84 hours, and the fractions collected contained 4 drops each. The excess DNA made the band very viscous, and it plugged the needle during collection of the drops. It was necessary to dislodge the plug, a procedure which gave as an artifact a second peak of absorption. However its activity was the same as the first peak. Once again every fraction containing c activity also contained mic, and the leading edge of the peak seemed enriched for c relative to mic.

A third experiment is illustrated in Figure 9. The parameters of the run were as before except that the temperature was much lower. This led to a skewing of the major peak probably owing to diffusion during collection of the fractions. The centrifugation lasted 84 hours. The absorption profile of this experiment along with the relative transforming activity of selected fractions is given in Figure 9. Again enrichment for relative c activity in the leading edge is apparent.

If, however, transformation frequency per OD unit of DNA was examined, it became clear that this effect represented a diminished density of mic activity, not an enhanced density of c activity, except in the very first fraction of the peak, where absorption measurements are less reliable. That mic appears to band at a lighter density than other markers has been observed before (Mindich and Hotchkiss, 1964a).

Selection of transformants of wild type cells by the various DNA fractions permitted analysis of their frequency of segregation. In the second experiment although transformants from fractions within the peak appeared normal, fractions from the leading edge yielded transformants with markedly enhanced segregant frequencies, as seen in Table XXIII.

Table XXIII: Segregant frequencies of transformants of various CsCl fractions.

Fraction no.:	58 (first active fraction)	60 (rising edge)	63 (trailing edge)	82 (artifact peak)
Mean segregant frequency	21.4%	26%	7.7%	5.1%
Number of transformants tested	12	17	6	2

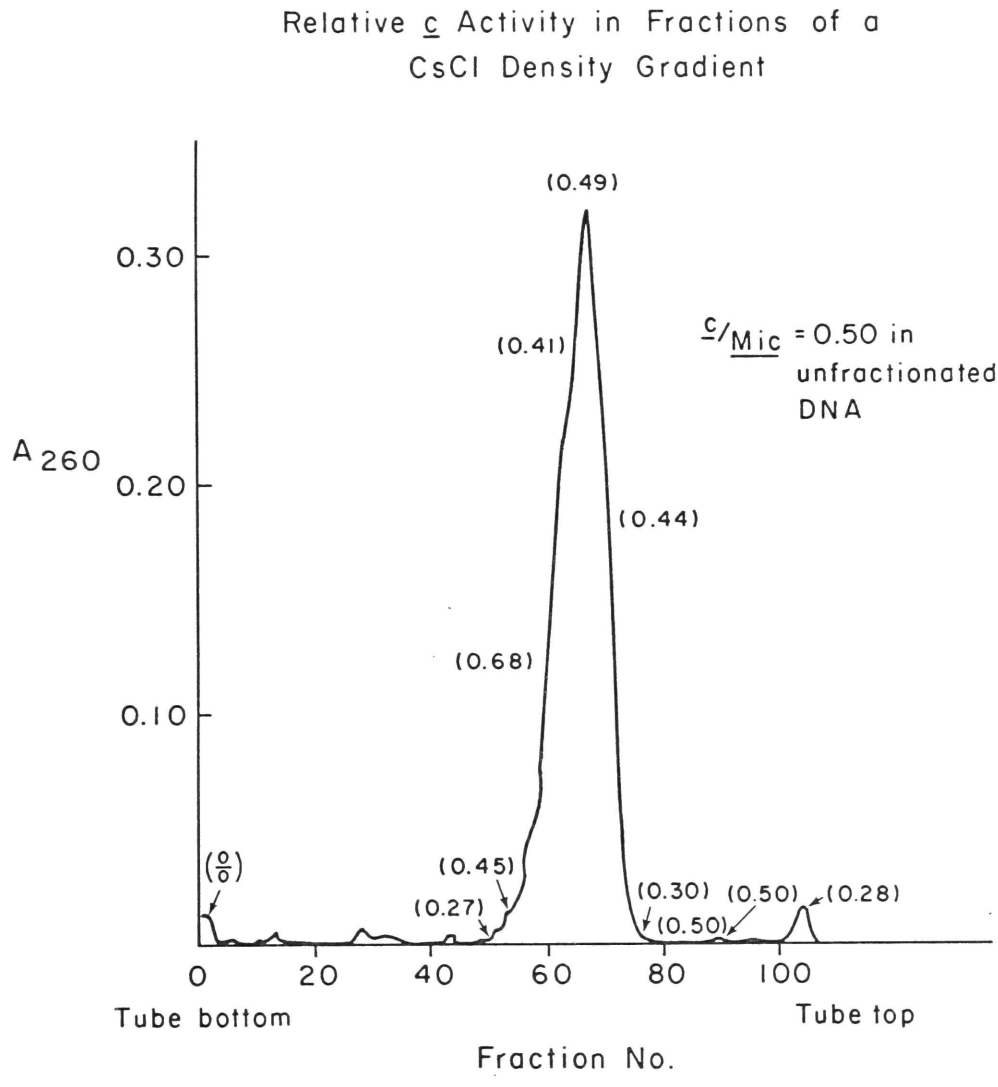


Figure 9: Fractionation of c DNA on a cesium chloride density gradient. See text for details of centrifugation. c/mic for the indicated fractions are given in parentheses.

However, testing of transformants of the third experiment for their segregant frequencies gave the results in Table XXIV.

Table XXIV: Segregant frequencies of transformants of various cesium chloride tractions, part 2.

Fraction no.	50	54	60	65	67	71	104
Mean segregant frequency	6.6%	3.1%	2.7%	3.3%	2.6%	3.5%	3.2%
Number of transformants tested	2	12	5	6	5	6	7

There is possibly an enhanced segregant frequency among transformants of the minor component in fraction 50--corresponding to very little of the DNA--but certainly not so dramatic as in the previous experiment and well within the normal limits of c.

Thus we must conclude that although c activity may be partially separated from mic activity by equilibrium centrifugation in a density gradient, this separation is more likely owing to properties of mic than of c. Subfractionation of c with respect to the segregant frequencies of its transformants may be possible, but owing to the lack of reproducibility of the results and the difficulty of their interpretation, this question is left open.

Adsorption of c containing DNA to methylated albumin-kieselguhr (MAK)

Over the years a number of attempts have been made to fractionate pneumococcal DNA chromatographically on the basis of one or several of its physical properties, and to relate the transforming activities of the resulting fractions to the properties determining the separation. Beiser and collaborators, using ECTEOLA, a cellulose-derived anion exchanger, were able to elute all the bound DNA, but could not localize transforming activity to any one fraction (Beiser, Pahl, Rosenkranz, and Bendich, 1959). Mindich and Hotchkiss used IRC-50, a polycarboxylate resin, and eluted the DNA with a continuous gradient of salt concentration. They were able to fractionate the pneumococcal DNA on the basis of size and base compo-

sition of the molecules. DNA from bacterial species of different base composition could be completely resolved, and high molecular weight RNA or denatured DNA were not preferentially retarded (Mindich and Hotchkiss, 1964b). On the basis of transforming activity of the various fractions obtained from a multiply-marked DNA, the authors suggested that the genome fragments bearing aminopterin resistance and str were richer in guanine + cytosine (GC) than those bearing sul, mic, or canavanine resistance, an order confirmed by measurement of buoyant density of the fractions (Mindich and Hotchkiss, 1964b).

Roger, Beckmann, and Hotchkiss in 1966 described a method of separation of native from denatured DNA by adsorption to a column of methylated albumin-kieselguhr (MAK) followed by stepwise elution with increasing salt concentrations. This method was based on reports of Sueoka and Cheng (1962) that stepwise elution from MAK separated DNA according to a number of its properties, including molecular size, base composition, and hydrogen bond content. DNA with a higher GC content or a greater double-stranded character eluted at a lower salt concentration (Sueoka and Cheng, 1962). It is the method of Roger, et al., that was applied to the attempted fractionation of c-containing DNA. Another possibility as a column substrate was hydroxylapatite, which also distinguishes native from denatured DNA (Bernardi, 1971). However, partially single-stranded molecules will elute from hydroxylapatite with native DNA, whereas on MAK they will elute at the denatured position (M. Roger, personal communication). Since the c abnormality is probably not so extensive as to encompass an entire DNA fragment (unless the DNA were to be sheared to a degree endangering its transforming activity), it was hoped that MAK adsorption could distinguish the hypothetical partially abnormal DNA fragment from the bulk of the DNA. However, since differential adsorption to MAK is still in an empirical state, no precise structural conclusion could be drawn from a successful separation. The adsorption of the postulated triple-stranded c DNA to MAK might be controlled by its greater charge density than a double-stranded structure. On the other hand the presence of unpaired ends might lend it a single-stranded character.

The preparation of MAK was carried out as described by Roger, et al.

(1966), using methylated albumin kindly supplied by Dr. Muriel Roger and Hyflo SuperCel for the kieselguhr. The MAK was extensively washed and equilibrated with a solution of 0.5M NaCl-0.02M K_2HPO_4, pH 6.8. Then the MAK was carefully poured into a 1 x 10 cm column and washed with the 0.5M saline-phosphate solution. All procedures were carried out at room temperature. Ten OD units of cK DNA were adsorbed to the column, and the column was repeatedly washed with 0.5M saline-phosphate until no more non-adsorbed DNA was removed. Then 9-ml. volumes of salt solutions of progressively increasing concentration were added, 0.6M, 0.7M, 0.8M, and 1.0M NaCl-0.02M phosphate, pH 6.8. Each salt solution was allowed to run down to the top of the MAK before the next was added. Fractions of about 3 ml each were collected and their ultraviolet absorption measured. The recovery of DNA was about 35% (it is known that native DNA is tenaciously bound to MAK). Native DNA was expected to elute at about 0.8M salt, while denatured elutes at 1.0M (Roger, Beckmann, and Hotchkiss, 1966). Transformation assays of the fractions were performed in the usual manner with a ten-minute exposure of the competent cells to low-plateau levels of DNA. None of the fractions obtained during washing with 0.5M saline-phosphate had any transforming activity, nor did the last two fractions eluted with 1.0M saline-phosphate.

Although only 8% of the total OD units were recovered in the fractions eluted with 0.6M saline - phosphate, these fractions were active in transformation, and appeared to be enriched in c transforming activity relative to mic. However, as in the centrifugation experiments, no fraction could be obtained which lacked completely either c or mic. From the fractions eluted with 0.7M salt or greater, the c/mic ratios were in the normal range, with a mean of 0.32 ± 0.07. Segregant frequencies of transformants of wild type cells by fractionated DNA were all normal.

The column fractionation was repeated using cSK DNA in an attempt to distinguish whether the apparent separation of c and mic activities represented a true enrichment of c activity or rather a retarding of mic. Calculations of transforming activity per OD unit tended to favor the latter interpretation, but introducing the str marker would confirm it. Half the previous column height was used, and 5 OD units of cSK DNA in

0.5M saline-phosphate were adsorbed. The same eluting salt concentrations were used--0.5, 0.6, 0.7, 0.8, and 1.0M--but their volumes were 5 ml each, and the fractions collected were 2 ml, 1 ml, and 2 ml for each eluting salt concentration.

The elution profile of this experiment is given in Figure 10. The recovery was about 65% at the point where the experiment was discontinued. Selected fractions indicated in Figure 10 were assayed for their transforming activity. A standard DNA dilution which placed the concentration in the low plateau region was used, and the exposure of the cells was fifteen minutes. The marker frequencies obtained are given in Figure 10. From these data it is clear that although the 0.6M fractions were enriched for c activity relative to mic, the c/str is perfectly uniform throughout the fractions tested, while mic/str is depressed in the first few fractions. Thus c cannot be said to behave unusually in its adsorption and elution from MAK.

Effect of treatment with formaldehyde on the c transforming activity

Formaldehyde has long been used as a histochemical fixative because of its ability to cross-link amino and sulfhydryl groups in proteins. But purified RNA from tobacco mosaic virus was also found to be inactivated by treatment with 1 to 2% formaldehyde (Fraenkel-Conrat, 1954). The inactivation was accompanied by a gradual increase in ultraviolet absorption of as much as 30% and a shift of the peak 3 to 5 nm to higher wavelengths. The reaction was complete in 48 hours at room temperature, 18 hours at 40°C. All bases in the RNA which contained a primary amino group were affected, presumably by formation of a labile Schiff's base (Fraenkel-Conrat, 1954). Under these conditions native DNA or the activity of Hemophilus influenzae transforming DNA is unaffected. This difference in reactivity is presumably due to the double-stranded configuration of DNA, because deoxyribonucleotides and ribonucleotides have similar reaction properties. These reactions are inhibited at pH values within the range of protonation of the primary amino groups, and appear to be base catalyzed at alkaline pH. Formaldehyde can react readily with denatured DNA, and shifts its melting curve to lower temperatures, but

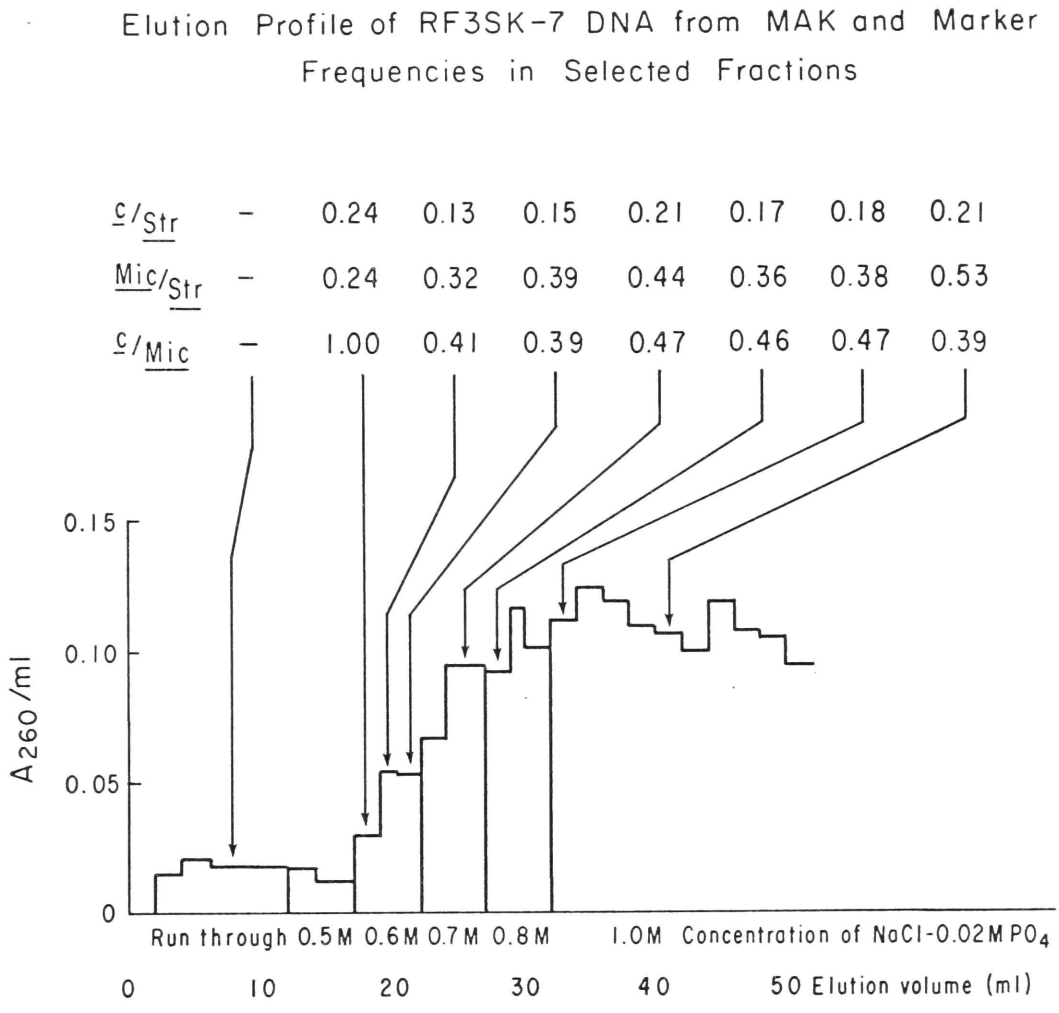


Figure 10: Fractionation of c DNA by differential adsorption to MAK. See text for details of elution.

does not itself cause denaturation (Grossman, Levine, and Allison, 1961) without an energy input of 35 kcal/mole between 35 and 45°C. (Haselkorn and Doty, 1961). No apparent damage to the phosphodiester backbone of polynucleotides results. The addition reaction to the primary amino groups is reversible and without harm to them.

Haselkorn and Doty devised a spectrophotometric method to measure the extent of the reaction, which was confirmed by measurements of binding of radioactive formaldehyde (Haselkorn and Doty, 1961). Marmur, et al., described a modification of this method to detect ultraviolet-irradiation induced damage in pneumococcal transforming DNA. They interpreted the increased reactivity with formaldehyde of irradiated DNA as evidence that the ultraviolet light has weakened the DNA secondary structure (Marmur, Anderson, Matthews, Berns, Gajewska, Land and Doty, 1961). Although the reaction with amino groups is reversible, Stollar and Grossman (1962) reported that when it occurs during thermal denaturation, and the reaction mixture is then cooled, the amino groups are no longer available for hydrogen bonding. Even after dialysis there is no appreciable loss of labeled formaldehyde (Grossman, 1968) although the ultraviolet absorption returns to the level characteristic of double-stranded DNA. Treated DNA cannot be renatured by slow cooling and it is of greatly increased effectiveness as an antigen in combination with antibodies to denatured DNA (Stollar and Grossman, 1962). In a summary of formaldehyde chemistry Walker (1964) concluded that both stable and labile bonds could form between nucleic acids and formaldehyde. The labile bonds are methylol addition products, while the stable bonds are methylene bis linkages, which remain after the removal of the formaldehyde by dialysis (Walker, 1964). For molecules thought to be partially denatured, the binding of radioactive formaldehyde may be a sensitive measure of the degree of denatured character (Kidson, 1969).

From the foregoing discussion formaldehyde treatment seemed a good tool to probe the possibility of the discontinuous ends in c DNA postulated by the triple-stranded model. Whether one entire strand at a time is unbonded, or the two strands share hydrogen bonds with the continuous strand, there should be a considerable number of free amino groups in

the region, available for reaction with formaldehyde.

The spectrophotometric method described by Marmur, et al., was the first approach tried. The reaction medium was 0.15M NaCl-0.015M sodium citrate. A Zeiss spectrophotometer was fitted with a circulating water supply to a jacketed cuvette holder, permitting precise control of the temperature. In addition to the blank cuvette for setting the instrument, there was a control without DNA, a cuvette containing 0.5 OD units/ml of cK DNA, and one containing 0.7 OD units/ml of a DNA bearing sul-d, str, and mic markers but no c. The absorbances were read at 260 and 270 nm at 25°C., then the temperature was raised to 51°C. and the absorbances measured again. Formaldehyde was added to the control cuvette yielding a final concentration of 2.8% and the absorption increment (negligible) was measured. Finally the same amount of formaldehyde was added to the two cuvettes containing the DNA samples. Absorption was measured at various time intervals for 18 hours. A plot was then constructed of the ratio $(OD_f - OD_{t_o}) / (OD_f - OD_t)$ as a function of time, where

OD_f = absorbance at the end of the reaction,

OD_{t_o} = absorbance at the beginning of the reaction, and

OD_t = absorbance at any time t during the reaction,

all measured at 270 nm. The curves for the two DNA's, though not identical, reflected the same course of reaction: there was a sharp initial rise in the first 30 minutes, a long period (100 minutes) of no appreciable change, then a gradually increasing slope to the end of the reaction. The DNA lacking c marker showed the more pronounced initial reactivity, probably owing to contamination with RNA. Thus, this method of measurement was insufficient to distinguish c DNA from wild type on the basis of its formaldehyde reactivity.

The next step was to devise a formaldehyde treatment method which would permit recovery of transforming activity. The only biological activities previously assayed after formaldehyde treatment have been infectivity of tobacco mosaic viral RNA (Fraenkel-Conrat, 1954), activity as a substrate for enzymic digestion (Grossman, 1968), antigenic activity (Stollar and Grossman, 1961), and ability to transform H. influenzae (reported by Fraenkel-Conrat, 1954).

I chose 0.05M borate buffer in 0.05M KCl, pH 8.3 as the solution in which to treat the DNA. This pH is well below the alkaline denaturation point of DNA, which at this ionic strength does not begin until about pH 11.5 (Ageno, Dore, and Frontali, 1969). The interstrand crosslinking reaction (usually between adenine residues) is favored by high concentrations of formaldehyde and non-alkaline pH. Grossman reports that 0.04M borate buffer pH 9 minimizes this reaction. Usually in the other cases where biological activity was measured, the reaction was stopped and formaldehyde reduced to non-interfering levels by a dilution. When the treated DNA was used as a substrate for exonuclease activity, the reaction was performed in glycine buffer, which had the additional advantage of providing many primary amino groups to react with residual formaldehyde (Grossman, 1968). I combined all these features, and stopped the reaction by diluting samples of the reaction mixture twenty-fold into 0.1M glycine buffer in 0.1 M NaCl, pH 8.1. Then the samples were dialyzed against 0.1M citrate-0.15M NaCl to remove the glycine and unreacted formaldehyde.

The experimental design was the following:
The same cK DNA used in the cesium chloride and MAK experiments was diluted to a concentration of 1 OD unit/ml into borate buffer (control) or borate buffer containing 1% formaldehyde (treated). The reaction volume was about 0.6 ml. Immediately and after 1, 10, 30, and 120 minutes 0.1 ml samples were diluted twenty-fold into glycine buffer and dialyzed against citrate-saline. All procedures took place at room temperature. After a period of dialysis ranging from 4 hours to overnight, samples were diluted to uniform ultraviolet absorption and assayed for transforming ability. Exposure of competent cells to the DNA samples was ten minutes. If the dialysis step were omitted and the transformation performed in the presence of only 0.005% formaldehyde, no activity for either c or mic could be recovered even for the sample taken immediately. Thus formaldehyde is exceedingly toxic to the transformation process.

The results of the first experiment are given in Table XXV. The numbers reported are the fraction of the activity of the control at 0 time.

Table XXV: Inactivation of DNA by treatment with formaldehyde, pH 8.3, at room temperature.

	mic	c(SA)	c(PAS)
Control 0	1.0	1.0	1.0
120 minutes	1.8	1.6	1.1
Treated 0	0.92	1.2	1.1
1 minute	0.86	1.0	1.0
9 minutes	1.2	0.95	1.2
29 "	0.83	1.1	0.88
120 "	0.57	1.0	0.84

The experiment was repeated treating at 49°C. and at pH 9.7 on the hypothesis that although a triple-stranded structure might be inaccessible to formaldehyde at room temperature, it might be more loosely bonded at elevated temperature or more alkaline pH. The results are similar to those in Table XXV.

It can be seen that the results are quite variable. Although the progression with time is irregular, the longest treatment does seem to give measurable inactivation. However, the extent of inactivation of c is no more than for mic. So either the DNA does not react with formaldehyde at all and some other factor is causing the apparent inactivation, or the inactivation is caused by the progressive entry of formaldehyde into the marker structure but no more into c than into mic. A third possibility is that formaldehyde binding does not interfere with transforming activity. In any event no differential inactivation of c may be seen, whatever the cause. Thus this approach too has failed to provide support for a loosely bonded structure.

From the earlier consideration of how the small extent of the merozygous region might mask any perturbations of physical properties resulting from a triple-stranded structure, such a structure cannot be excluded from consideration as a result of the experiments to test differential buoyant density and differential adsorption to MAK. The formaldehyde inactivation work is potentially a more powerful tool, but as can be

seen from the fluctuations of the transforming activity in the treated samples, the manipulations needed preliminary to assaying the samples are a considerable source of error.

Previous work by Abe and Hotchkiss (Hotchkiss, personal communication) involved sub-critical heat inactivation of c-containing DNA. Holding a DNA solution at an elevated temperature below the melting temperature of the double helix leads to local depurination. Presumably more loosely bonded regions would undergo such depurination more readily than those with a regular structure. However, c was found to be inactivated at a rate similar to other stable markers. Hotchkiss and Lane also attempted to fractionate c DNA according to its sedimentation velocity in a sucrose gradient, supposing that the increased flexibility of a loosely bonded triple-stranded region might tend to diminish its sedimentation velocity. Here again c was indistinguishable from stable markers (Hotchkiss, personal communication).

Taken as a whole, this body of negative results obtained by so many different methods begins to suggest that the triple-stranded model is not a very likely possibility for the structure of the c region. Dr. S.V.S. Kashmiri in this laboratory has also been working on the problem, and as some of his results have a significant bearing on this discussion, I include them here with his kind consent. Kashmiri studied the transforming activity of c and cd DNA in their native, denatured, and annealed forms. In all three states the activity of c and of its associated d and d^+ remained a constant proportion of that of the str marker. To reconcile this result with a three-stranded structure, one must say that triple-stranded native DNA, single-stranded denatured DNA, and (presumably) double-stranded re-annealed DNA all have equal average efficiency in introducing c into a recipient cell and maintaining its relationship with the d and str loci. loci. For this conclusion to be plausible, considerable modification of the triple-stranded model must be made (Kashmiri and Hotchkiss, manuscript in preparation).

Kashmiri has taken this investigation another step. After denaturing DNA from a c or cd strain, he separated the complementary strands, using their property of differential affinity for the ribo-copolymer, poly UG,

and their resulting differential density in an equilibrium cesium chloride density gradient (Hradecna and Szybalski, 1967). This fractionation gave only two well-separated peaks, neither of which was active in transformation by itself. When the two peaks were mixed and annealed, up to 50% of the original transforming activity was restored. In addition to preparing such homoduplexes, whose complementary strands originated in the same DNA, Kashmiri mixed DNA strands bearing different genetic markers to prepare heteroduplexes. A summary of some of his results appear in Table XXVI.

Table XXVI: Marker recovery in heteroduplex DNA*

	Heteroduplex structure	str	c	d	d^+	cd
I	c-d^+-str/c^+-d^+-str	67.5	26.4			
		64.6	21.8		59.0	
	c-d^+-str/c^+-d-str	49.6	19.2	85.1		
		64.0	30.9		49.2	
	c-d-str/c^+-d^+-str	85.4	47.9	59.1		35.2
	c-d-str/c^+-d-str	90.6	40.9			
II	c-d^+-str/c-d-str	69.6	70.7	105		78.2
		77.9	79.3		78.2	
III	c^+-d^+-str/c^+-d-str			92.3		
					77.5	

* These figures represent the mean of the activity of the two reciprocal forms of the heteroduplex as a percent of the mean of the activities of the corresponding homoduplexes. The two forms of the heteroduplex must both be constructed and tested because of the demonstrated strand bias in pneumococcal markers (Gabor and Hotchkiss, 1969), one strand having more transforming activity for a given marker than its complement has.

The first part of Table XXVI shows the results for heteroduplexes containing a c region in one strand and a non-c region in the other. Not only is the recovery of c activity greatly depressed compared to the str marker, but so is d or d^+ when borne on the same strand as c. That this effect is a property of the mismatch at the c locus is evident from the second part of the table, where both c and c-associated d or d^+ show recoverable activities at least as great as str in heteroduplexes with c in both strands. The third part of the table confirms that it is asso-

ciation with heteroduplex c that controls the activity of d, not some property of being heteroduplex at the d locus per se.

This is strong evidence in support of the tandem duplication model and rejecting that of the triple-stranded structure. It is hard to imagine how redistribution of c and c^+ alleles on heteroduplex DNA should be so specifically prejudicial to the strand containing c activity, especially considering that in the "heterotriplex" structure of the triple-stranded model, c has perfectly normal activity. If, however, the true structure of c is a tandem duplication, then complementary base pairing in a heteroduplex with a non-c DNA would demand looping-out of a single-stranded region containing the extra copy. The depressed transforming activity could be explained either as a decreased likelihood of such a structure forming in the annealing mixture, or a decreased probability of its surviving the various steps of transformation to become integrated intact.

In summary, a variety of treatments of the DNA from c and cd strains has revealed no evidence favoring the triple-stranded model and two very suggestive pieces of data supporting the model of a tandem duplication. Thus it is proposed that further studies of the c strain be designed to reveal further properties confirming or refuting this model.

VI
DISCUSSION

In the preceding chapters it was appropriate to discuss the results as they were presented, both to make the argument more lucid, and to suggest motivation for subsequent experimental steps. Therefore in this chapter I will briefly recapitulate the evidence developed, and then procede to some rather more speculative implications of those results in terms of the specific pneumococcal c mutation and, more generally, of duplications as structural elements of segregating systems.

In Chapter I a number of segregating prokaryotic systems with a variety of properties were described. Based on various manipulations of the systems, some authors attributed instabilities to genes causing non-specific mutation at high frequencies (Treffers, et al., 1954; Goldstein and Smoot, 1955; Zamenhof, de Giovanni, and Greer, 1958), some to the presence or absence at a particular locus of an episomal fragment which in the integrated form generated a mutant phenotype (Dawson and Smith-Keary, 1963; Hill, 1963; Taylor, 1963), and some to a merozygosis in which mutant and wild type alleles coexist within the cell and which segregates by separation of these alleles. Two variations of this last form of instability arise: those systems in which the duplicated material is borne on an extrachromosomal piece of DNA (probably Lederberg, 1951; Ames, et al., 1963; Curtiss, 1964; Schwartz, 1965; Soll and Berg, 1969) and those systems in which the duplicated region is located within the chromosome itself (Newcombe, 1953; Hopwood, 1967; Campbell, 1965; Horiuchi, et al., 1963; Hill, et al., 1969; Folk and Berg, 1971; Russell et al., 1970; Bernheimer and Wermundsen, 1969; Ravin and Takahashi, 1970).

Usually in the latter case, the duplication is thought of as a tandem duplication, because in the simplest models of segregation involving either a looping out or an unequal crossing over, only a duplication in tandem can segregate without loss of intervening non-duplicated-- and presumably essential--chromosomal material (Hershey, 1970). However evidence favoring a duplication in the first place, and particularly a

tandem duplication, is necessarily indirect (Hill, Foulds, Soll and Berg, 1969) except in the most favorable cases (Russell, et al., 1970).

A series of criteria for determining the nature of a segregating strain may be developed from considerations of logic and of previous work.

1) For an instability to have a genetic basis, it must be heritable; otherwise it is a physiological modification. Cloned populations must retain the instability over many generations in the absence of selection. Where a mating process is available, the instability must persist throughout.

2) If the source of instability is a mutator gene, one would expect mutation in the mutator gene itself to render the strain stable. This occurrence would be as rare as spontaneous mutation, so that a rapid rate of stabilization would rule out this possibility.

3) If the source of instability is an extrachromosomal controlling element, its loss should render the strain stable. The frequency of this loss would likely be many times greater than spontaneous mutation, but should not result in reassortment of host gene combinations.

4) If the source of instability is merozygosis, then both alternative forms of a duplicated allele should coexist in a single cell of the strain, and reassortment could occur on segregation. In this category two types of merozygosis are possible:

a) If the merozygosis is extrachromosomal, no linkage should be demonstrable between segregating genes and stable genes of the same strain.

b) If the merozygosis is contained within the chromosome, segregating markers should show linkage to stable markers.

In applying these criteria to the sul-c mutant strain of pneumococcus, we have seen (Chapter III) that the instability is transmissible not only through single colony isolation and many passages in the absence of selection, but also in transformation through the DNA of the strain, where it behaves as a single mutation. By all available criteria, the segregants arising from the c strain are stable wild type. The DNA of the c strain can carry a full complement of both allelic forms of a closely linked

locus, d, as determined by transformation, and these forms can segregate in non-parental combinations. Thus the instability must be a merozygosis, in which two sets of determinants for the d (and c) locus coexist. That this merozygosis is intrachromosomal is established from the linkage of c to str, a marker which cannot be demonstrated to be involved in instability.

Several further questions on the nature of "c-ness" may arise. Is c a mutation with a distinct phenotype which can be detected in the absence of instability? or is what we call "c" merely a duplication of the d locus? In Appendix A are described experiments which indicate that among 113 freshly isolated c transformants constructed under a variety of conditions and selected at the very lowest drug levels allowing clear distinction of transformed cells from recipients, all showed instability and to the same extent as documented for normal c or cd strains. From this, it appears that the mutation and the merozygosis are inseparable. However it cannot be excluded that the mutation, c, can exist without the merozygosis, but that it has an unsuspected phenotypic alteration. In any case there must be some structural abnormality, which can be designated the c mutation, that generates instability in transformants.

Consider the transformation of a d recipient by a c donor DNA. Three distinct types of transformants can result. Two of these, cd-10 and cd-13, can be imagined to arise by integration of the cd^+ donor DNA into a c^+d recipient chromosome. But what about cd-11? There is no basic reason to suppose that the recipient d strain is abnormal at the d locus, so it is probably haploid. The c donor possesses only d^+ determinants. But somehow, at an incidence of about half of all cd transformants, a cd transformant type can arise which now has an excess of d allele. According to either structural model under consideration, the initial integration of c must readily induce the recipient cell to amplify its own complement of the resident d allele. The reciprocal case also holds, that is, a wild type recipient transformed by cd-11 donor DNA can give c transformants. Since the recipient has the normal d^+ complement and the donor has only d and no d^+, the extra d^+ must be generated from the recipient cell. Such an effect is very similar to that seen by Hill, et al., when a duplicated genome segment is transduced into a normal recipient by a phage

whose carrying capacity is limited to only a part of the duplicated region. As long as a critical segment is transduced, the entire duplication can be regenerated, the recipient providing the markers which were not carried by the phage (Hill, Schiffer, and Berg, 1969).

Thus we can designate the c mutation as that structural alteration which permits generation of a duplicated d region and the enhanced drug-resistance concomitant to it. It may be fortuitous that the measured linkage of c to d is 0.66, and that the frequency of cd-11 among all cd transformants is about 0.5. It would be an interesting hypothesis that c entering unlinked to d (or to d^+) generates duplication of the recipient locus, but entering with its own linked d locus, can introduce it as a ready-made duplicate.

The reader might wonder why so much emphasis has been placed on relatively indirect evidence for the presence of both alternative alleles in the c strain, when a known enzyme is affected. Should it not be possible to isolate that enzyme and determine whether two populations with different properties may be found? or if only a single population, whether the alteration in property can be related to the known genetic properties? This approach could very well be useful and provide a different point of attack for the problem. However, the enzyme has proven difficult to isolate in a reproducible manner, and the bioassay used to measure the production of folic acid is cumbersome and difficult to quantitate. Preliminary studies of synthesis of folic acid in c cells in the presence of different concentrations of SA showed that at a SA level inhibiting 90% of wild type synthesis, the c strain was still able to make 85% of its normal folic acid complement (Hotchkiss, personal communication). On the simplest hypothesis of two enzymes produced in the c strain, one wild type, one mutant, one would expect c to be 50% inhibited at a SA concentration sufficient to inactivate wild type. However this evidence does not rule out presence in the c strain of determinants for two enzymes, with a more complex interaction between their regulation or activity and their expression as folic acid synthesis.

The alternative possibility is that the extra information contained by the c genome codes not for an entire extra enzyme, but for an extra

polypeptide segment within the existing folic acid synthetase. Such an altered enzyme need not be inactive, but would probably display an altered phenotype when exposed to selection. It is not surprising to find the active site involved in the abnormality, since a normal strain bearing a d mutation has an altered enzymatic activity, and since the merozygosis encompasses the d locus.

The extent of the merozygosis is limited to the region immediately around c and d, rarely involving even the a locus--believed to affect the same enzyme--so it is probable that information for only a part of that enzyme is duplicated. It might be predicted that substrains of c or new c or cd transformants could be identified, on the basis of an altered drug resistance, which might have a longer or shorter duplicated segment. In fact, an occasional cd type has been isolated which grows well at SA levels sufficient to starve a c strain but which can only give tiny colonies at the SA level at which the cd normally grows vigorously. Perhaps this transformant type has a smaller duplicated region which yields a less drastic phenotypic modification of the recipient strain. However this possibility is difficult to confirm because the cells which do grow at high levels of SA are now as resistant as normal cd strains to SA. Thus there could be an optimal range for the length of the duplication. Any form which becomes stabilized as a longer (see Chapter III, p.45) or shorter (see above) region might resolve to the optimal length or stabilize under the slightest selection pressure.

In Chapter IV it was established that the segregant frequency of a line or subline containing c was a characteristic property of that line. Although from experiment to experiment some variability in the segregant frequency could be seen, within a single experiment, the results were quite reproducible. Certain perturbations of the growth environment, including treatment with ultraviolet light, mitomycin C, and acridine orange, led to progressive and reproducible alterations in the segregant frequency, the first two agents increasing it, and the last depressing it. It was concluded that all three agents acted on the DNA of the strain either to enhance or diminish the probability of the DNA becoming involved in a recombination event. When c is introduced into a strain containing a

mutation analogous to the recB-C mutants of E. coli, its segregation ability is unaffected, suggesting that if a recombination event be involved, it is more probably intra- than interchromosomal.

Two aspects of this work require further comment: the lack of accumulation of segregants normally, and the failure of the extra segregants formed under stimulation of ultraviolet light or mitomycin C to replicate as segregants. On p. 62 it was pointed out that the segregation behavior of a c strain is anomalous, in that it does not exhibit the random fluctuation of a series of subpopulations which Luria and Delbrueck (1943) established to be normal for randomly occurring genetic alterations such as mutations. Thus either segregation occurs by a non-random mechanism or it differs in some other way from mutation. The former hypothesis is unlikely in view of the firmly established genetic characteristics of both parental and segregant types, and their apparently random generation in the absence of selection.

How then could the segregation event differ from generation of a mutation? Although both events are probably complex in terms of the number of steps involved, a mutation can usually not be expressed until its gene product has had a chance to accumulate enough to affect the cellular phenotype. By this time, the mutation event itself is irreversible for all practical purposes, mutation rates being so low as to make the possibility of reversion negligible. Here however segregation may differ from mutation. It occurs at a much higher rate than mutation; and although clearly the final segregation product can never revert to the c type, there could well be intermediate steps whose reversibility might be significant. Furthermore, one can make the assumption that what grows in NOB is any intermediate in the segregation process, rather than the final product alone. Then scoring a culture in NOB will detect not only those cells which have become stably segregant, but also those which have experienced even the first, and readily reversible, step of segregation. The latter are likely to be more numerous than the former, so that an accumulation of segregants could be undetectably slow. And the rate of occurrence of the first step (or its reverse) would be so high as to rapidly overcome any fluctuations in the proportions of segregants in a

series of small populations.

If the action of ultraviolet and mitomycin C were to stimulate this first step, it would be analogous to shifting the equilibrium of the reaction toward the product. When the stimulus stops and growth resumes, the normal equilibrium state would become re-established.

An attractive model involving more than one step is an initial ultraviolet-sensitive recombination removing the extra genetic determinants from the chromosome, but reversible in that reintegration could be permitted. The final segregation leading to stable wild type might not occur until the excised piece were lost from the cell, thus becoming physically incapable of re-integration.

A necessary concomitant of this hypothesis is an intermediate stage at which a clone would appear capable of developing both parental and segregant phenotypes. Perhaps this is just what is seen in Appendix B, in which clones selected in liquid medium containing NOB still possess SA resistance which diminishes in proportion as growth in NOB continues. Why selection in NOB-containing agar should not give this result is unexplained. Perhaps liquid medium permits a greater diffusion of nutrients, tending to favor types which are less favored in the confines of a clone embedded in agar.

In Chapter V two models for the underlying structure of the c strain are proposed and tested. A number of experiments failing to support the triple-stranded model, together with two strong pieces of evidence in support of a tandem duplication model, lead us to propose that the latter represents the true structure of the c genome more closely than the former. However, I must emphasize that either is an equally valid explanation of the phenotypic and segregation properties. Each has considerable flexibility, too, to accomodate variant forms not yet described. According to the triple-stranded model markers could be arrayed in a number of configurations not yet seen. Also the region of overlap could be extended or reduced. On the tandem duplication model, phenotypic modification could arise from changes in the extent of the duplicated segment. However, it seems as if the configurations so far described are by far the most common, and thus presumably the most favored.

Accepting the evidence favoring the tandem duplication, we may speculate on the events leading to its origin. The c strain was selected as a spontaneous mutation to SA resistance. Other unstable strains have arisen spontaneously (Hopwood, 1967; Campbell, 1965; Horiuchi, et al., 1963) or under the influence of particular kinds of mutagenic agents (Green and Krieg, 1961; Hill, 1963; Newcombe, 1953; Schwartz, 1965). Still others arose as the result of mating processes (Lederberg, 1951; Luria, Adams, and Ting, 1960; Bernheimer and Wermundsen, 1969, for example). Mating processes involve recombination; spontaneous mutation probably involves an unequal crossing over (Demerec, 1960; Magni, 1969); and mutagenic agents such as ultraviolet light, ethylmethanesulfonate, and acridines generate mutations involving insertions and deletions which might well have a similar basis (Drake, 1969). Thus most cases of merozygosis originate in processes which probably have recombination as a common element. That so many of them are presumed to require recombination to segregate could be thought of as a reversal of the generation step.

A second feature held in common by the various merozygotic strains is the kind of selection under which they arose. In most cases the selection was for the ability to grow in the absence of a specific nutrient (Hill, et al., 1969), the ability to use a specific nutrient as the sole source of carbon (Lederberg, 1951), or the ability to survive a relatively benign drug selection. All of these selections have in common that they are gradual, starving sensitive cells as their requirement for nutrient becomes more acute rather than killing them outright. The sulfonamide selection, which effectively starves a cell for folic acid, may be considered as being of this type. Perhaps cells generate duplications fairly regularly and these duplications are only "stabilized" under these rigorous growth conditions. Or perhaps cells at a certain state of sickness become more active in rearranging their genomes in an attempt to cope with the stringent conditions. In any case, it seems reasonable that a selection which allows several divisions before cell death might well permit adjustment of individual cells to meet the challenge. Clearly this is pure speculation, since the only kind of evidence which may be adduced is the sort of correlation invoked above.

Several times I have attempted to regenerate the c merozygosis by using a combination of homologous transformation, ultraviolet irradiation, and gradual SA selection, without success. Thus the phenomenon is more involved and less probable of spontaneous occurrence, at least for the sulfonamide resistance locus, than had been hoped.

From the point of view of evolution in higher organisms, gene duplication followed by mutation in one copy has been proposed as a basic mechanism permitting an increase in the content of variability without a corresponding sacrifice of potentially advantageous function which would be the result of mutation alone. Considerable evidence consistent with such an evolutionary mechanism has been assembled from amino acid sequence studies of hemoglobins, cytochromes, immunoglobulins, polypeptide hormones, and isozymes of a variety of eukaryotic species. Such a mechanism could represent a significant factor in prokaryotic evolution as well. The work of Soll and Berg (1969) on nonsense suppressors and the body of data implicating merozygosis in a number of suppressor strains suggest that genes determining species of transfer RNA are capable of great functional flexibility, providing that duplicate copies can perform the functions already established.

In summary, then, the study of merozygotes can be rewarding in terms of understanding basic genetic mechanisms, and allelic interactions within the cell. They may also provide useful models for the steps of evolution.

APPENDIX A

Evidence that the c phenotype and the property of instability are inseparable

The purpose of this series of experiments was to attempt to distinguish whether the c mutation, defined here as a mutation giving rise to the characteristic resistance to SA and PAS, can ever be separated from the property of instability. Theoretically this question could be approached either by isolating unstable derivatives and looking among them for absence of drug-resistance, or by isolating drug-resistant derivatives and examining them for loss of instability. Practically, the latter approach is more accessible.

The question arises because of the implications of our symbolic representation of the c and c^+ alleles as determining drug resistance or sensitivity respectively, and of co-existing in the merozygotes. Taken literally, this symbolism could be logically extended to imply that, just as stable c^+ (wild type) segregants can occur, so should stable c (drug resistant) types. These could be detected as clones with the full c drug resistance, but limited NOB resistance. Normally strains derived from c with the characteristic drug resistance contain about 5% NOB-resistant cells (see Chapter IV).

The impetus for this approach came from preliminary results of Dr. Kashmiri, who isolated transformants of d cells induced by native, denatured, and annealed-denatured DNA from the c strain. Among transformants selected according to their PAS resistance, one obtained by treatment with annealed donor DNA was unable to grow in levels of SA greater than 150 μg/ml, suggesting that the c donor has been able to replace the d recipient allele with a d^+. Thus, this clone must be a c clone. But it was unable to segregate to wild type (Kashmiri, unpublished results).

This result seemed to be confirmed in my hands. DNA bearing c and mic markers was denatured with 0.1 M NaOH, then neutralized. A sample of this denatured DNA 0.8 OD units/ml in 0.5M NaCl, was annealed by

heating at 65°C. (boiling methanol) for one hour, then quenching in an ice bath. The resulting DNA solutions, native, denatured, and annealed, were used to transform wild type and d recipient cells using the standard transformation assay and a ten-minute exposure to the DNA. Transformants were selected in liquid culture containing 50 μg/ml of PAS for either recipient, or 50 μg/ml or 600 μg/ml of SA for wild type or d recipient respectively. These drug concentrations are lower than the maximum resistance expressed by c or cd strains, to include the possibility that a "stable c" might have a different phenotype from the normal unstable c. Single colonies of these transformants were picked and rejuvenated, then screened for the properties of full drug-resistance or instability.

Among the first 17 c transformants of wild type tested, all appeared to have normal segregant frequencies (mean = 6.6 ± 1.9%) whether they were selected in SA or in PAS or whether they arose from native, denatured, or annealed DNA. However among 18 transformants of the d recipient, 8 showed no resistance to NOB. Six of these showed full resistance to 600 μg/ml SA, indicating them to be similar to the classical cd-11 type. The other two, however, appeared to lack resistance to SA at 600 μg/ml, though they resisted 150 μg/ml, as does a true c. Both of these arose from PAS selection of a transformation involving denatured DNA, a circumstance which seemed reasonable from some points of view to favor stable integration of the c. In the denatured state, separate from influences of the other DNA strand, a c strand might be less likely to regenerate the instability.

When 22 more clones of the same transformation were selected in 50 μg/ml PAS, fully 15 had similar properties to the two in the previous selection. These properties survived subcloning. When cultures of two of these putative "stable c" types were used to prepare DNA, and this DNA was used in a transformation assay, their resistance to PAS was readily transferred to either wild type or d recipient cells. However they were unable to enhance the SA-resistance of the d recipient, and the SA resistance induced in a wild type recipient was more characteristic of d than of c. Neither were any of these last resistant to PAS. None of the PAS-resistant transformants of wild type were at all resistant to SA. This array of properties suggested that what was originally selected

was not an altered c, but rather a PAS-resistant alteration of the original d recipient.

This was confirmed by transformations in which the "altered d" was used as a recipient in transformation. DNA from true d strains was unable to enhance its SA resistance, while c DNA was able to do so.

The next question was whether production of this "altered" phenotype could be observed in transformations by other than denatured DNA in recipients other than d. Thus many more single colonies from the original transformed cultures of the wild type recipient by the various DNA solutions were selected. Among 15 transformants induced by native DNA, 13 by denatured, and 10 by annealed DNA, selected in SA, all appeared to be normal c. Ten transformants by native DNA selected in PAS also appeared normal. But 6 out of 14 from denatured DNA and 2 out of 12 from annealed DNA and selected in PAS showed 100% resistance to NOB. This is an anomaly corresponding to the one observed using the d recipient: it appears to be an alteration of the basic recipient properties to the PAS-resistance typical of the c strain, with little other alteration. The introduction of this PAS-resistance is compatible with full retention of other basic properties of the recipient, including its SA and NOB resistance or sensitivity.

This modification is unlikely to be involved in determining the properties of c, since it can exist in an NOB-resistant strain, whereas the PAS-resistance of c is invariably accompanied by NOB-sensitivity. Also it appears to be unlinked to the d locus, in that a transformant for the PAS resistance is rarely transformant for d and vice versa. So the basic question of whether c and instability can be separated has been answered in the negative, at least for these 113 colonies.

However, this side issue of the PAS-resistant modification arising in transformations by denatured and, to a lesser degree, annealed c DNA was pursued. The key was provided when it was noted that a low background of PAS-resistant colonies always arises in selections involving PAS. In transformation using native DNA, the transformation frequency is quite high, and most observed PAS-resistant colonies result from true transformation events. However annealed DNA is less efficient, and

denatured DNA may have only 1 to 5% native activity. In these cases it is necessary to correct PAS frequencies for the background. Most of the work reported here had involved native DNA, so this scoring anomaly had not been a problem. Now I tested the recipient cultures and found them all to contain low levels of spontaneous PAS-resistant colonies. These colonies were phenotypically identical to the PAS-resistant modification occurring in the PAS-selected "transformants." Furthermore the proportion of the total PAS-resistant frequency that could be attributed to this spontaneous background was very close to the proportion of the selected transformants which appeared not to be true c but possessed the modification. Although the normal wild type strain is sensitive to 5 μg/ml SA, both spontaneous PAS-resistant types and those which arose in the transformations exhibited a cross-resistance to about 20 μg/ml SA. This helps to explain how the "altered d" type had a SA resistance which could be confused with c. That the spontaneous PAS-resistance was a heritable property of the cell and not a long-lived physiological consequence of PAS selection was demonstrated by its survival through several passages and single colony isolations in the absence of PAS.

When DNA's from several of the spontaneous colonies (P11, P12, P13, P16, P17) were compared with DNA from one of the strains arising from the transformation by denatured c DNA (c65), the following efficiencies of transformation of the PAS-resistance were measured:

Table XXVII: Efficiency of PAS-resistant transformation by DNA from spontaneous and transformant PAS-resistant colonies.

	P11	P12	P13	P16	P17	c65
PAS/str	1.9	1.0	0.28	0.25	0.42	0.32
	2.6	0.91	0.21	0.22	0.24	0.34

From the results in Table XXVII it can be seen that the efficiency of PAS resistance in c65 DNA is not unlike those in some of the spontaneous colonies. Thus we must conclude that the modifications arising in the transformation experiments had nothing to do with the presence or the degree of denaturation of the c DNA.

APPENDIX B

Properties of c transformants selected from liquid culture versus agar plates

When a culture of cells bearing the c mutation is scored in liquid medium containing the drug NOB, the usual way to measure segregant frequency, there is some initial difficulty in deciding how to evaluate the resulting array of colonies. In addition to a rather constant number of colonies of uniform large size, there is usually a variable number of colonies of various sizes, as a "background." Their frequency and size depend on the concentration of NOB in the medium, the duration of the overnight incubation period, and the amount of dilution the cells in that particular culture have experienced. The large colonies on the other hand remain constant in numbers over NOB concentrations ranging from 10 to 40 μg/ml; they reflect dilution in a regular manner representative of a hypothetical "true" concentration of NOB-resistant colony-forming units in the culture being scored; and the uniformity of their size is unaffected by the duration of the incubation period. It is this latter type that are reported as "NOB-resistant segregants." To avoid confusion, background is partially controlled by incubating all assays a uniform 12 hours, using 20 μg/ml NOB as a standard concentration, and noting any apparent discrepancies in a dilution series.

In agar plates the problem of "background" is much less. Whether the selection is more strongly in favor of "true" segregants, or whether it is more difficult to see the "background" cannot be stated.

Customarily the segregants have been thought of as those pre-existing in the c culture at the time of scoring, while the "background" has been taken to represent segregation events occurring in the scoring tube before the NOB had killed the sensitive progenitor. This would explain their variable size (the number of divisions in NOB prior to formation of a segregant clone) and their dependence on cell concentration (presumably delayed starvation by NOB when a larger number of sensitive cells were present).

When a single resistant colony is selected from NOB, it is reasonable to suppose that it might be contaminated at a low level with invisible macrocolonies of incompletely starved sensitive cells. The resistant colony itself might conceivably have helped to sustain such sensitive contaminants, perhaps through cross-feeding. However even this explanation seems inadequate to explain the results summarized in Table XXVIII, in which three different sorts of c-containing cell lines were subcloned in medium containing NOB.

Table XXVIII: Summary of effects of selection in liquid culture on the retention of non-segregant characteristics.

Strain	Cloning series	Observations
stock c 5% NOB-resistant	step I	10 single colonies selected in NOB; all 100% NOB-resistant, each SA-resistant from 2.0 to 35%.
	step II	1 of these which had 11% residual SA resistance used to prepare 5 single colonies in NOB; all 100% NOB-resistant, 4 100% SA-sensitive, 1 3.3% SA-resistant.
	step III	a single colony in NOB from the unique colony in II now showed 100% NOB-resistant, 100% SA-sensitive.
stock cd-13 1% NOB-resistant	step I	10 single colonies selected in NOB; all 100% NOB-resistant, each SA-resistant from 0.12 to 8.8%.
	step II	1 of these which had 2.8% residual SA resistance used to prepare 5 single colonies in NOB; all 100% NOB-resistant, all 100% SA-sensitive.
Scd-5 0.4% NOB-resistant (transformant of c recipient by str-d DNA)	step I	3 single colonies selected in NOB; each 10 to 50% NOB-resistant, each 40 to 95% SA-resistant.
	step II	1 of these which had 18% NOB resistance, 84% SA resistance used to prepare 6 single colonies in NOB; all 100% NOB-resistant, each SA-resistant from 3.7 to 21%.
	step III	3 of these further subclones in NOB; all 100% NOB-resistant, all 100% SA-sensitive.

Between each of the selection steps there was a two-hour rejuvenation in the absence of drug. An estimated 16 divisions occurred between any single cell and its resulting clone. An estimated 3 divisions occurred

during rejuvenation.

All of the lines retained the SA-resistance characteristic of the NOB-sensitive parent line at least into the second subcloning, while two of them were not completely sensitive to SA until the third subcloning. Table XXIX, in contrast, illustrates the result of selection from agar plates containing NOB. Most of the colonies have entirely lost their SA resistance during the initial subcloning.

Table XXIX: Summary of effects of selection in agar plates on the retention of non-segregant characteristics.

Strain	Cloning series	Observations
stock c 3.3% NOB-resistant	step I	9 single colonies selected in NOB; all 100% NOB-resistant, 8 100% SA-sensitive, 1 0.5% SA-resistant.

Those NOB-resistant colonies recovered spontaneously in the absence of selection were 100% sensitive to SA.

Practically, these results dictate agar selection whenever the properties of segregants are to be studies without further purification. However they are also suggestive from the point of view of the mechanism of segregation. Since it is hard to imagine an NOB-sensitive contaminant surviving three subcloning steps in NOB, the only alternative explanation is that during segregation a cell passes through a phase where it is resistant both to SA and to NOB. The presence of one or the other drug in the medium would then be sufficient to weight the balance in favor of that form. An attractive model for this stage is the step in segregation in which recombination has excised the duplicated segment of the genome from the chromosomal continuum, but it has not yet been lost from the cell. In the presence of SA it would have the capacity to re-integrate, regenerating the original structure; under NOB selection such integration would be unfavorable, and the segment would tend to be lost. Admittedly this scheme is highly speculative and dependent on elimination of the alternative contamination hypothesis.

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