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GENETIC RECOMBINATION IN PNEUMOCOCCAL TRANSFORMATION: THE ROLE OF INTEGRITY AND HETEROGENEITY OF DEOXYRIBONUCLEATE MOLECULES, AS REFLECTED IN KINETIC ANALYSIS

ROCKEFELLER INSTITUTE DOCTORAL DISSERTATION

JOAN LOUISE KENT, B.A.

1963

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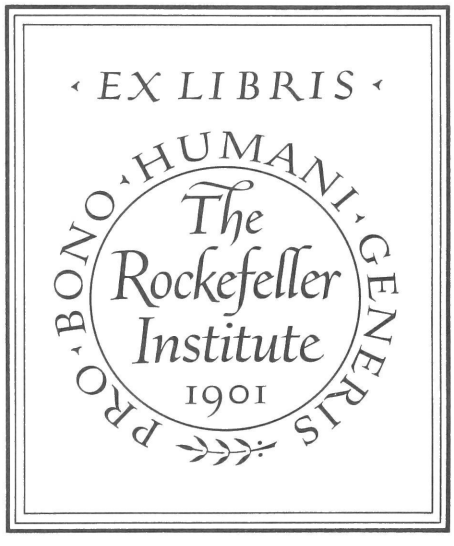

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Genetic recombination in
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GENETIC RECOMBINATION IN PNEUMOCOCCAL TRANSFORMATION:

THE ROLE OF INTEGRITY AND HETEROGENEITY OF DEOXYRIBONUCLEATE MOLECULES, AS REFLECTED IN KINETIC ANALYSIS.

A thesis submitted to the Faculty of The Rockefeller Institute

in partial fulfillment of the requirements

for the degree of Doctor of Philosophy

by

Joan Louise Kent, B.A.

Acceptable for Publication
Rollin D. Hotchkiss
Professor, The Rockefeller Inst

15 April 1963

The Rockefeller Institute

New York, New York

PREFACE

This study was undertaken during my tenure as a graduate fellow in the Laboratory for Genetics at the Rockefeller Institute. I wish to express my appreciation to Dr. Detlev W. Bronk for providing this unique environment in which it has been both a privilege and a pleasure to study and to partake of the broad, stimulating educational experience so readily available to students.

To Dr. Rollin D. Hotchkiss who has been my research adviser, I am deeply indebted for his persuasive, persistent optimism and encouragement in so many phases of graduate study. Through the continual intellectual stimulation which he has freely dispensed, he has guided me into the ways of science. I wish especially to thank Dr. Muriel Roger who initiated me into the study of macromolecules and provided generously of her time, advice and materials during the course of this investigation. In addition, I would like also to acknowledge the help of Dr. Maurice S. Fox for the introduction into kinetic analysis.

For their patient and efficient help in preparation of the final manuscript, I am grateful to Miss Sonia Wohl for her inestimable assistance in preparing the bibliography, as well as to Miss Elizabeth Bowman, Mrs. Hannah Simon, and Mrs. Sally Ann Clauss.

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ABSTRACT

Pneumococcal transformation has been used to study intramolecular genetic recombination. A kinetic technique is developed whereby the cumulative number of multiply-marked transformants is measured as a function of the duration of exposure to a multiply-marked DNA. Transformants accumulate with time at a rate characteristic of the particular markers and the number of DNA particles involved, thus singly-marked transformants accumulate linearly with time of exposure to DNA, as do some multiply-marked transformants. Other unlinked pairs of markers accumulate exponentially with time, by a second-order process, at a rate very close to that expected from random interaction of one cell with two DNA particles. Linked markers can be shown to reside in one DNA particle by this method, because the pattern of accumulation is undistinguishable from that observed for single markers. Linkage frequency can be expressed as the ratio of the linear rate of accumulation of multiply-marked transformants relative to singly-marked transformants.

Kinetic analysis has been made of the fate of each factor in a linear array of three genetic markers within one DNA particle. The linear rate of accumulation in time of each of the several kinds of recombinant progeny acquiring any one, two, or three of these markers demonstrates the frequent and regular occurrence of complex intramolecular events in pneumococcal transformation. One such complex event results in acquisition of the outer two or "bracketing" markers but not the central factor from the linear array present in the single donor DNA particle which initiated the transformation. The frequency with which these particular recombinations appear suggests that there is very little interference between events occurring over the entire interval. Furthermore, as judged by clonal analysis, the recombination is a unique event within one division cycle of the cell and gives rise to pure clones of transformants after growth in non-selective medium; heterozygous cells are not detected within these clones. The redistribution of alleles between donor DNA and recipient cells can affect the relative frequency of complex to simpler intramolecular recombinations over a range of about two-fold.

By changing the conditions under which a cell population is brought to competence for transformation and then transformed, the complex intramolecular recombinational events can be essentially eliminated even though the DNA preparation has not been changed in any way. Such events are, therefore, a reflection of cellular processes and not alone attributable to the state of a DNA preparation.

The effect of in vitro modification of DNA structure on the behavior of linked marker groups and on the frequency of occurrence of complex intramolecular recombinations is examined by the kinetic method. The rate of loss of biological activity during subcritical heat inactivation (giving localized submolecular lesions) is determined by the number of markers being introduced into the transformants and not by the genetic (and physical) relationship of these markers within DNA particles. Collapse of secondary structure in DNA, due to critical heat denaturation, causes only a slight change in adsorption affinity of the preparation for competent cells, but markedly reduces the ability of the DNA to penetrate into cells, and may also lead to some inert particles unable to adsorb to the cell surface. Nevertheless, linkage relationships of markers can be only slightly affected in otherwise good preparations. Renaturation restores some of the ability to penetrate cells and also much of the biological activity to previously denatured DNA preparations, but does not increase linkage frequencies above those of the denatured material. Annealing of mixtures containing genetically distinct DNA's creates no new biologically active hybrid particles, as determined quantitatively by kinetic experiments and by clonal analysis of transformant progeny. These data are discussed and interpreted as showing that secondary structure of DNA influences not its adsorption but primarily its penetration into the cell, whereas the specific structure along polynucleotide strands probably influences the frequency of recombinational events at individual sites.

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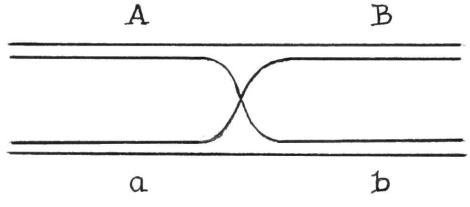
A. THE PROBLEM OF GENETIC RECOMBINATION

The reassortment of genetically controlled traits among the progeny of given individuals is a major tool in genetic analysis. In early and classical work on the genus Drosophila (reviewed in Morgan, Bridges, and Sturtevant, 1925), proof of localisation of genes within chromosomes brought with it the realization that there are many genes per chromosome (Drosophila has only four types of chromosomes per cell) and that these genes may be ordered in a linear array within individual chromosomes. The association of two genes within the same chromosome is known as linkage and is experimentally defined by the non-random reassortment of these genes among the progeny; thus, two genes located within separate chromosomes assort randomly, but two linked genes tend to reoccur as a pair among the individuals of the progeny population. Linkage is observed by the preservation of pairs or groups of genes in progeny of the next generation, and genetic recombination is responsible for separation or reassortment of two genes initially linked. For example, consider a linear array of two linked genes in one pair of Drosophila chromosomes in a female fly:



If no recombination occurs, then each chromosome of this pair passes unaltered into individual progeny of the next generation either as array AB or ab; if recombination, or crossing-over, does occur, then the reassorted Ab and aB of the recombinant chromosomes are found in individuals among the progeny. Types Ab and aB are found in equal proportion within the progeny population. The frequency of appearance of recombinant chromosomes among the progeny depends on the particular pair of linked markers chosen.

Recombination, itself, is a function which provides for orderly and limited variation in the genetic potential of individual organisms within populations. Within the cell, it is unique to genetic material; no other

sub-cellular component is known to undergo material reassortment in this manner. Since the measurement of recombination frequencies (or, alternatively, linkage frequencies) is critical to genetic analysis, the mechanism of the recombination is a central issue in genetics. The mechanism(s) of recombination remain enigmatic. With the advent of microbial genetics after 1940, genetic analyses could be performed at much higher resolution because of newly available selective techniques (Beadle and Tatum, 1941) for the recognition of rare recombinant progeny; although several new phenomena and problems have emerged in consequence, these studies have not added materially to an understanding of mechanism and process.

Many intriguing parallels in phenomenology exist between the chromosomal and molecular levels of analysis of the genetic apparatus, but with little indication of congruency in the (unknown) underlying mechanisms. Although the tremendous difference in structure, size, and complexity between the chromosome and the molecule makes extrapolation hazardous, nevertheless, similar principles seem to apply to both levels. These can be listed, briefly.

Deoxyribonucleate (DNA) serves as genetic material whether used in the purified molecular form as genetic donor (e.g., pneumococcal transformation) or as organized within chromosomes in the cell. Genetic recombination occurs on both levels of organization and is accompanied by the physical exchange of material, although it is not yet feasible to correlate recombinational frequency data between the two levels. The genetic material is arranged in a linear array, making possible the construction of consistent genetic (recombination frequency) maps. These maps express the general proposition that recombination frequency (map distance) is inversely related to actual physical distance between genes. A recombinational event is generally believed to result in production of two equivalent but reciprocal entities.

Recognition that DNA functions as genetic material has permitted the development of molecular genetics. As early as 1924, Feulgen and Rossenbeck had demonstrated that DNA is localized within chromosomes in cells, although its function was then unknown. The isolated, biologically active pneumococcal transforming principle was later shown to have chemical properties like DNA (Avery et al, 1944). There followed the demonstration, by means

of cytochemical techniques, that the amount of this material present in the nucleus of a diploid cell is constant and characteristic for a given species, varying from approximately twice that present in the nucleus of a haploid cell of the same species to the double of this amount in a dividing cell (Boivin et al, 1948; Mirsky and Ris, 1949). The double helical structure for DNA, in which two polynucleotide chains are joined by hydrogen bonding between specific pairs of purine and pyrimidine bases, was proposed by Watson and Crick (1953 a,b) on the basis of X-ray diffraction data and analytical chemical studies. The arrangement of DNA and protein within chromosomes is unclear at the present time, and this gap significantly limits studies on the macromolecular level of resolution for structure and function of chromosomes.

The universal occurrence of genetic recombination on both the chromosomal and molecular levels can be established. It occurs in all genetic systems, and genetic recombination data have been exploited to make consistent (chromosome) maps in e.g., Drosophila, Zea mays, Neurospora, Aspergillus, Escherichia coli, and bacteriophages. Documentation of unequivocally intramolecular recombination comes from the transformation systems in which soluble purified DNA acts as genetic donor; using a linked pair of markers, which are inferred to reside within the same DNA molecule, Hotchkiss and Marmur (1954) found that the two markers can either be transferred together into transformant progeny or else separately to give singly-marked transformants, analogous to a recombinational (or cross-over) event; see experimental sections D-1 and D-2 for fuller discussion of intramolecular recombination.

The fundamental generalization that recombination frequency (or genetic map distance) in a given system can be related to the actual physical (chromosomal) distance between markers is one of the principles of classical genetics. Correlation of the genetic (frequency) map order with microscopically observed and genetically determined position of chromosomal bands (physical distance) in the giant chromosomes in salivary glands of Drosophila (Bridges and Brehme, 1944, plates 2 and 3; Painter, 1934) provided conclusive proof that genes are arranged in a unique linear array within chromosomes, which can be determined by either genetic or cytogenetic analysis. Only these giant polytene chromosomes, present in non-dividing cells,

are suitable for cytological studies in Drosophila, but they are assumed to have the same structure and arrangement along the long axis as the chromosomes in other cells. There is essentially complete agreement between the two methods on the order in which genes are arranged, but the genetically measured distance may represent the cytologically measured distance imperfectly or even with significant distortion. For microbial systems, cytological investigation is extremely difficult, but other types of experiments have contributed to acceptance of the distance-frequency generalization. In the E. coli conjugation system, male (HFr) bacteria can be grown in radioactive phosphorus and stored for varying time periods to allow radioactive decay, before mating with unlabeled female (F^-) strains; by correlating the relative rates of disappearance of HFr genetic markers from the progeny as a function of their position on a linear genetic map, the general relationship between genetic and physical distance was demonstrated for the bacterial chromosome (Fuerst, Jacob, and Wollman, 1956; Jacob and Wollman, 1961, pp. 217-219). More pertinent to the molecular level are the observations of Nomura and Benzer (1961), who demonstrated that the presence of a genetically defined "deletion" within a very short region of a coliphage genome corresponds to a decrease in recombination frequency (i.e., shrinkage of map distance) from 4.7% to 0.7% between outside markers within the same gene (same functional unit, cistron, i.e.). The construction of consistent genetic maps in organisms such as N. crassa, A. nidulans, E. coli, and several bacteriophages is compelling evidence for the general additivity of recombinational distances in linear arrangements of genes in these and perhaps most other organisms.

The most significant advance made towards understanding genetic recombination by those geneticists who worked with higher organisms is the demonstration that recombination (or crossing-over) is accompanied by physical exchange of material. A correlation of cytological and genetic crossing-over was determined through the use of morphological chromosome markers in Zea mays (Creighton and McClintock, 1931), since phenotypically recombinant progeny contained morphologically recombinant chromosomes in which large segments had been transferred. Similar correlations are inferred from the chemical analysis of progeny formed by recombination between isotopically labeled parental types in microbial systems. For example, material is transferred from both parents to recombinant phage particles, and the amount of

material transferred is related to the genetic distance (map distance) between linked markers, as judged by the density of recombinant phage relative to that of N^{14}- or N^{15}-labeled parental phage (Meselson and Weigle, 1961; Kellenberger et al, 1961). Similarly, Fox and Hotchkiss (1960) and Fox (1962a) have shown that P^{32}-labeled DNA used as donor in pneumococcal transformation preserves, in nascent transformants, an undiluted relation between its physical (P^{32}) and its genetic (streptomycin resistance) marker. Thus any hypothesis for the mechanism of recombination must include provisions for breakage-and-reunion of the participating genetic entities.

Analysis of the large populations of progeny that result from each Drosophila mating, reveals that reciprocal recombinants occur in equal numbers (reviewed in Morgan et al, 1925). The demonstration that individual recombinations yield reciprocal and complementary chromosomes, upon exchange of physical material, requires the recovery of all the progeny of a single genetic cross. Upon examination of single asci of Neurospora crassa, in which all eight ascospores are present in a unique linear array, it is readily demonstrated that most genetic recombinations are indeed reciprocal (Dodge, 1930; Lindegren, 1933).

However, certain persistent anomalies often occur in genetic analyses performed on groups of tightly linked markers. These have been interpreted to represent molecular rather than chromosomal dimensions and have been of interest in the hope that they reflect molecular mechanism(s) of recombination (Hotchkiss, 1957c, 1958). In many organisms, unexpectedly high frequencies of multiple recombinational events occur over short regions of the genome. These have been observed repeatedly in A. nidulans crosses (Pritchard, 1960) and in analysis of progeny of single bacteria after biparental phage infection (Hershey, 1958; Chase and Doermann, 1958). Furthermore, the individual recombinational event can apparently be non-reciprocal over small regions; this is the interpretation for the occurrence of (rare) aberrant tetrads (Perkins, 1962; Pritchard, 1960; Mitchell, 1956) in which the reciprocal recombinants occur in such unequal ratios as 3:1, 5:3 or 6:2 among the eight ascospores. Whether their rarity makes these aberrant tetrads more or less significant is not clear. Even among populations, as in the phage progeny from a single infected bacterium, the unequal recovery of reciprocal recombinants can be interpreted to result from non-reciprocal

recombinations (Hershey and Rotman, 1949). In addition, there are certain types of genetic exchange that are of necessity non-reciprocal because only one viable product can result from the interaction of two unequal entities, e.g., conjugation in E. coli, or transformation of bacteria by purified deoxyribonucleate, or transduction of bacteria by bacteriophage.

To explain in molecular terms the recombination data obtained over short regions of the genome, an alternative classical hypothesis of genetic recombination (Belling, 1933) has been revived by microbial geneticists (Lederberg, 1955). The copy-choice mechanism, as now formulated, requires that recombination occur during replication of genetic material. Replication of DNA (i.e., at the molecular level) is held to be semi-conservative, so that each strand of a double helix acts as a template along which its complementary partner is synthesized (Meselson and Stahl, 1958). For genetic recombination, an error in copying would occur during the replication of the genetic material by means of a switch between two neighboring but non-homologous templates. Non-reciprocal recombination could occur after such a switch, although there need not be physical exchange of material between the (parental) templates. This hypothesis has had wide appeal, primarily because many authors believe that it tends to explain their data with fewer assumptions than the breakage-reunion mechanism.

Not only is there little direct evidence in favor of copy-error in recombination, but available data suggest that recombination and replication can indeed be separated. In higher organisms, cytological and radioautographic techniques have been used to demonstrate that duplication of DNA within chromosomes can occur before synapsis of homologous chromosomes, which is necessary for crossing-over, and recombination and replication would then be separate processes in time (for review, see Rhoades, 1961). On the molecular level, Fox and Hotchkiss (1960) have suggested that less than 5% net DNA synthesis need occur under conditions allowing completion of genetic recombination between DNA and the pneumococcal genome, as judged by the new genetic properties of DNA extracted from nascent transformants formed 1) in the presence of 5-fluorodeoxyuridine in concentrations much greater than required to severely inhibit cell growth and 2) in the presence of added inorganic P^{32} whose incorporation into DNA would indicate net synthesis. Although localized DNA duplication in the region of the re-

combination cannot be excluded, DNA synthesis does not seem to be required, and it appears that physical exchange of material occurs (Fox and Hotchkiss, 1960). The copy-error hypothesis alone is insufficient to explain the data.

The basic mechanism(s) of recombination is unknown, and the list of experimental variables influencing recombination frequencies is both long and diversified. Thus, physiological variables such as temperature, medium or diet, and irradiation can alter recombination frequencies in Drosophila (reviewed in Morgan et al, 1925); temperature of growth affects linkage in N. crassa (Rifaat, 1959); and temperature, medium and physiological state of the cells alter intramolecular recombination frequencies in pneumococcal transformation (Hotchkiss and Evans, 1958). Localized genetic factors can affect crossing-over in adjacent segments of DNA molecules (Ravin and Iyer, 1962; Green, 1959) or in regions of the same or other chromosomes (Sturtevant, 1919; Schultz and Redfield, 1951). And many genetic anomalies of chromosomal morphology (such as inversions, duplications, translocations, deletions) as well as the presence of chromosomal centromeres modify recombination frequencies, although many of these effects can be attributed to abnormal synapsis between heterologous segments of a chromosome pair during meiosis. Such aberrations have seldom been defined on a molecular level.

The study of unequivocally molecular and intramolecular recombination, with which this investigation is concerned, is uniquely accessible in transformation systems since one component of these is chemically purified DNA. With these systems, it has now become possible to determine which genetic exchanges may be characteristically molecular, as distinct from chromosomal, in origin.

B. THE PNEUMOCOCCAL TRANSFORMATION SYSTEM AS RELATED TO THE STUDY OF RECOMBINATION

Exposure of a suitable population of pneumococci to purified deoxyribonucleate extracted from another genetically marked strain of the same organism results in the appearance of progeny bacteria (up to 25%) that possess genetic properties previously carried only by the soluble DNA. The "transformants" not only develop the phenotypic characters introduced by the DNA, but also a permanent or genotypic formulation of these new traits which is passed on to the daughter cells of transformants, when these divide. The interaction between bacterial cells and DNA which results in the appearance of bacteria with altered genotype and phenotype is called transformation. It has been observed in several genera of bacteria, but studied principally in Diplococcus pneumoniae, Hemophilus influenzae, and Bacillus subtilus. Ravin (1961) has provided a comprehensive review of the literature of transformation.

Transformation was initially obtained with pneumococci when Griffith (1928) was able to recover living, encapsulated pneumococci from mice which had been inoculated with cells of an unencapsulated strain and with heat-killed cells of an encapsulated strain. It soon became possible to carry out transformations in vitro (Dawson and Sia, 1931), but the nature of the transforming principle remained unknown until 1944, when Avery et al demonstrated the purified material to be like DNA in chemical properties. In further demonstration, Hotchkiss (1949) showed that extensively purified transforming principle contained little or no protein ($< 0.02\%$) and that all of the amino acid present in acid hydrolysates could be quantitatively accounted for by breakdown of adenine. With the introduction of drug-resistance markers and other techniques by Hotchkiss (1951, 1957c), quantitation became possible by selective scoring of transformants; this advance opened the way not only for the study of cell interaction with DNA under defined conditions, but also for an inquiry into the homogeneity of a population of DNA particles as extracted from one bacterial strain.

A typical transformation experiment involves mixing DNA with cells, terminating the interaction with deoxyribonuclease, and allowing expression of the new phenotype during a growth period before selective scoring. The

process of intramolecular genetic recombination can be studied with special advantages deriving from the nature of the transformation system. The facts pertinent to this correlation are basically three-fold.

First, there are two independent and separable components - the cell population and the chemically purified DNA. Each can be controllably and specifically influenced before the initiation of transformation. For example, pneumococci are receptive to DNA only under specific physiological conditions which have to do both with the stage of the culture in the bacterial growth curve (Hotchkiss, 1957c) and with the phase of the individual cells in the bacterial division cycle (Hotchkiss, 1954). The receptive state, known as competence, may be varied both quantitatively (yield of transformants) and qualitatively (relative frequencies of types of transformants recovered). On the other hand, purified DNA can be subjected to in vitro degradation by such means as heat, ultraviolet irradiation, shearing forces, P^{32} decay, or phosphodiesterase; the effects on the treated material may then be bio-assayed on a standard, competent cell population. If the molecular basis of the degradation is known, it is possible to correlate structure with function of the material; in experiments to be discussed here, the induced molecular abnormalities are tested for their effect on genetic recombination.

Second, the initiation of irreversible interaction of the two components is kinetically controllable as a function of the concentration of each component, and of the duration and temperature of the interaction. The initiation of transformation has in fact been analysed by analogy to the classical Michaelis-Menten formulation for enzyme-substrate interaction (Fox and Hotchkiss, 1957). A clone of transformant progeny can be recovered from interaction of one cell with one molecule of DNA. After infection of a single bacterium by a single phage particle, on the other hand, a more complicated sequence of events ensues during which a growing and changing population of phages undergoes several rounds of replication with exchange of genetic material before any mature phage progeny can be recovered; the yield of phage per bacterium is usually much greater than the input particle so that the result of an individual recombinational event cannot be directly determined.

Third, there are available several biochemically independent markers, some of them having specific genetic relation to one another. Exposure of a competent population to a multiply-marked DNA yields transformants to one or more markers. If the number of multiply-marked transformants is in excess of that expected by random double transformations of one cell, the markers are said to be genetically linked; this concept is developed more fully in section D-1 in conjunction with the discussion of kinetic analysis.

The experimental work presented and discussed here falls into three general categories. In the opening section, kinetic analysis, as formulated by other workers for the initial reaction of cells with singly-marked DNA preparations, is extended to include multiply-marked transformants for groups of markers in different genetic relationship to one another. It thereby becomes possible to show in a direct manner that linked markers are present within one DNA particle, whereas unlinked ones are not, and to determine linkage frequencies. In the second section, where kinetic measurements are used for quantitative determination of linkage frequencies in three-factor crosses, the regular occurrence of complex intramolecular recombinational events is demonstrated for various configurations of alleles. In the third section, two attempts are made to define further the structural requirements necessary for DNA to participate in genetic recombination; the effects of thermal treatment resulting in localised, submolecular damage or in disorganization of secondary structure for whole DNA particles are examined by the time kinetic technique for measuring linkage frequencies. By the use of an annealed mixture of genetically distinct DNA's, the possibility of forming new, biologically active species of DNA in vitro is investigated. Data obtained with these modified DNA preparations suggest that continuity of structure along polynucleotide chains is of more immediate significance than intact secondary structure in a DNA particle for participation in actual recombinational events with homologous segments of cell genome.

C. MATERIALS AND METHODS

I. All the transformation experiments described were performed with strains of Diplococcus pneumoniae, primarily the unencapsulated wild-type strain R_1 (a derivative of R36A of Avery et al, 1944) with singly drug-resistant strains derived from it by mutation and multiply-marked strains obtained from these by transformation. The drug resistance properties used were selectively detected under the following conditions:

streptomycin resistant (S) cells were selected in growth medium containing 150μg/ml of dihydrostreptomycin (Pfizer & Co.). The colonies arising therefrom were counted (scored) as described below.

micrococcin resistant (K) cells were selected at 10mμg/ml of the drug, which was a gift of Dr. Norman Heatley, Oxford University.

sulfanilamide resistant strains were those of Hotchkiss and Evans (1957, 1958) in which the several mutations form part of one linked, complex locus. The cell types used in this study are:

a+, scored in 15μg p-aminosalicylate/ml plus 40μg p-nitrobenzoic acid/ml.

+d plus ad, which grow well at 50μg sulfanilamide/ml. This group is referred to as d.

ad, selected at 250μg sulfanilamide/ml.

ad^+ plus $+d^+$, scored in 20μg/ml p-nitrobenzoic acid. This group is referred to as d^+ and represents the wild-type allele of d.

The three markers Sad are linked, in that order, and the marker K is not linked to any of the others, as shown in Section D-1.

Genetic map for three linked markers; distances are not proportional.

II. Deoxyribonucleate was prepared by the Hotchkiss modifications (1957a) of the method of Avery et al (1944). Cells were grown to high density in fresh beef heart infusion-neopeptone broth. After lysis of a concentrated cell suspension with sodium deoxycholate at pH 8.0, the preparation was deproteinized with chloroform and isoamyl alcohol by the Sevag procedure, followed by precipitation of DNA fibers from 50% alcohol. The fibers were then redissolved in citrate-saline. After two deproteinization steps, RNA was removed by incubation with 50μg/ml of ribonuclease at 37°C for 30 minutes before the final, third deproteinization; this enzyme preparation was a gift of Dr. Moses Kunitz of the Rockefeller Institute. After sterilization in 70% alcohol, DNA solutions were stored at 4°C in 0.02M phosphate-saline buffered at pH 6.8; preparations remained fully active for at least one year. The average yield was 0.7mg of purified DNA per liter fully-grown culture, representing the bulk of the DNA. Concentration of the stock DNA solutions was determined by "hyperchromicity" or the increase in optical density at 260mμ following exposure to 0.1N NaOH, as described by Hotchkiss (1957b). DNA preparations were obtained from strains RF6S-7 (streptomycin resistant and sulfanilamide +d resistant: DNA 249) RF4S-7 (streptomycin resistant and sulfanilamide a+ resistant: DNA278), RF64K-7 (sulfanilamide ad resistant and micrococcin resistant: DNA 250), RSt-7 (streptomycin resistant: DNA 272) and the multiply-marked drug resistant strain RF46SKCNAD (resistant to streptomycin, micrococcin, high concentrations of sulfanilamide and to several other drugs: DNA 237, 259, 264, 281); these strains were those used in the laboratory of Dr. R. D. Hotchkiss.

III. Competent populations of Pneumococcus to be used as recipients in transformation were grown at 37°C to about 2×10^7 viable units/ml in a supplemented enzymatic casein hydrolysate medium, as described by Fox (1959). Freshly-grown cultures were synchronized by cooling to 25°C for 15 minutes (Hotchkiss, 1954) and stored in 10% glycerol at -20°C (Fox and Hotchkiss, (1957); yields of 5-20% transformants to streptomycin resistance were obtained routinely after thawing such cultures at 0°C and removing the glycerol. These preparations retained activity for as long as six weeks after freezing at -20°C. Occasionally, freshly-grown pneumococcal populations were used as recipient cultures immediately after the temperature shock synchronization, but the transformation yields were less predictable than with batches of

cells prefrozen at a suitable time. The strains used as recipients were: the wild-type R_1 (++), and several sulfanilamide resistant strains RF4-7 (a+), RF6-7 (+d) and RF64-7 (ad).

IV. In a typical transformation experiment, a frozen competent culture was thawed at 0°C, and an aliquot filtered on a millipore filter (Millipore Filter Corporation, porosity 0.45μ) for resuspension in a chosen volume of cold enzymatic casein hydrolysate medium, modified by supplementation with calcium chloride (10^{-3}M) and with bovine serum albumin (0.2%, Fraction V, Armour & Co.); calcium and serum albumin are optimal for transformation at concentrations greater than required for growth and development of competence (Hotchkiss and Ephrussi-Taylor, 1951). The concentrated culture was preincubated for 15 minutes at 30°C before addition of DNA. DNA, usually at a final concentration of less than 0.25μg/ml, was added, with mixing, at 30°C and zero time (Fox and Hotchkiss, 1957). Occasionally, the transformation was performed at 37°C; the time course of accumulation of transformants is significantly different at 30°C (Fox and Hotchkiss, 1957) than at 37°C (Hotchkiss, 1954). At measured time intervals of less than 30 minutes, aliquots were removed to two volumes of meat infusion medium (Hotchkiss and Evans, 1958) which contained 0.75μg/ml deoxyribonuclease (Worthington pancreatic deoxyribonuclease, once crystallized); this dilution terminates the exposure to DNA, as the enzyme inactivates any DNA not already taken up by the cell population. The aliquots were then incubated at 37°C for 90 minutes to permit phenotypic expression of new genetic traits, prior to selective scoring.

V. Assays by selective scoring for transformants were performed by the method of Hotchkiss (1957c), in which serial dilutions of expressed cultures are made into meat infusion broth containing 2 to 5μg/ml of horse or rabbit anti-pneumococcal (anti R) serum globulin and appropriate drugs. After 16 to 20 hours incubation at 37°C in small test tubes, the agglutinated progeny of individual transformants resistant to the selective agent can be counted as micro-colonies on the bottom of the tubes, and the titer of transformants per ml in the expression tube calculated. The percent transformation to a given marker(s) is calculated from the ratio of the number of transformants obtained after expression to the viable count of the population at the time

of exposure to DNA. This will represent about four times the percentage based on cell count, since Pneumococcus grows with a chain length of about four cells per viable unit under these conditions. In a few experiments, selective scoring was performed in solid medium. The medium and drug concentrations were unchanged, but 1% sterile defibrinated horse or rabbit blood and 1% agar were added. The bacteria were either added in an upper layer of agar or dispersed throughout the medium. Colonies were counted after 16 to 20 hours incubation at 37°C.

The number of multiply-marked transformants expected to occur after random interactions, or collisions, between one cell and several particles of DNA is calculated as the product of the individual probabilities of transformation to a single marker. Let t_1 and t_2 represent the number of transformants recovered per ml of transformable culture to each of two, separate markers, and let t_{12} represent the number of transformants per ml which have acquired both markers. If the population has a viable count equal to N/ml, then the expected number of multiply-marked transformants is expressed as:

$$E_{12} = \frac{t_1 \times t_2}{N},$$

which may be compared with t_{12} observed. If N' were set equal to the total cell count/ml, then $E'_{12} \cong \frac{1}{4}E_{12}$, because of the chain-like growth of Pneumococcus. E_{12} is used in the calculations, however, since it represents a measured quantity.

VI. For thermal inactivation of DNA, the subcritical and critical inactivations were distinguished by the temperature used and the duration of treatment.

Subcritical degradation was performed according to the method of Roger and Hotchkiss (1961) by immersing DNA solutions in a refluxing water-ethanol bath at 85°C; the DNA was heated at 10μg/ml in 0.02M phosphate saline, buffered at pH 6.8. At approximately hourly intervals, heated aliquots were removed to tubes pre-chilled in iced-water and were subsequently assayed. Such samples were apparently stable for as long as a week at 4°C. In five hours of heating under these conditions, the DNA retains about 40% of its initial activity for streptomycin resistance.

Critical heat inactivation, or melting, was carried out by the method of Roger and Hotchkiss, 1961). DNA samples at 25μg/ml in 0.02M phosphate

saline, buffered at pH 6.8, were heated at 92.3°C or at 95.5°C for 15 minutes in a refluxing ethanol-water bath. An aliquot was then removed to a tube pre-chilled in iced-water and -- upon assay -- had a residual activity of 1% to 5% of the initial value for the streptomycin resistance marker; this is designated the denatured sample. Another parallel aliquot was "annealed", via slow cooling after denaturation, either by immersion for 60 minutes in a boiling methanol bath at 65°C, followed by slow equilibration to 30°C, or else by slow equilibration of the sample to room temperature from 75°C (about 2 hours); this is the renatured sample and has a typical recovery of 40% to 60% of its original value for the streptomycin resistance marker. The heated samples did not change in activity during a few days at 4°C.

D-1. THE KINETIC ANALYSIS OF ACCUMULATION OF MULTIPLY-MARKED TRANSFORMANTS

Introduction.

Because the pneumococcal transformation system is amenable to highly quantitative experimentation, the data can be analysed kinetically. Several factors are important in quantitation: 1) the use of pancreatic deoxyribonuclease to break down active DNA in solution provides a precise and irreversible termination to the interaction (McCarty et al, 1946); 2) the selective scoring techniques introduced by Hotchkiss (1951, 1957c; Hotchkiss and Evans, 1957, 1958, 1960) make possible accurate determination of the number of transformants recovered per marker; 3) the discovery that, at 30°C, the increase of transformants can be linear with duration of exposure to DNA for periods up to 90 minutes allows precise rate measurements (Fox and Hotchkiss, 1957) under a variety of conditions. For example, the rate of accumulation of transformants to streptomycin resistance can be a linear function of the concentration of competent cells and of DNA. Since the initiation of irreversible transformation is considered to result from collision between cells and DNA particles, it becomes meaningful to analyse the kinetic data as if it had been obtained for non-biological physicochemical systems.

In a transformation experiment, there are two components, the DNA at variable concentration and the cell population at variable density. In these experiments, the cell density is usually held constant so that the choice of experimental variables is limited to the concentration of DNA or the time interval allowed for the interaction; in either case, one measures the accumulated number of transformants (the reaction product). The total number of (effective) collisions which occurs during an experiment, and hence the potential number of transformants, can be limited by the arbitrary selection, through experimental design, of either independent variable.

The equivalence of kinetic data obtained with either time of interaction or concentration of DNA as the independent variable can be demonstrated by setting up rate equations for the accumulation of each transformant type.

Assuming:

1) a collision model for irreversible initiation of transformation.
2) one DNA particle as sufficient to initiate a transformation of a cell to any or all genetic traits on it.

3) markers for S and K on separate different particles of DNA.

4) more than one DNA particle can interact with one competent cell.

Let N = non-transformed (with respect to S) cell count/ml
= 1.6×10^8/ml. N decreases $<$ 10% during the experiment.

(DNA) = concentration in μg DNA/ml
= 0.2μg DNA/ml if held invariant. Fox (1957) has observed that approximately one bacterial equivalent of P^{32}-DNA is fixed by a cell population when one transformant to one marker appears in the progeny; one pneumococcal equivalent represents about 2×10^{-9}μg DNA. Under typical experimental conditions, not more than 3×10^6 streptomycin resistant transformants/ml are recovered after exposure of about 1.6×10^8 cells/ml to 0.2μg DNA/ml at 30°C for 30 minutes; about 3% of the total DNA is fixed (removed from solution) by the cell population under these conditions.

S = number of streptomycin resistant transformants/ml and increases linearly with time of exposure to DNA at a typical rate of 4×10^4 S/minute, as shown in the experimental section.

K = number of micrococcin resistant transformants/ml and increases linearly with time at a typical rate of 3×10^4 K/minute.

SK = number of transformants/ml resistant to both drugs. These transformants accumulate quadratically in time, and the number observed at any time $<$ 10% S or K, as demonstrated in the experimental section.

t = duration of exposure to DNA. $0 \leq t \leq 35$ minutes, if variable.

$$\left.\begin{array}{l} N + DNA \xrightarrow{k_a} S \\ S + DNA \xrightarrow{k_c} SK \end{array}\right\} \text{ or } \left\{\begin{array}{l} N + DNA \xrightarrow{k_b} K \\ K + DNA \xrightarrow{k_d} SK \end{array}\right.$$

The above diagram represents the significant pathways by which competent cells (N) may become SK transformants. A cell may readily acquire the markers S and K by successive interaction of one cell with more than one DNA particle, as discussed and demonstrated in the experimental section. If the two markers had to be acquired simultaneously (because of limitations in cellular behavior), the accumulation of SK transformants would be linear in time but at a very low rate commensurate with or smaller than calculated on the basis of random three-body collisions over small, finite time in-

tervals. The observed fact that the accumulation of SK transformants is quadratic in time justifies the scheme of successive interactions, as does the experiment shown in Figure 5 and discussed in the text. The two pathways in the diagram represent successive interactions of one cell with two DNA particles; the pathway representing simultaneous interaction of one cell with two DNA particles (three-body collisions) is assumed to contribute negligibly.

For an experiment in which t is the independent variable, the following rate equations apply:

$$dS/dt = k_a N \ (DNA)$$

$$dK/dt = k_b N \ (DNA)$$

$$dSK/dt = k_c S \ (DNA) + k_d K \ (DNA)$$

By integration between limits,

when $t = 0$, $S = 0$, $K = 0$, $SK = 0$
$t = t$, $S = S_t$, $K = K_t$, $SK = (SK)_t$

and under the conditions that N and (DNA) decrease negligibly in the interval from 0 to t and hence are taken as constant in this interval.

$$S_t = k_a N \ (DNA) \ t \qquad (1)$$

$$K_t = k_b N \ (DNA) \ t \qquad (2)$$

$$(SK)_t = \frac{k_a k_c}{2} N \ (DNA)^2 t^2 + \frac{k_b k_d}{2} N \ (DNA)^2 t^2$$

but $k_a \simeq k_d$ and $k_b \simeq k_c$ (see Figure 5 and text)

$$(SK)_t = k_a k_b N \ (DNA)^2 t^2 \qquad (3)$$

These three equations state that the number of transformants which has accumulated during time of interaction t is directly proportional to t in the case of singly-marked transformants (S or K) and is directly proportional to t^2 in the case of the doubly-marked transformant SK (assuming S and K to be on separate different particles of DNA).

Similarly, the accumulation of transformants is proportional to the DNA concentration for the singly-marked transformants (equations 1 and 2) and to the square of this concentration for SK transformants (equation 3).

If a parallel set of rate equations were set up for an experiment in which t is held constant and in which (DNA) is selected as the independent variable, then the same three equations would be obtained on integration. We, thereby, demonstrate the equivalence of the two experimental designs in kinetic studies. The three equations (particularly the first two) resemble expressions for the number of collisions occurring between unlike particles per unit volume under the stated conditions. Under these experimental conditions and with respect to the DNA component, the observed kinetic order of interaction is identical to the molecularity of the interaction, as represented by the value of x, (see equation as written below).

In previous experiments, the yields had been measured for streptomycin resistant transformants at a constant time of exposure for varying cell density (Fox and Hotchkiss, 1957) or for varying DNA concentration (Thomas, 1955; Fox and Hotchkiss, 1957; Hotchkiss, 1957c). For varying DNA concentration, the results at 30°C are similar to earlier work by Thomas (1957) and by Hotchkiss (1951, 1957c) at 37°C; note that at 30°C (but not at 37°C), the accumulation of singly-marked transformants is linear with time. For graphical analysis of the data obtained at varying DNA concentration for constant time of exposure, the logarithmic form of the three equations had been used:

$$\log (Y) = \log (k_y Nt) + x\log (DNA)$$

or

$$\log (Y) = k' + x\log (DNA)$$

in which Y = number per ml of the measured category of transformants.

x = 1 for equations (1) and (2) and x = 2 for equation (3) at low (DNA) to bacterial density ratios. x = 0 in both cases if DNA is present in very high relative concentration, because the invariant cell population has been saturated in its ability to react with DNA.

A saturating curve, initially linear, is obtained on logarithmic co-ordinates in such experiments (Figure 1, e.g.) when log (Y) is plotted against log (DNA). Since the slope in the linear region (x) is approximately equal to 1 for the single marker to streptomycin resistance, it is concluded that one particle of DNA is indeed necessary and sufficient to initiate the series of events leading to a clone of S transformants. Since this slope is about equal to 2 for the SK transformants, two DNA particles are required to interact with one cell to produce this type of transformant. The same interpre-

tations are made from kinetic analysis of the observed linear rate of accumulation for streptomycin resistant transformants and the observed quadratic acculation for doubly-marked SK transformants with duration of exposure to DNA at 30°C.

Since the accumulation of singly-marked transformants can be linear with time at 30°C, Fox and Hotchkiss (1957) measured the rate of accumulation as a function of DNA concentration or of cell concentration in the system (see also Thomas, 1955).

Fox and Hotchkiss (1957) interpret the saturating rate curves as consistent with the following model:

$$B(S) + DNA \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B(S)\ DNA\ complex \xrightarrow{k_3} B(tr)$$

in which B(S) is the concentration of bacterial adsorption sites, presumably proportional to the bacterial concentration.

DNA is the concentration in μg/ml.

B(tr) is the concentration of cells in which transformation has been irreversibly initiated.

This formal scheme is analogous to the Michaelis-Menten formulation for enzyme-substrate interaction, and it is equally valid for molecular and cellular components.

By appropriate evaluation of the transformation data, the three constants were estimated by Fox and Hotchkiss (1957). Since k_2/k_3 is approximately 30, DNA which has already been adsorbed to a bacterial site is much more likely to be released back into solution than to continue on in the sequence of the productive events. The calculated estimate of the number of DNA particles adsorbed by a cell at saturating DNA concentration is 33 and 75 (by two methods) for the bacterial culture used; the value of this number is approximate but serves to emphasize that more than one particle of DNA can be readily adsorbed by a cell, although only one is necessary to initiate irreversible transformation. The kinetic model does not define the biochemical nature of the adsorption process; in further experiments, Fox and Hotchkiss link the maintenance of such sites to protein synthesis in resting populations. See Ravin (1961) for a review of this question, which will not be considered here.

In summary, the formal kinetic scheme provides for a rapid reversible interaction between components followed by a slower irreversible step. At 30°C, the population is metabolically stabilized which permits more meaningful rate experiments, and it is affirmed that only one particle of DNA is necessary for transformation to a marker, although many more may be adsorbed by the cell.

It had earlier been established that, like protein, deoxyribonucleates make up a class of compounds which vary, in specific and subtle ways, according to the source. For DNA preparations from heterologous sources, it had been amply established by the extensive work of Chargaff (1955) that the purine and pyrimidine base composition can vary significantly among DNA samples from various sources, although the purine:pyrimidine ratio is always one; nevertheless, DNA, as a type of chemical compound, is uniformly identifiable by its general properties such as overall chemical constituents bonded by specific linkages, high molecular weight, and anomalously high viscosity.

DNA as extracted from one source - in this case Pneumococcus - also consists of a population of molecules with different properties. Genetic heterogeneity within one preparation was demonstrated by Hotchkiss (1951) in studies on transformation to capsule type, and to streptomycin and penicillin resistance; although resistance to either drug alone is transformed in relatively high yield, the incidence of transformation to both markers within a single clone is close to that calculated for simultaneous or successive random events, which suggests that two independent DNA particles are required. The results of this experiment imply that the genome of a cell consists of more than one kind of DNA particle but says nothing about the number or nature of "chromosomes". Since 1951, chemical heterogeneity within a DNA preparation has been suggested by genetic activity in fractions obtained by methods discriminatory for base composition of particles, especially by the critical heat denaturation of DNA. When the measurements are performed on biologically active DNA, the various genetic activities can be differentially inactivated by the temperature to which the DNA is heated, where as much as 4°C may separate the point of 90% inactivation of one marker and the onset of inactivation of another (Roger and Hotchkiss, 1961).

In the presentation of a mixture of effective particles to a competent bacterial population, molecular heterogeneity results in competition for a limited number of bacterial adsorption sites, thereby limiting the yield of transformants to any single genetic marker. Such competition can be unequivocally demonstrated (Hotchkiss, 1954) in a mixture of differentially marked pneumococcal DNA's or of heterologous DNA's (e.g., calf thymus) at saturating concentrations; the decrease in yield for a given marker (inhibition) is quantitatively correlated with the relative concentration of the marker-bearing DNA in the mixture. It is concluded that pneumococcal adsorption sites are non-specific with respect to genetically different molecules of intact DNA. (See Section D-3 for further discussion of the characteristics of bacterial adsorption of modified DNA).

An individual, chemically and, therefore, genetically distinct particle of biologically active DNA is smaller than the entire genome but may, nevertheless, contain information for several (unrelated) biochemical functions. For each bacterial transformation system, there have been reports of genetic linkage between markers regulating apparently unrelated functions; these will be listed only, since they have been recently reviewed in detail by Ravin (1961). These reports include those of linkage between streptomycin resistance and mannitol utilization (Hotchkiss and Marmur, 1954; Hotchkiss, 1956), and between streptomycin resistance and sulfanilamide resistance (Hotchkiss and Evans, 1958) in Pneumococcus; between streptomycin resistance and canavanine resistance (Goodgal and Herriott, 1957) and also between streptomycin resistance and kanamycin resistance (Stuy, 1961) in H. influenzae; between indole independence and sucrose synthesis as well as β-galactosidase synthesis and sucrose synthesis (Spizizen, 1959) and between aromatic amino acid and histidine synthesis (Nester and Lederberg, 1961; Nester et al, 1963) in B. subtilis.

The criteria required for demonstration of genetic linkage were stated by Hotchkiss and Marmur (1954) to be the recovery of an excessive proportion of multiply-marked transformants under specified conditions. The expected number, or proportion, of such transformants is calculated on the basis of the probability of the random occurrence of simultaneous or successive transformation of one cell with more than one DNA particle (i.e., a reaction bimolecular with respect to DNA). If t_2/viable count per ml is the frequency

of transformants to a given marker, then ($E_{12} = t_1 \times t_2$/viable count) represents the probable number of cells transformed both to marker 1 (on molecule 1) and also to marker 2 (on molecule 2); in other words, it relates the individual probabilities for transformation to each of the single markers. Hotchkiss and Marmur pointed out that an excessive proportion of multiply-marked transformants should occur 1) independently of DNA concentration (since the collision frequency of cells with two marked DNA particles would decrease abruptly and characteristically with DNA concentrations to give a different slope in the linear response range) and 2) independently of which allele of the marker is present in the recipient or the donor strain (since specific sites which could be occupied by one of many alleles at any given time are being considered). Both of these criteria are satisfied in the streptomycin-mannitol system, when one multiply-marked DNA preparation is used; for a mixture of DNA's, each marked with only one of the two genetic traits, the recovery of multiply-marked transformants is very close to that predicted by random double events (Hotchkiss and Marmur, 1954). Goodgal has dealt with these properties in some detail (1961). For a multiply-marked DNA, the yield of doubly-marked transformants may be one thousand times that predicted by random double events.

Hotchkiss and Marmur (1954) conclude by suggesting a "mode of linked association within the DNA particles of the doubly-marked strain." Since transformants can be genetically marked for either or both of the linked markers, the authors emphasize that only discrete segments of DNA particles need be used in the genetic recombination, so that a process analogous to crossing-over in higher organisms could occur on the intramolecular level.

Kinetic experiments on initiation of irreversible transformation are thus the preferred means of demonstrating linkage between genetic markers. DNA concentration had been chosen as the independent variable in previous experiments. The data now presented introduce the measurement of transformant yield as a function of the duration of exposure to DNA to demonstrate and quantitate the presence and the degree of linkage between genetic markers within DNA particles. The results correlate well with the models of transformation as initiated by collision events and provide an independent means of obtaining consistent and precise data on linkage frequencies. More specifically, the kinetic order of reaction of cells and DNA with respect to duration of the initial interaction can be equated with the molecularity of the event, i.e., with the number of DNA particles required to produce a multiply-marked transformant.

Experimental Results.

In Figures 1 and 2 are presented typical titration curves for singly- and multiply-marked transformants recovered as a function of DNA concentration. First, it can be seen that the yield reaches a saturating level at the same DNA concentration, within any one experiment, for any selected category of transformants. This result can be correlated with the observation of Lerman and Tolmach (1957) and Fox (1957) that the amount of P^{32} from labeled DNA which is fixed by a competent population of pneumococci is always proportional to the transformant yield; the competition of DNA for surface adsorption sites is obvious. Second, there are two patterns for accumulation of transformants. One pattern is obtained for singly-marked transformants to S or d and to the pair ad so that the ratio of ad transformants among all the d transformants remains constant (the linkage frequency); the logarithmic slope of the line is approximately one for all three classes of transformants, indicating that only one DNA particle is required. For the pair SK, however, the slope is very close to two, as expected when two particles (one for S and one for K) interact with the cell. This results in a ratio for SK to S transformants constantly decreasing with lower DNA concentration. The number of SK transformants recovered is always close to that calculated for random double events. By such titrations, it is, therefore, possible to distinguish between linked and unlinked pairs of markers and to measure the linkage frequencies (Hotchkiss, unpublished; Goodgal, 1961).

In time kinetic experiments, cells are exposed to DNA at $30^{o}C$ for increasing periods of time; in a plot of the cumulative number of transformants against the duration of exposure, a linear rate is always obtained for a single marker at any DNA concentration (Figure 3). The accumulation is first-order in time, as in DNA concentration. However, for the accumulation of marker pairs, the pattern may be first-or second-order in time, depending on the relationship of the two markers, the DNA concentration, and the nature of the DNA preparation used.

The drug resistance markers for streptomycin (S) and for micrococcin (K) resistance behave, under all conditions, like two completely independent factors. For example, the accumulation pattern for doubly-marked transformants from one DNA is exactly parallel to that calculated for ran-

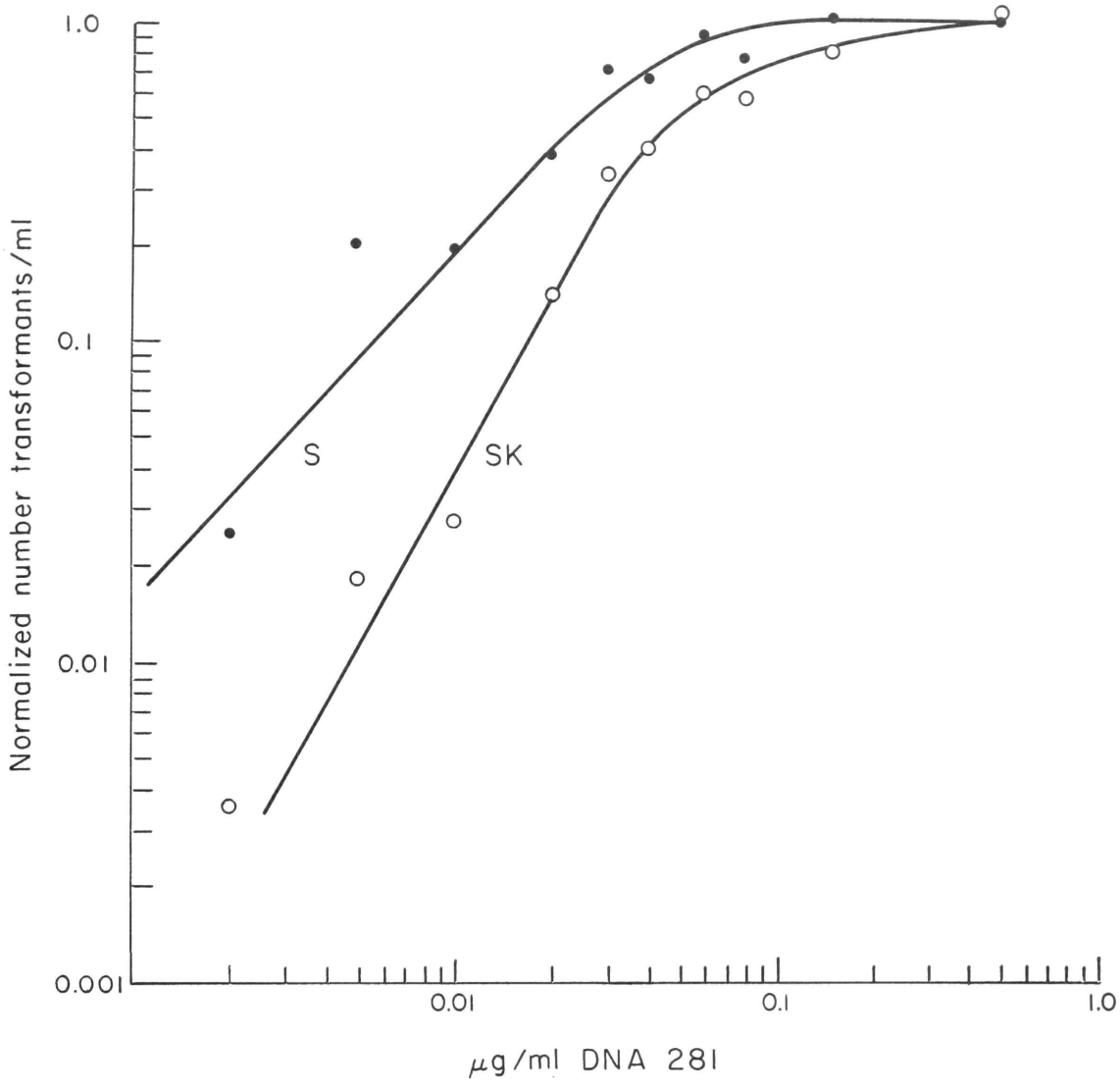


Figure 1. Yield of transformants recovered as a function of DNA concentration during exposure, normalized to maximum value.

The experiment was performed at 30°C, allowing a 30 minute exposure to DNA for a cell population approximately 3×10^7 viable count/ml. The absolute number of SK transformants recovered was always slightly less than that calculated for random double events between one cell and two DNA particles. S - streptomycin resistance marker; K - micrococcin resistance marker.

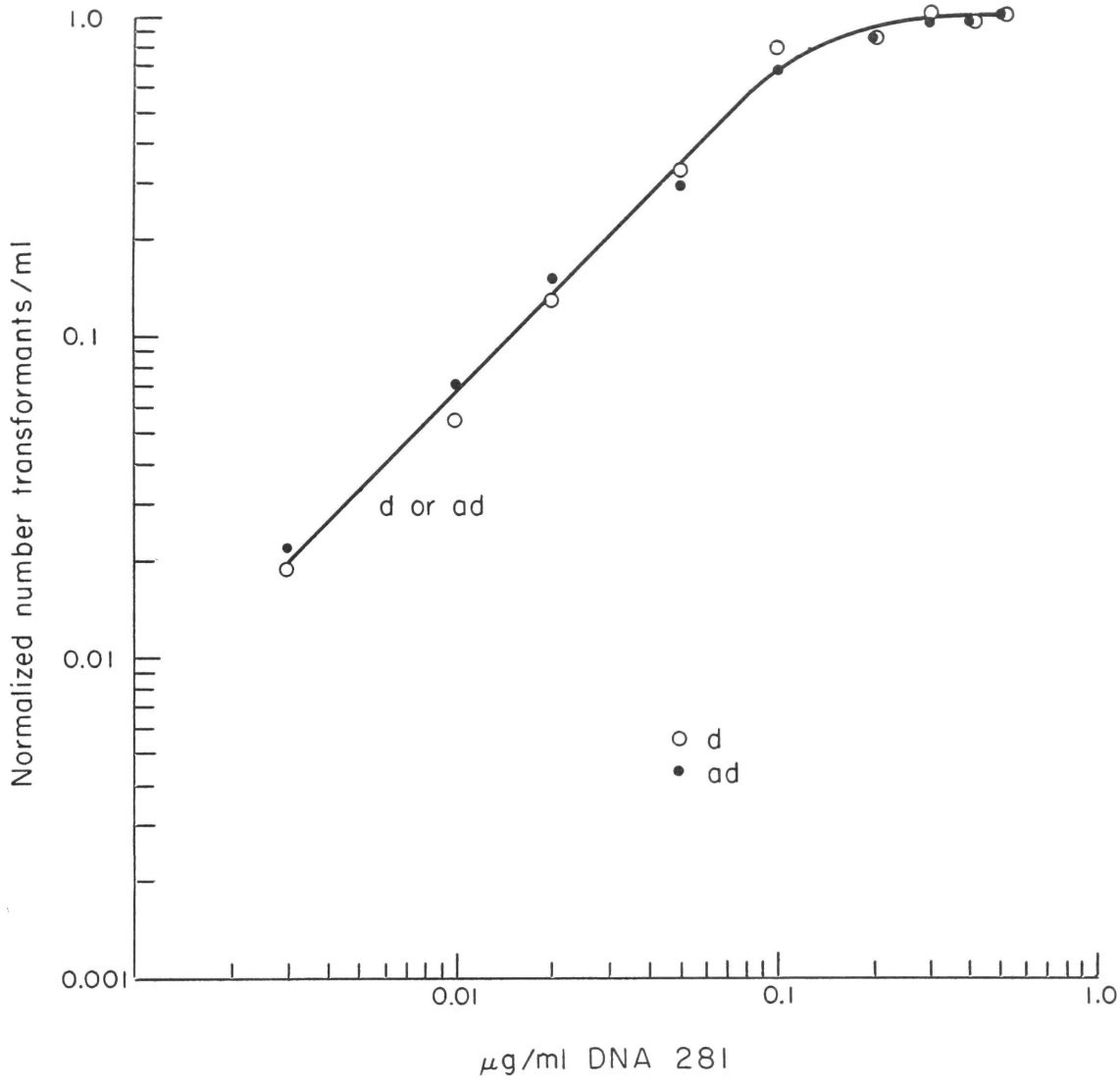


Figure 2. Yield of transformants to linked markers recovered as a function of DNA concentration during exposure, normalized to maximum value.

The conditions were equivalent to those described in Figure 1. ad controls high level sulfanilamide resistance and its component d controls a distinct, lower level of sulfanilamide resistance. The multiply-marked ad transformants constitute 50% of the singly-marked d transformants at each DNA concentration.

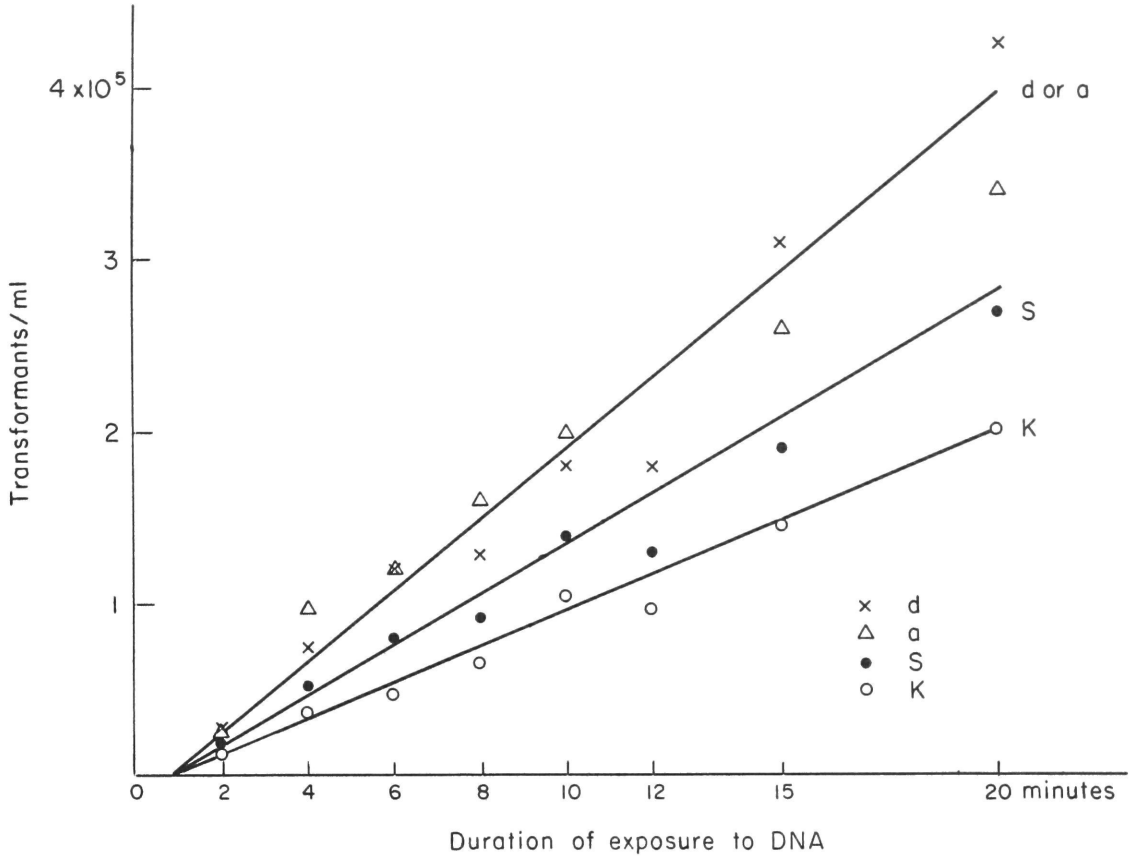


Figure 3. The linear rate of accumulation of singly-marked transformants during exposure, at 30°C, to a multiply-marked DNA.

DNA, at 0.2μg/ml was added at zero time to a competent culture of about 3 x 10^7 viable count/ml; aliquots were removed at the stated intervals to medium containing deoxyribonuclease in order to terminate the exposure, and the samples were allowed to develop resistance for 90 minutes at 37°C before selective scoring. The markers are: d and a, two different and distinguishable markers affecting resistance to sulfanilamide; S - streptomycin resistance marker; K - micrococcin resistance marker.

dom double events throughout the exposure period (Figure 4), although it should be noted that the absolute number of SK transformants can be lower than expected. Nevertheless, Figure 4 suggests that a cell already undergoing (irreversible) transformation is not thrown out of the competent state or otherwise blocked from undergoing additional rounds of transformation either simultaneously or successively for other markers. This point is demonstrated more clearly in the experiment represented in Figure 5 where, after a short exposure to S-marked DNA, a competent cell population is diluted into a large excess of K-marked DNA; the number of S transformants does not increase during the second exposure, but the linear accumulation rate of both the K and the SK transformants after addition of K-DNA demonstrates that cells which have previously encountered S-marked DNA particles are as readily accessible to K-particles as the general population. This point is further emphasized in Table I, where it is shown that pre-incubation with pneumococcal DNA for two exposure periods of different duration over a thirty-fold concentration range did not affect transformability of the population upon addition of a second DNA added in excess; these findings coincide with those of Stuy (1962) but are contrary to those reported by Goodgal and Herriott for H. influenzae (1961).

In conjunction with previous calculations of Fox and Hotchkiss (1957) on the large number of adsorption sites per cell and on the high probability of release of adsorbed DNA from these sites, the data of Figure 4 and of Figure 5 are interpreted to mean that the initiation of irreversible transformation within a cell does not affect its competence with respect either to surface adsorption (no occlusion) or with respect to further (internal) sites used in recombination. Therefore, a cell can indeed acquire DNA markers from more than one DNA particle. Accordingly, data obtained with DNA mixtures may be directly compared to those from single multiply-marked DNA preparations.

The accumulation of SK transformants is always exponential with time, as shown in Figure 6 for three concentrations of one DNA preparation and in Figure 7 for an equal mixture of singly-marked DNA's; distribution of the two markers between two genomes has no effect except to decrease the relative concentration of marked particles within the population. In

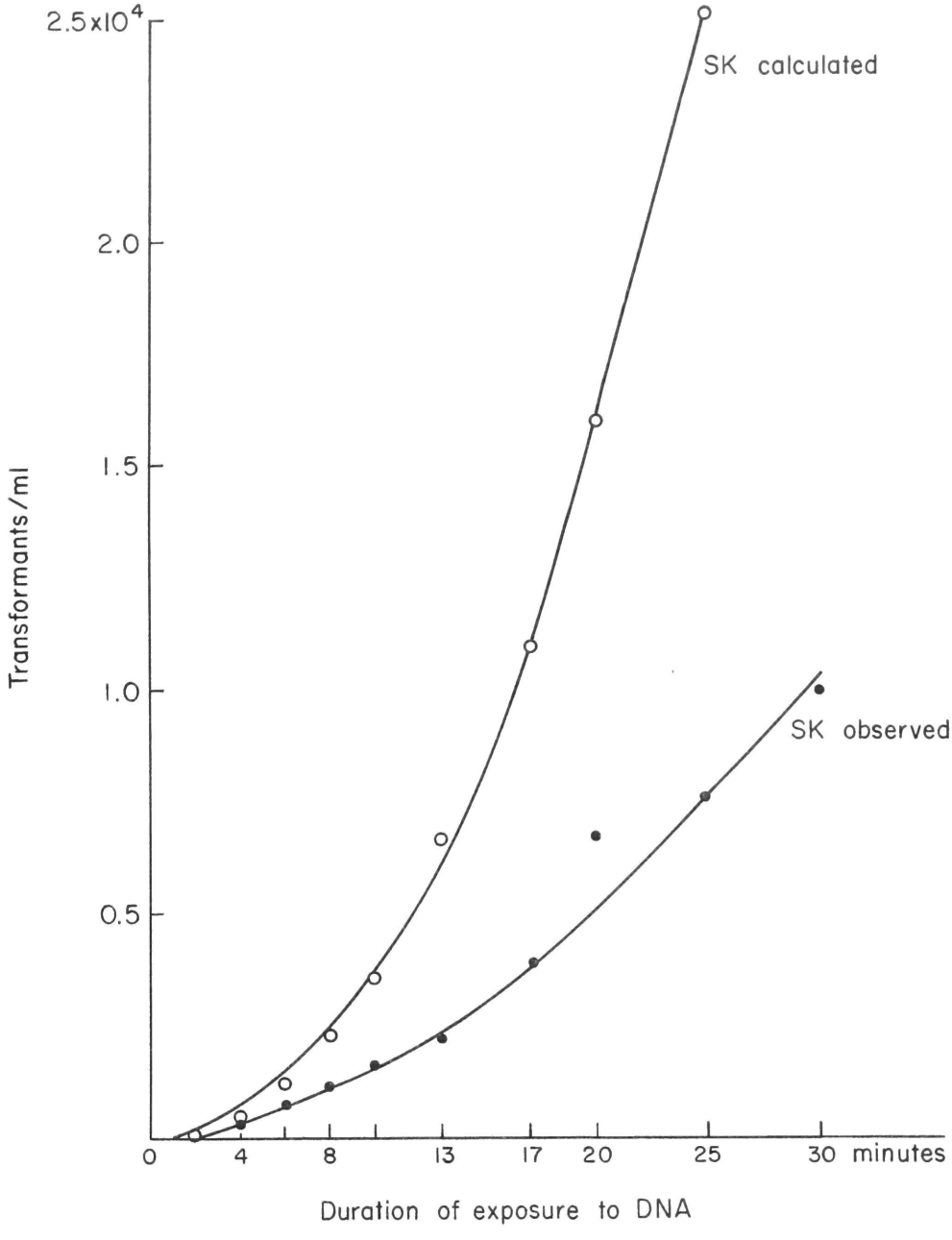


Figure 4. A comparison of the observed accumulation during DNA exposure of multiply-marked SK transformants with the pattern calculated for random double events.

A competent culture at about 4 x 10^7 viable count/ml was exposed to 1.0μg/ml DNA at 30°C. The expected number of multiply-marked transformants was calculated, as described in the text, from the observed values for singly-marked transformants for S and for K. S - streptomycin resistance; K - micrococcin resistance. When corrected from viable count to total cell count, the observed yield of SK transformants is approximately equal to the calculated value.

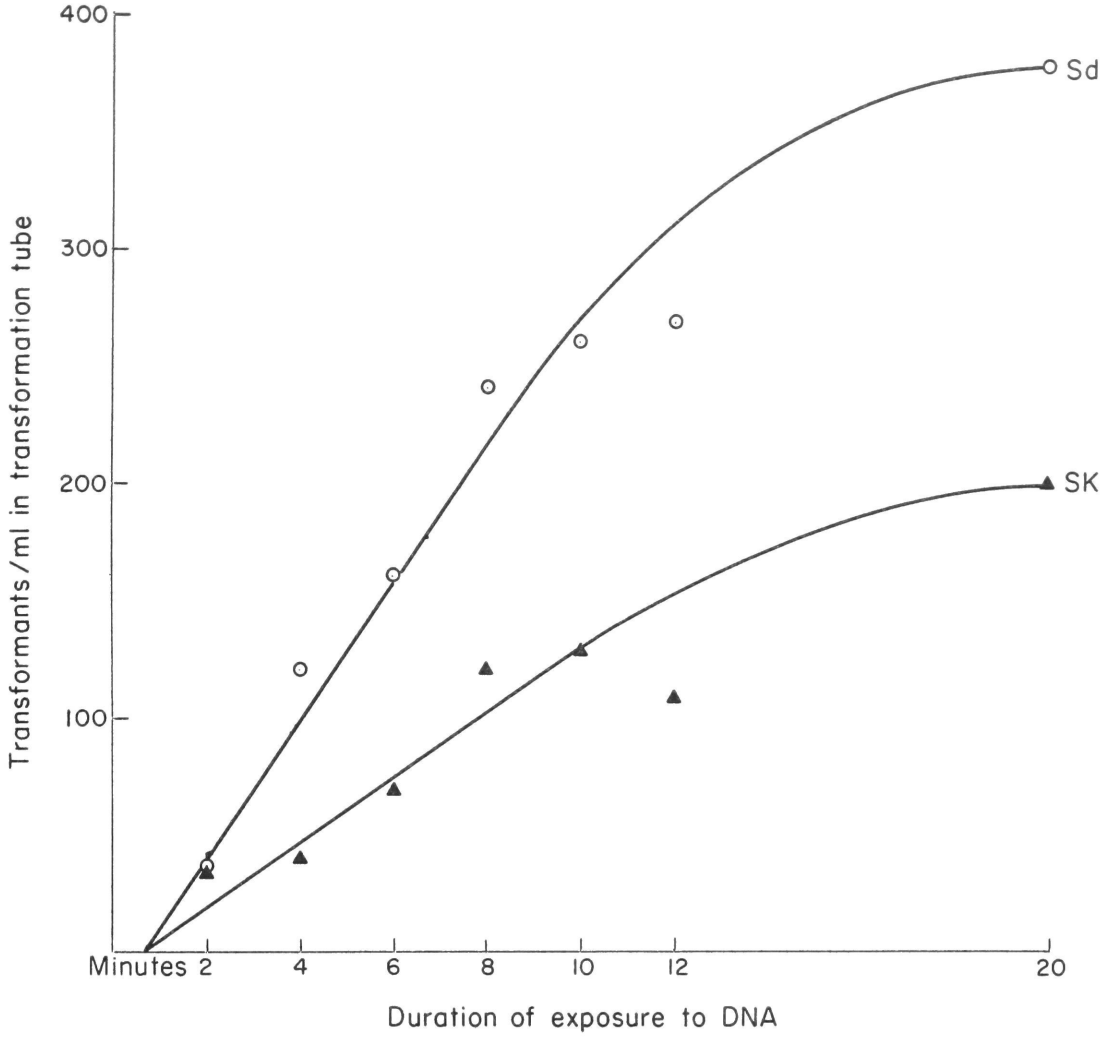


Figure 5. Linear accumulation of transformants after successive exposure to differentially marked DNA's.

A freshly grown and synchronized population at 1.1×10^7 viable count/ml was exposed to 0.1μg/ml S-DNA for 10 minutes at 30°C, giving a 2.5% yield of S transformants. The culture was then diluted 10-fold into fresh prewarmed medium at 30°C containing 0.5μg/ml of Kad-DNA; at this stage the Kad-DNA was in 50-fold excess, and therefore negligibly few new S transformations were initiated. At progressively longer intervals after addition of the Kad-DNA, aliquots were removed to deoxyribonuclease containing medium for expression. The markers are: S - streptomycin resistance; K - micrococcin resistance; d - sulfanilamide resistance. If present in one multiply-marked DNA, S and K would be unlinked, but S and d would be linked. In the final transformation tube, there were 2.8×10^4 S nascent transformants/ml; d transformants accumulated at a linear rate of about 4.8×10^3/minute, and K transformants accumulated at a linear rate of about 3.0×10^3/minute.

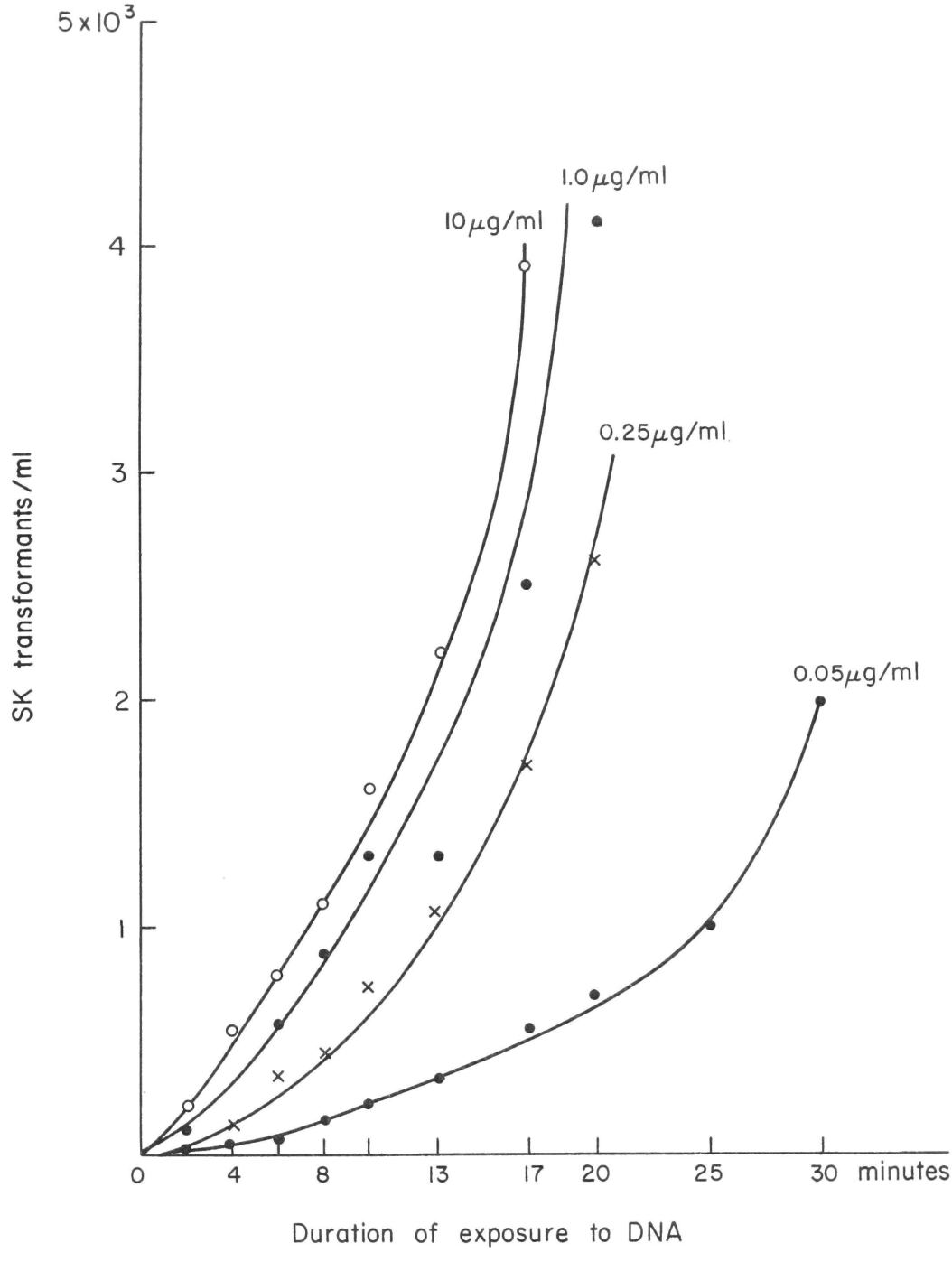


Figure 6. The exponential pattern of accumulation of multiply-marked SK transformants as a function of the duration of exposure to DNA.

A concentrated, competent culture at 4 x 10^7 viable count/ml was exposed to multiply-marked DNA at 30°C, and samples were withdrawn at intervals to expression medium containing deoxyribonuclease. Under these conditions 0.25μg/ml DNA gives a saturating yield (see Figure 1). The markers are: S - streptomycin resistance, and K - micrococcin resistance.

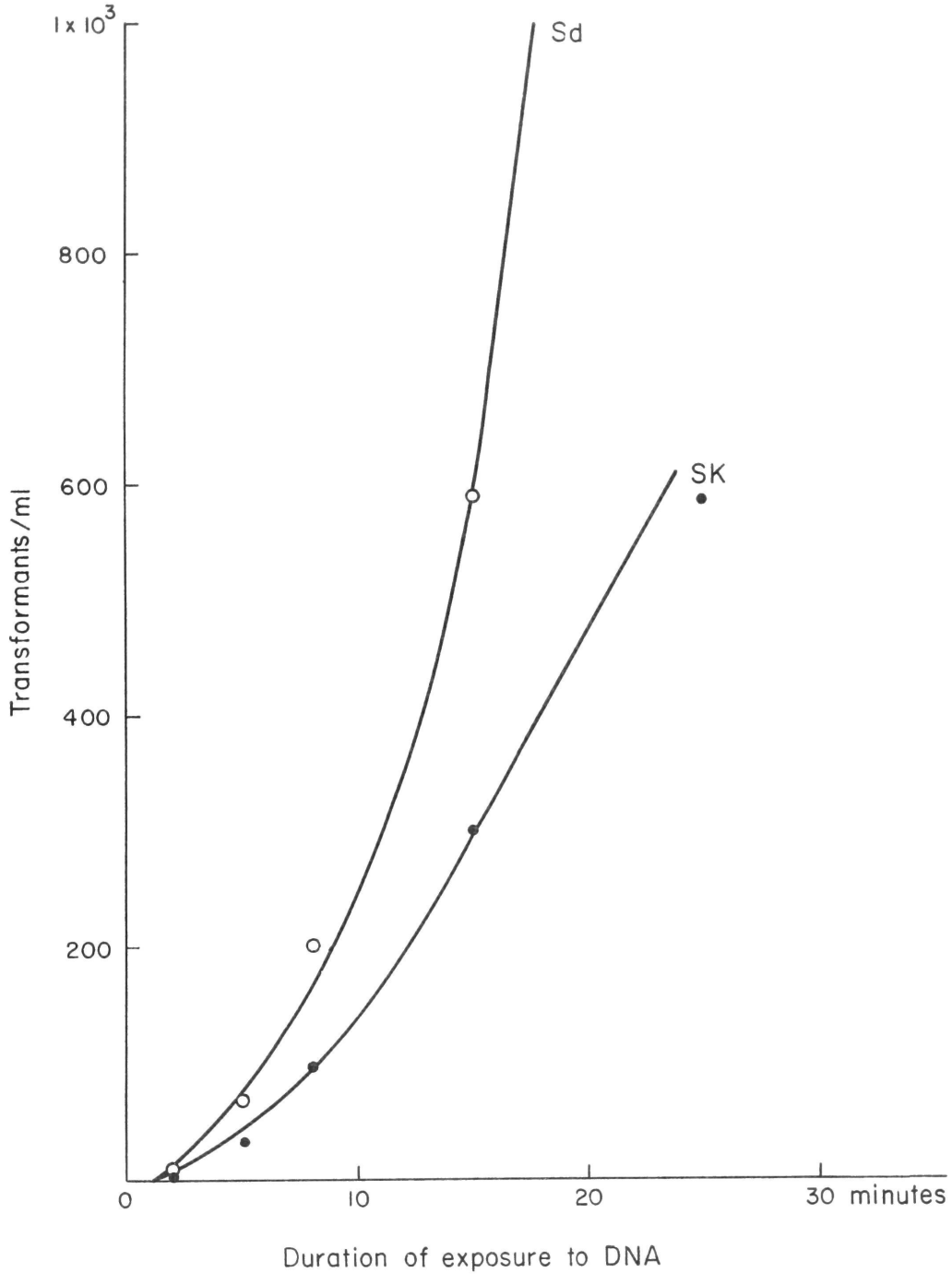


Figure 7. The accumulation of multiply-marked transformants in the presence of a mixture of two DNA's, each one bearing only one of the two markers selected.

The culture was exposed to a total concentration of DNA above that required to saturate the yield. Kad-DNA and S-DNA were mixed in 1:1 concentration before addition to the culture; 0.22μg ml of each DNA was present in the transformation tube.

TABLE I

The effect of exposure to DNA on competence of pneumococcal populations.

tube #	DNA-S μg/ml	minutes exposure	S/ml	DNA-K K/ml
1	0	10	0	1.4×10^3
		20	0	1.2×10^3
2	0.003	10	370	1.5×10^3
		20	500	1.2×10^3
3	0.01	10	710	1.1×10^3
		20	1450	760
4	0.03	10	1550	1.2×10^3
		20	1900	1.3×10^3
5	0.10	10	5400	1.1×10^3
		20	8900	1.2×10^3

A competent culture at 1×10^6 viable count/ml was preincubated for 15 minutes at 30°C before addition of the first or S-DNA for either 10 or 20 minutes. The culture was then diluted ten-fold into fresh prewarmed medium containing 0.09 μg/ml K-DNA to which it was exposed for 10 minutes at 30°C. Transformant yield is calculated relative to this diluted sample. The markers are: S - streptomycin resistance; K - micrococcin resistance.

Figure 8, the accumulation is specifically shown to be second-order since the square root of the number of SK transformants per ml is a linear function of time at several DNA concentrations (Figure 6 data, replotted).

Thus S and K are found to be unlinked by the time kinetic method, since a bimolecular process is required for SK accumulation under all conditions. For other pairs of markers, however, the accumulation may be initially first-order in time, and the markers are, therefore, said to be linked (i.e., the requisite initial interaction is monomolecular).

A first-order increase in time is shown for several pairs of markers, at high DNA concentration, in Figure 9, viz., streptomycin resistance (S) and either of the sulfanilamide resistance markers a or d, the high level sulfanilamide resistance pair ad, and the triply-marked group Sad resistant to both streptomycin and high sulfanilamide concentrations. Similar data are obtained for low concentrations of DNA in that range where the transformation yield is proportional to DNA concentration. Even at high DNA concentrations, data from early time points are approximately equivalent to those obtained with longer durations of exposure at low concentration, in terms of the probable opportunities for effective collisions between competent cells and DNA. A calculation of the expected number of two-step Sd transformants shows the observed number to be considerably in excess (Table II) at both low and high DNA concentrations throughout the experiment. Therefore, it is concluded that a competent cell can simultaneously acquire linked markers by a monomolecular process not available for unlinked markers.

After long exposure to DNA, upward curvature appears in the graph of accumulation of doubly-marked Sd transformants (Figure 10); this acceleration is attributed to a superimposed accumulation of a significant number of transformants which occur as the result of random double (bimolecular) events. Through the use of a mixture of singly-marked DNA's, it is demonstrated that Sd transformants can accumulate by a process second-order in time (Figure 7). By thus providing the markers from different genomes and ipso facto on different DNA particles, we may have an accumulation indistinguishable from that of unlinked markers; therefore, the upward curvature seen in Figure 10 is attributable to a superimposed bimolecular process.

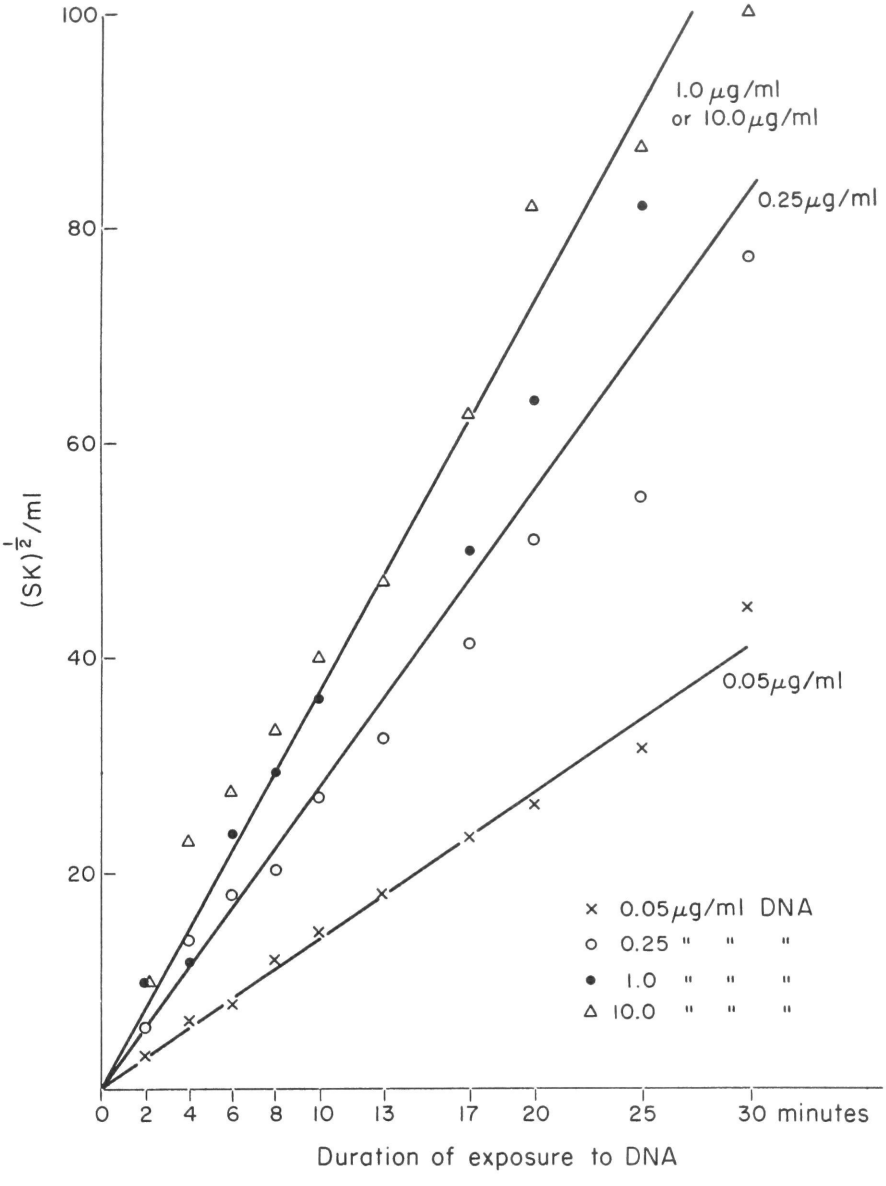


Figure 8. Second-order accumulation in time of SK multiply-marked transformants.

The data shown in Figure 6 are replotted here to show that the accumulation is second-order; the abscissa is now $(SK)^{\frac{1}{2}}$/ml.

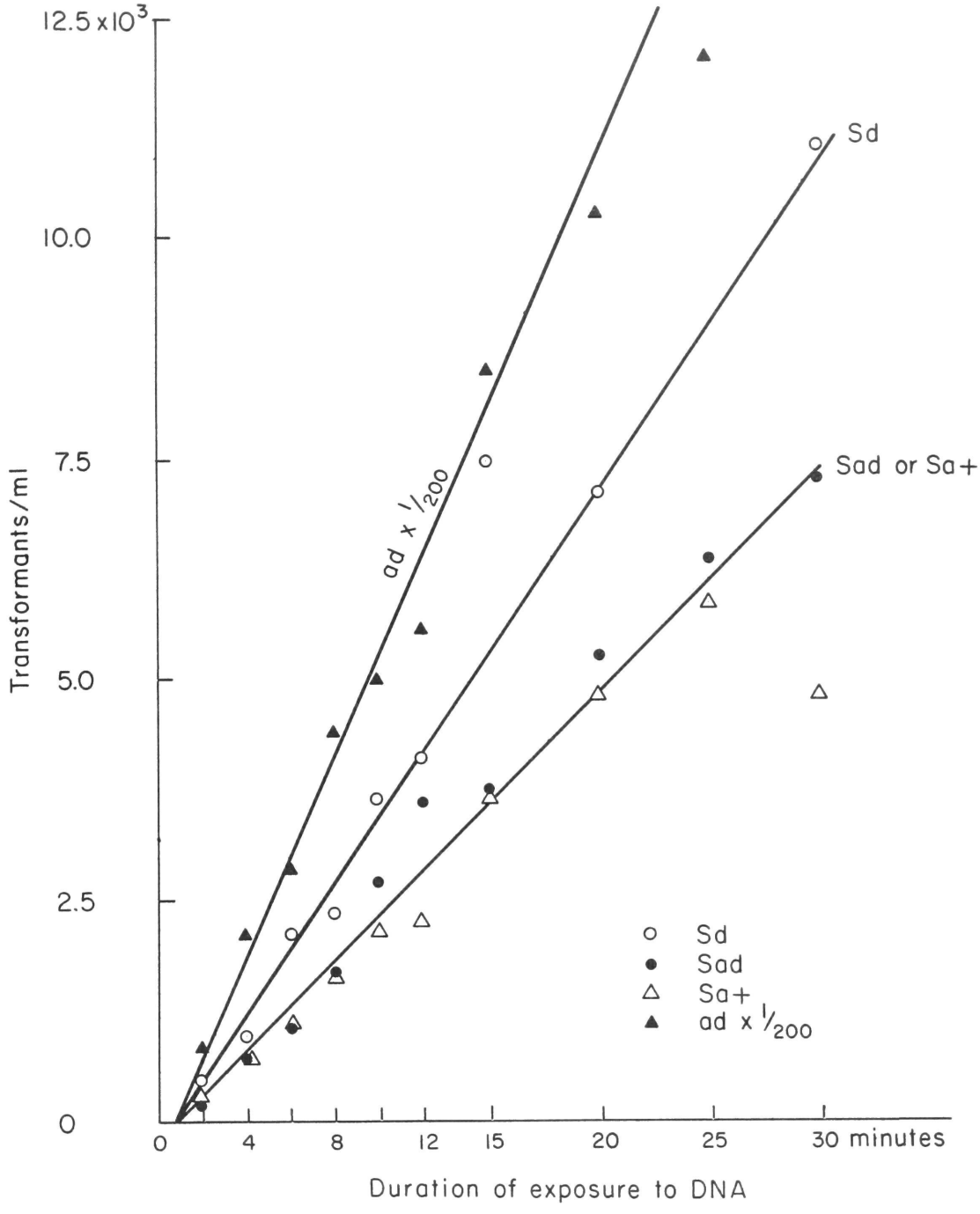


Figure 9. The linear accumulation with time of multiply-marked transformants to linked markers.

A concentrated, competent culture at 4 x 10^7 viable count/ml was exposed to 0.2μg/ml of a multiply-marked DNA, at 30°C. The markers are: S - streptomycin resistance; a+, d, ad, - different and phenotypically distinguishable markers for resistance to sulfanilamide.

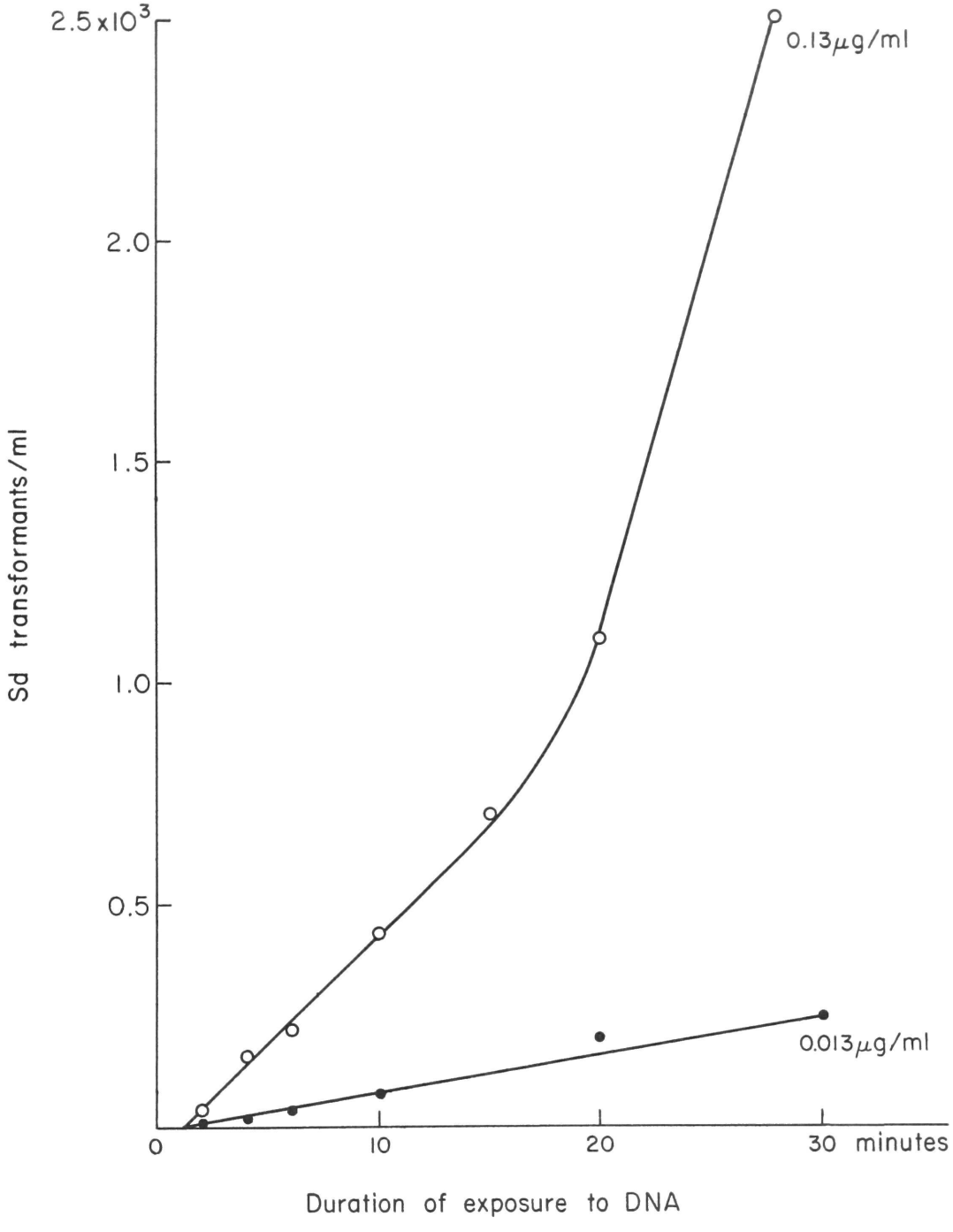


Figure 10. The accumulation of Sd multiply-marked transformants with time at high and low DNA concentration.

A competent culture at 1.0 x 10^6 viable count/ml was exposed to a saturating concentration of 0.13μg/ml DNA and an identical aliquot to a concentration of 0.013μg/ml, which is in the linear response range. The experiment was performed at 30°C.

TABLE II

Ratio of Sd transformants observed: calculated for random doubles at high and low DNA concentration.

DNA concentration μg/ml	Duration of DNA exposure in minutes					
	2	4	6	10	20	30
0.13	7	7	4	3	2	3
0.013	28	13	10	6.5	4	2.3

A competent culture at 1 x 10^6 viable count/ml was exposed to DNA at 30°C.

Recombination with a DNA particle does not affect cellular capacity for a subsequent interaction with a differently marked copy of the same particle at a neighboring segment of the cell genome. By successive exposure of a competent cell population to S-marked DNA (10 minutes) followed by a large excess of Kd-marked DNA, it is shown that both d and Sd transformants accumulate linearly from the time of addition of the second DNA (Figure 5). The ratio of SK:Sd transformants is equal to the K:d recovery ratio, which strongly suggests that on the intramolecular level there is no interference with a secondary round of transformation over measurable time intervals. Equivalent results are obtained after successive exposure to DNA's marked with a and d, respectively, although these two markers remain about 50% linked if present in the same genome and are, therefore, presumed to be closer together within one DNA particle than S and d.

Although a linear rise in number of multiply-marked transformants (to linked markers) begins very shortly after exposure to a DNA bearing linked markers, nevertheless, their rate of appearance depends markedly on the particular markers involved. The usual definition of linkage frequency as the ratio of transformant yield of paired to single markers can be expressed alternatively as the ratio of the linear rates of accumulation of transformant classes. Thus, if ad transformants accumulate at the rate of 1×10^4/minute and all of the d transformants at the rate of 2×10^4/minute, then the linkage frequency of ad:d is stated to be 50%. In this manner, Sd is found to be 2-8% linked to S, but both the low and the high linkage frequency can readily be measured and distinguished from the unlinked behavior of the pair SK by this technique. In addition, rate measurements are quantitative enough to permit ordering of three linked markers on one map; thus, the total rate of accumulation of all Sa transformants is often greater than the rate for all Sd transformants; therefore, by classical genetic mapping the order is Sad (Table III), as reported by Hotchkiss and Evans (1958) on the basis of other experiments.

After the demonstration that genetically linked and unlinked pairs of markers accumulate in recipient cells in characteristically different time patterns from a single preparation of an intact, multiply-marked DNA, the examination of "bad" preparations of pneumococcal DNA revealed that linkage properties of marker groups are more sensitive criteria of DNA quality than

TABLE III

Ordering of three markers by rates of accumulation.

markers	ratio relative to S
S	1.0
Sa	.052
Sd	.040

Conditions of exposure are: a wild-type culture at approximately 2 x 10^6 viable count/ml exposed to 0.02μg/ml Sad-DNA (264) at 30°C for increasing intervals up to 30 minutes.

the biological activity of a single marker. Since linkage groups occupy larger segments of a given particle than the single markers of which they are composed, this result is not particularly surprising. As a result, however, it should be possible to destroy linkage in a DNA preparation by localized (i.e., submolecular) degradations within the DNA structure, thereby forcing the accumulation pattern for transformation of linked markers to become second-order (bimolecular) in time. Such an experiment would demonstrate all of the phenomena reported for mixtures and for single DNA preparations in the behavior of one treated DNA preparation. It would also prove that a given physico-chemical method of DNA degradation acts to produce submolecular lesions.

Subcritical heat inactivation was the method selected; this is the slow, acid-catalyzed inactivation process occurring at temperatures below the critical, or transition, value (Roger and Hotchkiss, 1961; Ginoza and Guild, 1961; Greer and Zamenhof, 1962). At pH 6.8, there is about 40% survival of the sulfanilamide resistance d marker in five hours (Figure 11). Several workers have interpreted such inactivation to result from hydrolysis of the acid-sensitive N-glycosidic bond between purine bases and the sugar. Purine, but not pyrimidine, bases have been isolated after prolonged heating of DNA under similar conditions (Greer and Zamenhof, 1962). If the mechanism is depurination as suggested, the backbone chain would remain unbroken, unlike the case of decay of P^{32}-labeled DNA in which it could readily occur (Levinthal and Davison, 1961). The inactivation would thus be localised within a molecule and yet one of undiminished size. Independent of mechanism, however, are the findings of Roger and Hotchkiss that the linked pair ad is inactivated at a faster rate than the single d marker, and this difference indicates that segments rather than whole molecules are affected. Fox (1962b) has obtained similar differential rates of decay in P^{32}-labeled DNA.

In the inactivation of a multiply-marked DNA as shown in Figure 11, the single d marker and two marker pairs (SK and Sd) decay exponentially after an initial lag. The rate of exponential decay of d is about half that of either pair, in agreement with the data of Roger and Hotchkiss (1961) on the pair ad. In addition, the similar inactivation patterns of the linked pair Sd and the unlinked pair SK, which are revealed under experimental conditions designed to measure linkage, demonstrate that primarily only localised regions of particles are affected by inactivation.

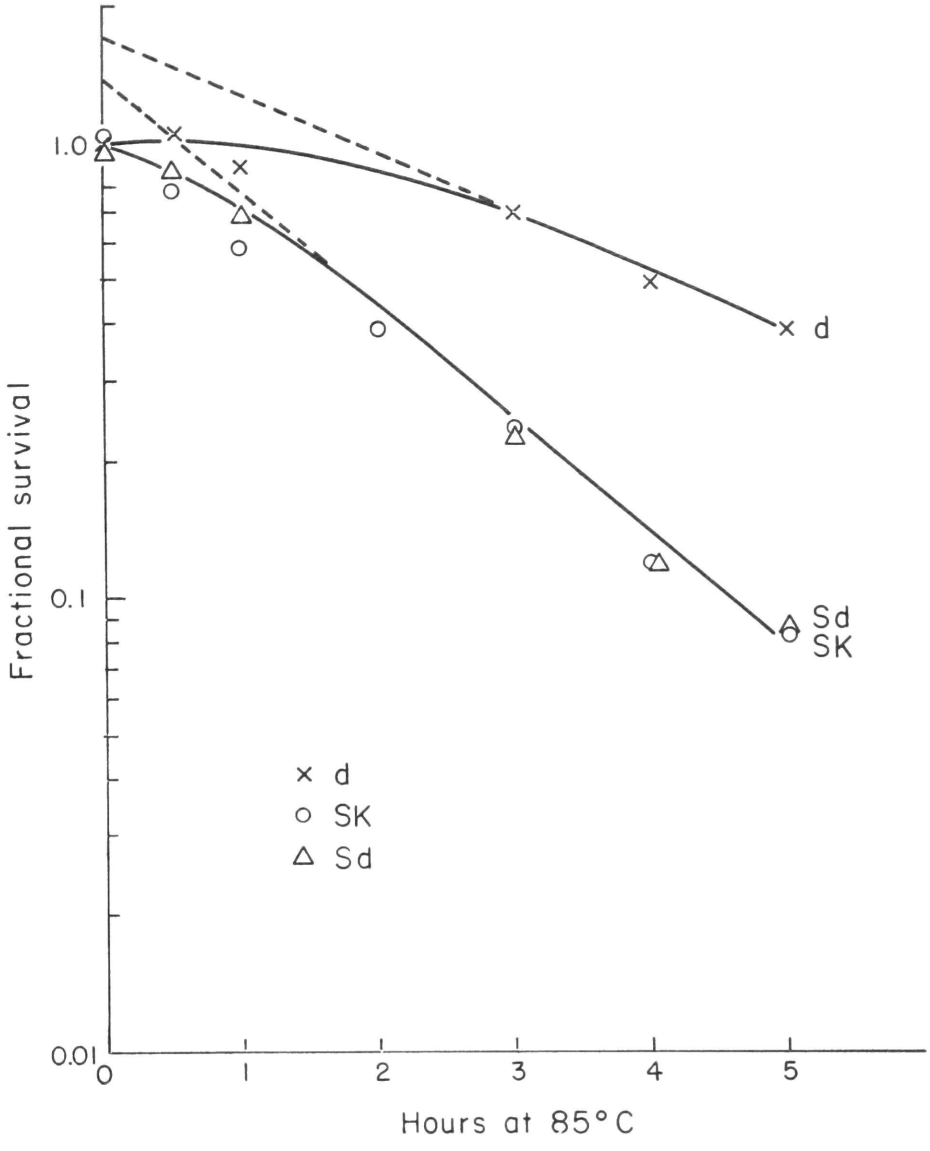


Figure 11. Survival of transforming activity after sub-critical heat inactivation of multiply-marked DNA.

The bio-assay was performed on a concentrated competent culture at a viable count of 4×10^7/ml. The culture was exposed for 5 minutes at 30°C to 0.02μg/ml of a DNA aliquot which had been heated at 85°C. The markers are S - streptomycin resistance; d - sulfanilamide resistance; K - micrococcin resistance. SK is an unlinked pair, whereas Sd is a loosely-linked pair. The exponential rate of inactivation of the muliply-marked transformant types is 1.8 times the exponential rate of inactivation for the singly-marked d transformant.

With DNA samples subcritically inactivated for varying periods of time, progressive changes appear in the accumulation patterns of markers in recipient cells. The single d marker continues to accumulate linearly with time but at a rate which continues to decrease for increasingly prolonged heating (Figure 12A) to mimic a dilution of the active marker within the total DNA population. On the other hand, the linked pair Sd at first accumulates linearly with time at saturating DNA concentration although at slower rates; as the heating is prolonged, however, Sd transformation becomes second-order in time (Figure 12B). This is interpreted to represent the fact that independent inactivation of either S or d destroys the linked pair but not the other single marker so that the relative concentration of active Sd molecules in the population quickly becomes low; nevertheless, particles singly-marked for either S or d are still present in relatively high concentration and can give rise to Sd molecules by a bimolecular process.

Such a subcritically heated preparation of DNA would consist of a mixture of particles each inactivated at random for one or more markers. Even an extensively heated DNA should show a residual linear accumulation rate for Sd transformants provided the DNA concentration is low enough to eliminate the superimposed pattern of Sd random doubles. In Figure 13, it is demonstrated that particles having both S and d markers can survive five hours of subcritical heating. The observed yield of Sd transformants increases linearly with time at low DNA concentration and can be 100-fold that calculated by random double events, at short exposure intervals. The data are shown in comparison to the yields obtained with an intact sample of the same DNA, for which the accumulation is linear at both high and low DNA concentration. Similar destruction of linkage is observed for several other linked pairs of markers, the accumulation becoming second-order in time (Figure 14); the unlinked pair SK continues to accumulate exponentially with time irrespective of the extent of inactivation and also at constantly decreasing yields. All of these results are in accord with the clear-cut reaction between cells and intact regions of one or more DNA particles, as previously formulated.

It is concluded that the kinetic order of accumulation in time of multiply-marked transformants is dependent upon the genetic and physical relationship of these markers to one another within the isolated sample of

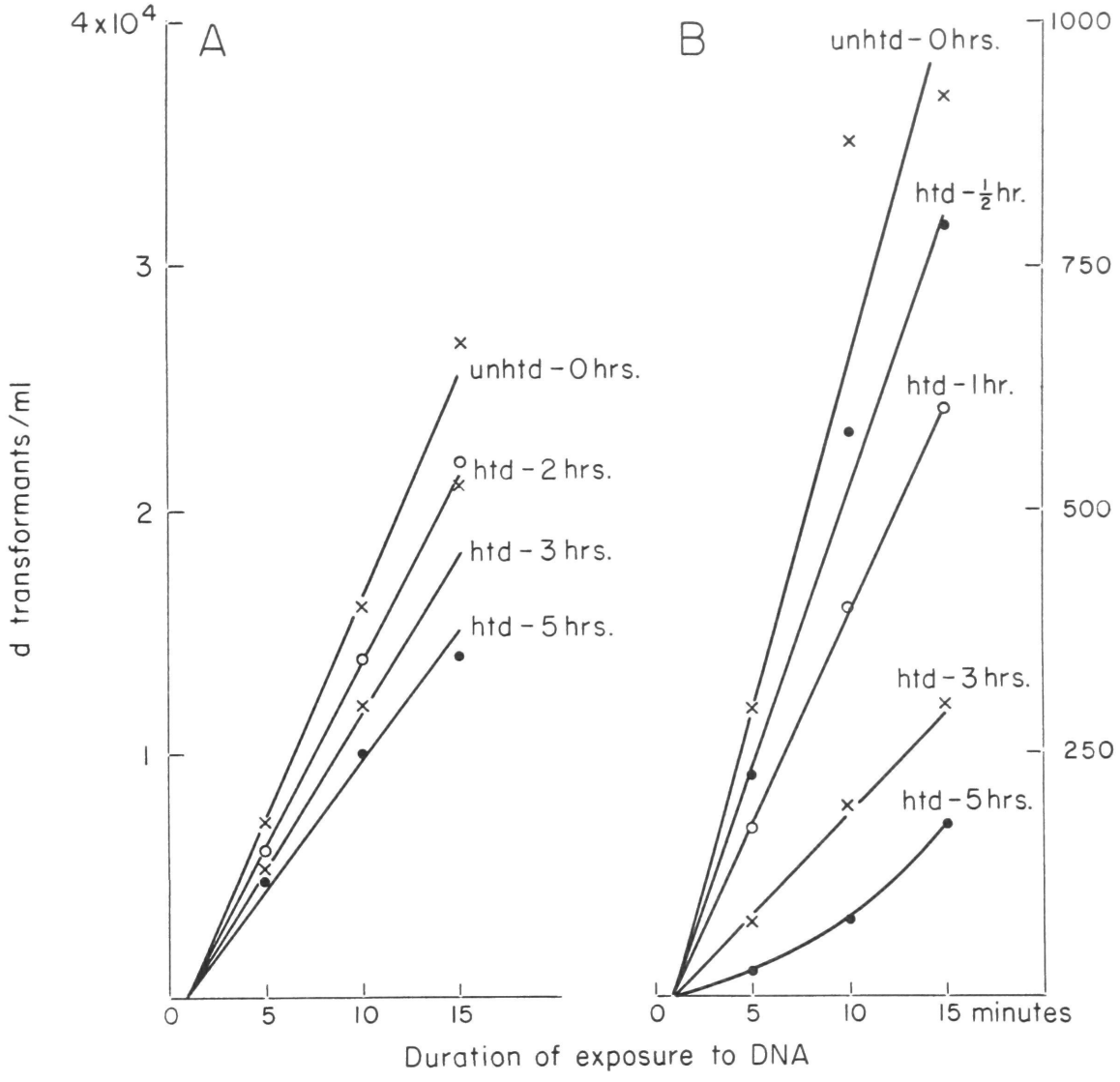


Figure 12. The accumulation of transformants during exposure to sub-critically heat inactivated samples of a multiply-marked DNA.

Each bio-assay was performed at 30°C, using 0.14μg/ml DNA and a competent population at 2 x 10^6 viable count/ml. Each DNA sample had been heated at 85°C for several hours, as noted on the figure. The markers are: S - streptomycin resistance, and d-sulfanilamide resistance.

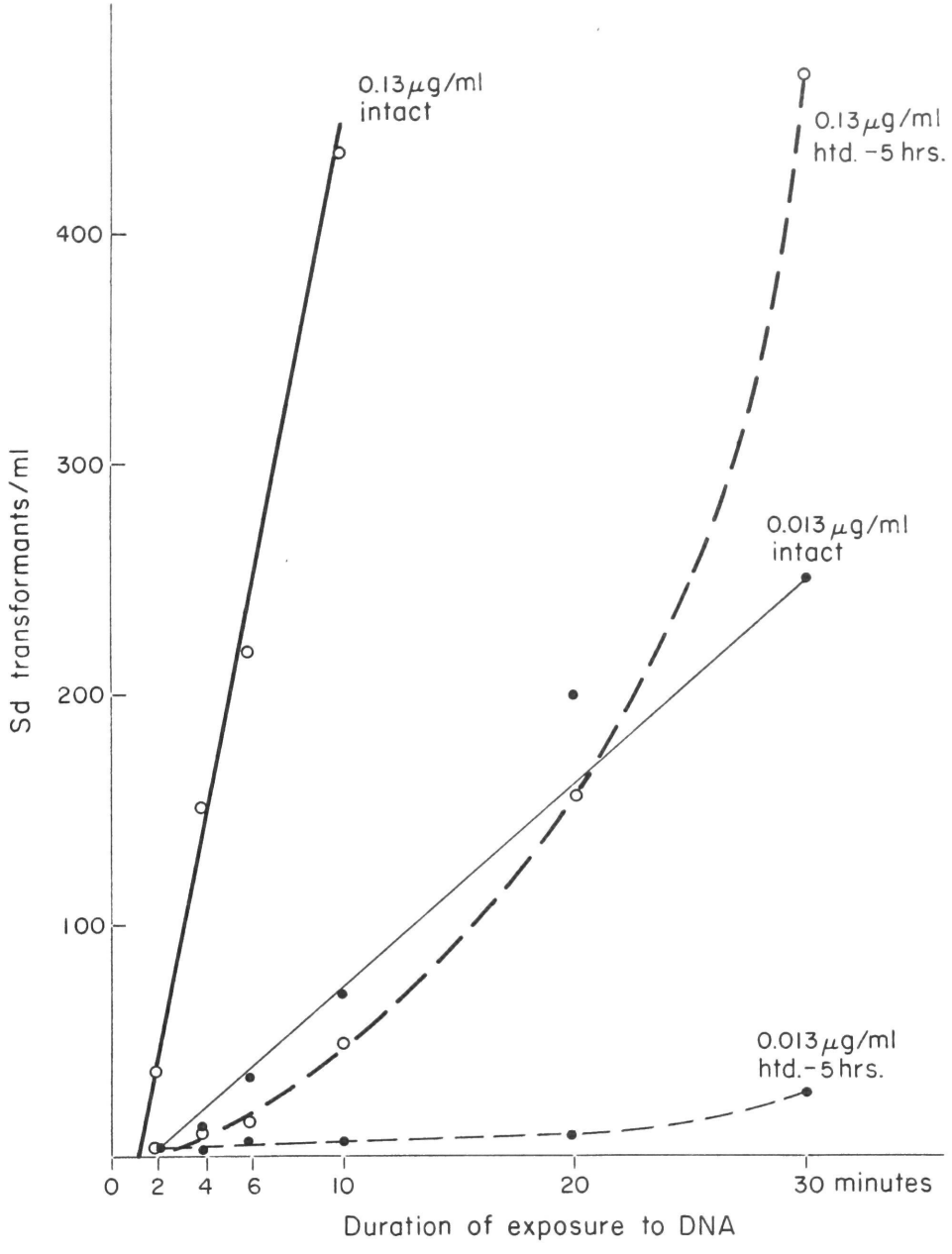


Figure 13. The effect of extensive sub-critical heat inactivation on the pattern of accumulation of Sd transformants from a multiply-marked DNA.

Each bio-assay was performed at 30°C, using a competent population at about 2×10^6 viable count/ml. An unheated aliquot of DNA was used as control (——) in contrast to a sample which had been heated for 5 hours at 85°C (----). The higher concentration of 0.13μg/ml gives a plateau yield while the lower concentration of 0.013μg/ml is within the linear response range. The S marker lost 60% of its biological activity in 5 hours exposure to this temperature. The markers are: S - streptomycin resistance, and d - sulfanilamide resistance.

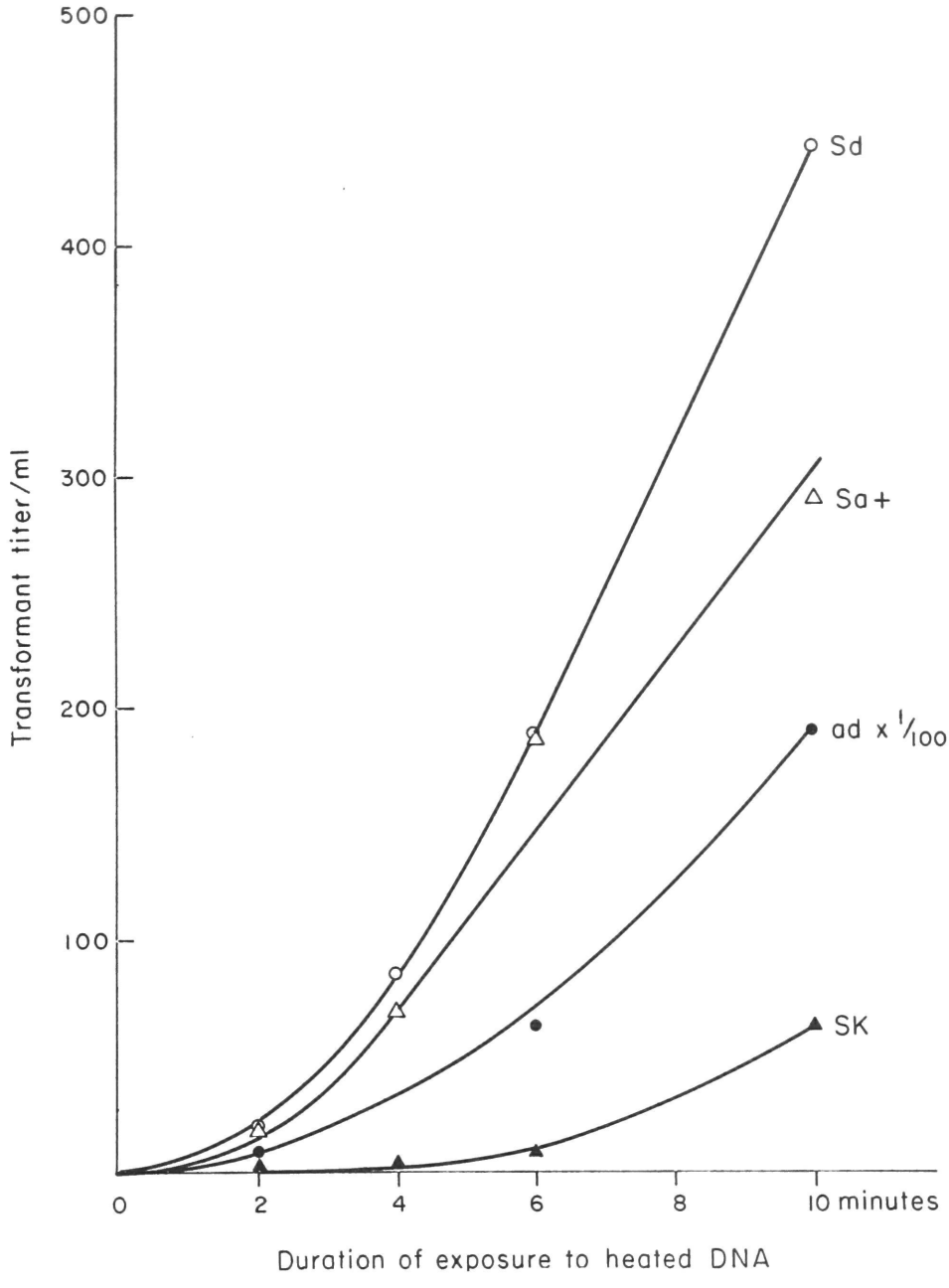


Figure 14. The accumulation of multiply-marked transformants upon exposure to subcritically heated, multiply-marked DNA.

A concentrated competent culture at 4 x 10^7 viable count/ml was exposed to 0.25μg/ml DNA, which had been heated for 4 hours at 85°C. The markers are: S - streptomycin resistance; K - micrococcin resistance; d, a+, ad - different and phenotypically distinguishable markers for resistance to sulfanilamide. In an unheated preparation, the pair SK is unlinked and accumulates exponentially with time, but each of the pairs Sa+, Sd, and ad, is linked and accumulates linearly with time; the marker a is 50% linked to d in an unheated sample.

the bacterial genome, i.e., to co-existence within the same particle. When they become physically unlinked, the genetically linked markers can be forced to accumulate like the unlinked pair SK. Unlinking can be accomplished either by the use of mixtures of singly-marked DNA's or by the use of appropriately damaged multiply-marked DNA. An unlinked pair, on the other hand, does not behave like a linked pair under any conditions. Furthermore, linkage frequencies can be precisely assessed from kinetic data, and ordered genetic maps of DNA particles can be obtained having, at least in principle, greater reliability than those obtained from arbitrarily chosen single exposures of cells to DNA.

D-2. THE IDENTIFICATION AND CHARACTERIZATION OF COMPLEX INTRAMOLECULAR RECOMBINATIONAL EVENTS IN THREE-FACTOR CROSSES

Introduction.

Unexpected behavior in recombination has been observed for closely linked loci in a wide variety of genetic systems (see Section A). These aberrancies are expressed by three distinct phenomena: 1) unexpectedly high frequencies of multiple recombinations over short distances (in phage recombination or in Aspergillus or Drosophila crosses) and 2) the rare recovery of aberrant tetrads from fungal crosses and 3) the infrequent recovery of progeny bacteriophage heterozygous over short regions of the genome after biparental infection of a single bacterium. Note that "short" refers to a distance computed from recombination frequencies, and that the proportionality between physical distance and map distance may be different for unlike genetic systems. The analysis of a three-factor cross for a linear array of markers within one DNA particle can be made in the pneumococcal transformation system in order to explore the relation between anomalous recombination data and unequivocally intramolecular recombination.

Negative interference is defined as the occurrence of multiple recombinational events at higher frequency than calculated for their random and independent occurrence in separate segments of the genome (Haldane, 1919). This effect has been reported in Drosophila (Chovnick, 1958), Neurospora (St. Lawrence, 1956) and yeast (Leupold, 1958), but studied primarily in bacteriophage and Aspergillus. Analysing T_4 bacteriophage progeny after biparental infection, Chase and Doermann (1958) used two-, three- and four-factor crosses to demonstrate that the number of excess recombinants recovered from multiple events increases abruptly within total map intervals allowing less than 3% recombination and is inversely related to the length of the map interval. This excess is observed for triple as well as double recombinations, indicating that higher-order exchanges also occur in unexpectedly high frequency over small regions; a five- to seven-fold excess of double recombinations is obtained over map intervals from 2.7% to 7.0% in length, but the excess can be thirty-fold in an interval of 0.71% and two hundred-fold in a four-factor cross requiring three recombinations within

an interval of 0.91%. By kinetic analysis, Edgar and Steinberg (1958) showed that these multiple exchanges occur during one event. Nevertheless, all the mutants can be ordered unambiguously in a linear array on a genetic map.

In Aspergillus nidulans crosses, similar types of data are obtained for meiotic and mitotic recombination of chromosomes. A hundred-fold excess of double recombinations can occur in map intervals corresponding to less than 0.1% recombination, but there may be no interference in three-factor crosses spanning map intervals of a few percent (intra-cistron: Pritchard, 1955, 1960; Strickland, 1958b). Although usually limited to segments permitting a few tenths of one percent recombination (Pritchard, 1955), negative interference has been reported in an interval allowing 15% recombination and containing more than one cistron (Calef, 1957). From analysis of mitotic recombination data, Pritchard (1960) has concluded that localised negative interference operates and that recombination is frequently reciprocal. The loci can always be arranged in a unique linear array to correspond to the data.

On the other hand, Maccacaro and Hayes (1961) have shown that negative interference can operate over segments up to 12% of the entire chromosome in the E. coli conjugation system; this length corresponds to about ten coli-phage equivalents of DNA. Using a multiply-marked male donor, they observed that the probability of a second cross-over is inversely related to the map distance from the initial cross-over within this segment. There is a significant difference in the genetic data obtained when three closely linked loci of E. coli are transferred by transduction rather than by conjugation, as pointed out by Maccacaro and Hayes (1961). After selection for the two outer markers, the intermediate unselected marker is virtually never excluded from recombinants after conjugation (Cavalli, Lederberg and Lederberg, 1953; Maccacaro and Hayes, 1961), although it is excluded with significant frequency after phage-mediated transduction (Lennox, 1955; Gross and Englesberg, 1959). These three loci span only about 1% of the total chromosome, but the distance between the outer two loci for threonine and leucine synthesis approaches the maximum length of a transducible fragment (Lennox, 1955). Therefore, the appearance of the outer loci in a recombinant after transduction would be associated with recombinational events occurring quite close to them, and negative interference could occur;

in conjugation, acquisition of these traits is more likely to reflect recombinations occurring much farther away along the chromosome, according to Maccacaro and Hayes (1961). The physical distance between loci is obviously not the only factor governing multiple recombinational events; since the nature of the transduced fragment is still ambiguous, more detailed interpretation is impossible.

In an aberrant tetrad, a 3:1 ratio occurs among the four or eight meiotic products of an ascus resulting from a cross between parents heterozygous for two or more loci. The usual ratio is 1:1 for reciprocal recombination to one marker. The aberrant tetrads could reflect a non-reciprocal recombination or an inaccurate reciprocal recombination (Pritchard, 1960). The frequency of these unusual tetrads varies significantly from species to species. In Saccharomyces, intracistron recombination may be predominantly non-reciprocal (Roman, 1956; Leupold, 1958); in Aspergillus a frequency of 0.2% has been reported (Strickland, 1958 a,b); in Neurospora the frequency may be intermediate (Mitchell, 1956 a,b; Case and Giles, 1958b). The route by which these tetrads are produced is unclear, as is their direct relevance to the problem of genetic recombination.

The discovery and study of partial phage heterozygotes (Hershey and Chase, 1951) has led to the suggestion that they are an intermediate stage in bacteriophage recombination (Levinthal, 1954). About 2% to 4% of the progeny phage from a biparental infection are heterozygous over a given short section of the genome and segregate for the two parental alleles at these loci upon subsequent monoparental infection of bacteria. These heterozygotes are unstable because they produce mainly pure segregants upon growth and only a few heterozygotes (2% to 4%). Levinthal (1954) has estimated the heterozygous segment to be a map interval allowing 5% to 10% recombination, which is comparable to the region of high negative interference in phage recombination measured by Chase and Doermann (1958). Levinthal (1954) found that phage particles heterozygous for a central marker are usually recombinant for the outer loosely linked markers in a three-factor cross. His calculations suggested, further, that the heterozygotes occur frequently enough to account for all recombinants among loosely linked markers, and his data show that experimentally induced changes in recombinant frequency occur parallel to similar changes in heterozygote frequency.

These data were interpreted, by Levinthal, as consistent with a molecular "overlap" region for the structure present in heterozygotes. Subsequent segregation, during growth, could lead to phage recombinants, according to the Levinthal hypothesis. On the other hand, Steinberg and Edgar (1962) found that recombinants for closely linked markers are not necessarily recombinant for outside markers. Nevertheless, most of the data are consistent with an opposite hypothesis that partial heterozygotes represent an alternative to genetic recombination rather than a necessarily intermediate stage.

In explanation of the localised anomalies, Pritchard (1955, 1960) has offered the hypothesis of "effective pairing" for small segments of the genome. Within small regions occurring discontinuously and perhaps randomly over the genome, "effective pairing" would permit recombinational events to take place at high frequency per unit physical distance and thereby account for localised negative interference; outside these segments, no recombination would be occurring and genetic exchanges would average out over long distances. This contrasts to the alternative explanation that recombination in one segment increases the relative probability of recombination in an adjacent segment, thereby causing negative interference. Pritchard (1960) computes the mean length of an "effective pairing segment" to be a few tenths of a map unit for Aspergillus and five to ten map units in phage and estimates the "true length" at a few cistrons in both organisms. He notes further that "effective pairing" might be distinct from the cytological pairing observed in meiosis and could, therefore, occur at the time of DNA duplication during meiosis prior to generalized synapsis; if true, this rationalization would reintroduce the mechanism of copy-error in acceptable models of chromosomal recombination. However, Pritchard's hypothesis is independent of the copy-error vs. breakage-reunion controversy because it is primarily a steric definition of an earlier stage in recombination.

The study of recombinational anomalies per se has not succeeded in casting much new light on either the fundamental mechanism(s) of recombination or the structural requirements for pairing and recombination of the two interacting entities. The linear arrangement of genetic loci in a uniquely determined array is usually possible even over small regions.

Only in the transformation system can recombination at present be studied on an unequivocally molecular level. The distinction of intramolecular recombination is significant because it limits the ambiguity of interpretation of the data, i.e., a number of (inconclusive) attempts have been made to organize and interpret genetic data by the analogy that long and short distances correspond to the chromosomal and molecular level, respectively (for review, see Pontecorvo, 1958).

In higher organisms, chromosomes are the gross functioning entities, and even in microbial systems it is necessary to deal with whole chromosomes (as for fungal crosses and bacterial conjugation) or else with whole bacteriophages which may contain more than one molecule of DNA. In conjunction with genetic evidence, recent data from electron microscopy suggest that the entire genome of coliphage T_4 is contained within one chromosome (Cairns, 1961), but this is not necessarily equivalent to one continuous DNA molecule. In phage transduction between bacterial hosts, the structural relationship between the transduced fragment and the phage genome remains equivocal, except in the case of λ dg phage (Morse, Lederberg and Lederberg, 1956 a,b). In contrast, purified pneumococcal transforming DNA, as extracted from cells, is composed of molecular entities and not structured biological organelles, such as a chromosome; no information is available on the organization of pneumococcal genetic material on the supra-molecular level in vivo.

Although linked pairs of markers occur within one particle of a multiply-marked DNA preparation, singly-marked transformants are recovered most frequently. Linkage is never complete, and even for the tightly linked high level sulfanilamide resistance loci ad, only half the d transformants are ad. It is possible to maintain, therefore, that entire particles are usually conserved during recombination, but that individual ones have been broken during purification or during entry into the cell before recombination; if such a breakage were random along particles, then the linkage frequency would still be proportional to distance because a scission would be more likely to separate loosely linked markers. The response of linkage frequency to the physiological state of the cell (Hotchkiss and Evans, 1958) shows that random breakage cannot be the only factor, but it is incapable of entirely ruling out the participation of breakage as one influence. In a three-factor cross, the omission of a central DNA marker from transfor-

mants marked for the outer loci can definitively demonstrate the occurrence of intramolecular recombination, if it can be shown that only one molecule of DNA is participating. It would then also substantiate the interpretation that linkage data on two-marker systems reflects similar intramolecular recombinational events.

The kinetic analysis of irreversible initiation of transformation to linked markers, as discussed in Section D-1, demonstrates that the interaction of one cell with one DNA particle is sufficient to give rise to either a singly- or multiply-marked clone of transformants. The time kinetic technique will now be extended to three-factor crosses in order to demonstrate complex, intramolecular recombinational events. However, the initiation of irreversible transformation in a cell by adsorption of one DNA particle cannot be interpreted to determine a single event within that cell. In other words, having entered the cell together (i.e., on one DNA particle) two linked markers could nevertheless recombine with the resident bacterial genome in two different (independent) events, separated in time as well as in space. Such successive rounds of transformation by an adsorbed DNA particle were not observed by Hotchkiss (1956), but have been suggested by certain findings of Ephrussi-Taylor (1960).

Moreover, it is known that adsorption of DNA neither is equivalent to nor does it guarantee genetic recombination and the production of transformant progeny. For example, it was mentioned earlier that DNA from heterologous sources such as calf thymus competes on an equal basis, by weight, with pneumococcal DNA for bacterial adsorption sites (see Section D-1). The irreversible fixation of P^{32}-labeled DNA, as defined by insensitivity to deoxyribonuclease treatment, presumably relates to some process occurring after surface adsorption; for a pneumococcal system, Fox (1957) and Lerman and Tolmach (1957) have shown that the total amount of P^{32} fixed is proportional to the yield of transformants to one marker. Lerman and Tolmach (1957) demonstrated, nevertheless, that Pneumococcus will irreversibly fix labeled E. coli DNA or pneumococcal DNA to the same extent. In studies on interspecific transformation in Hemophilus, Schaeffer (1958) has shown that the transformation yield for a single marker is lower if the DNA originates in another species; nevertheless, the extent of P^{32}-DNA-fixation by the same cell population is independent of the source of the labeled DNA. There-

fore, the physical "fixation" of DNA cannot be directly equated with (functional) genetic recombination.

If the observed, multiply-marked recombinants occurred after successive, delayed rounds of transformation, then mixed clones should be produced by linked markers simultaneously adsorbed, but successively recombined. The experiments on three-factor crosses reported in this section demonstrate that complex intramolecular recombinations occur regularly as a single event in time within one division period. The essential absence of interference and of segregation or heterozygosis in transformant progeny is shown for the intramolecular recombinations that occur in ordinary pneumococcal transformation.

Experimental Results.

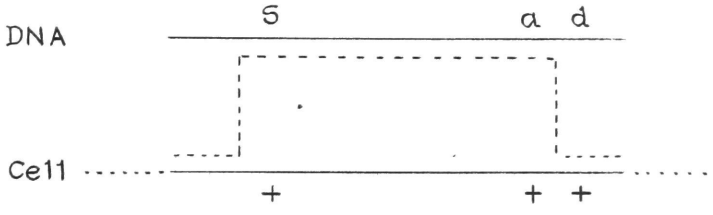
With the introduction of a three-factor cross, time kinetic experiments are further refined to determine the fate of the central marker of a linkage group under conditions when the outer or "bracketing" markers appear in transformant progeny as the result of transformations initiated by single DNA particles; such crosses have only occasionally been observed and have not been analysed previously in transformation experiments. If a three-factor array of markers is incorporated as a whole unit into transformant progeny, then the recombination may be described as a switch event between the smaller incoming DNA particle and the larger bacterial genome. If, on the other hand, the central marker is left out, then the recombinational event must be more complex and must involve a greater number of switches between the two entities. These situations are diagrammed in Figure 15 for the linear array Sad of the markers for streptomycin and sulfanilamide resistance. It is to be noted that the term switch does not imply any mechanism for genetic recombination but refers instead to the formal acquisition of genetic markers from a different genome.

Although the organization of the cell genome is less well understood than the structure of the incoming DNA particle, and one cannot determine the complexity of a recombinational event unambiguously, nevertheless, the schematic model of Figure 15 is useful in illustrating the difference in the events which occur in giving rise to the several types of transformants. If there is no interference, then "multi-switch" events, in which a central marker of a linear array is omitted from transformant progeny, should occur at a different frequency than "single switch" events, which include all the markers within the interval spanned. The reasoning here is identical to that used in calculating the number of multiply-marked transformants arising from random double events, i.e., the probability of co-occurrence of two independent events is taken as equal to the product of the individual probabilities. The occurrence of an even number of switches between any two loci would go undetected; since S and a are loosely linked, there may be opportunities for more switches in this interval than between the loci a and d, which are more tightly linked and presumably closer together.

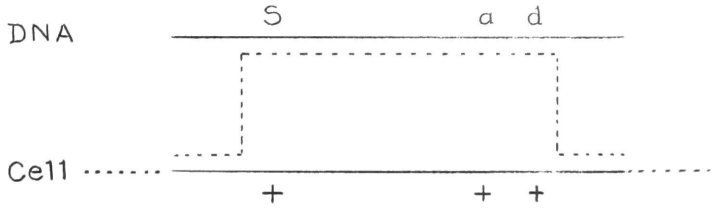
The terminology used in three-factor crosses includes the following symbols and conventions. The loci are listed in the genetic map order by

Figure 15. Diagrammatic representation of "switches" occurring during genetic recombination.

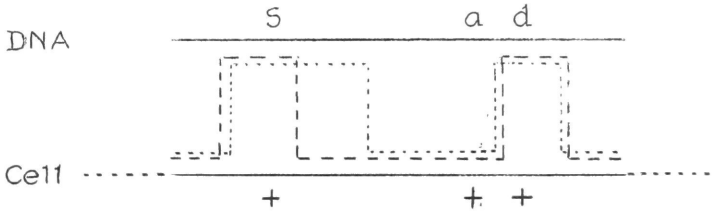
A. A cross-over event resulting in a Sa+ transformant.



B. A cross-over event resulting in a Sad transformant.



C. A more complex, or "multi-switch", cross-over event resulting in a S+d transformant of different phenotype and genotype: 2 alternatives.



the appropriate letter or by the + symbol to indicate the wild-type allele. Brackets enclose the symbols which represent the genetic constitution of recipient strains in transformation, e.g., (+++) refers to a wild-type strain. The donor strain from which DNA has been extracted is identified by underlining the appropriate symbols; Sad defines a DNA multiply-marked for resistance to streptomycin and to high-level sulfanilamide. The transformant progeny are represented by the same convention as the DNA so that the S+d type consists of those cells resistant to streptomycin and to low concentrations of sulfanilamide. In interpreting the data, it is essential to remember that many but not all types of transformants can be selectively scored. In addition to S progeny, these include a+, ad, and +d plus ad; all the d transformants (+d plus ad) can be scored in low sulfanilamide concentrations, but the frequency of +d progeny can be determined only by subtraction of the ad yield from the total d yield; therefore, the category designated as Sd includes both S+d and Sad cell types. In addition, the wild-type allele of the d locus (d^+) can be selectively scored.

In a transformation of a wild-type pneumococcal recipient (+++) by an Sad DNA, at saturating concentration at 30°C, all the linked multiply-marked transformant types which can be selectively scored accumulate linearly from the time of DNA addition (Figure 16); these types are Sa+, Sd, and Sad. In this system (Figure 16), the S+d transformant is the one resulting from a multi-switch recombination, but it cannot be singly selected. Its accumulation is estimated by subtracting the linear rate of accumulation of the Sad type from the rate observed for all transformants marked for both S and d. About 40% of those transformants in which the two outer markers appear fail to show the central marker phenotypically (Table IV). Over many experiments utilizing several different preparations of DNA and many cell populations, this ratio has ranged from 0.5 to 0.7. Figure 16 also reaffirms the fact that all types of linked transformants can result from the interaction of one DNA particle with one cell independently of the presence of intervening markers.

The direct demonstration of multiple switches requires that the resultant transformant be directly detected. Therefore, the experiment described above was repeated using a different allele configuration, namely an S+d DNA on a (+a+) recipient which yields an Sad genotype after a multi-

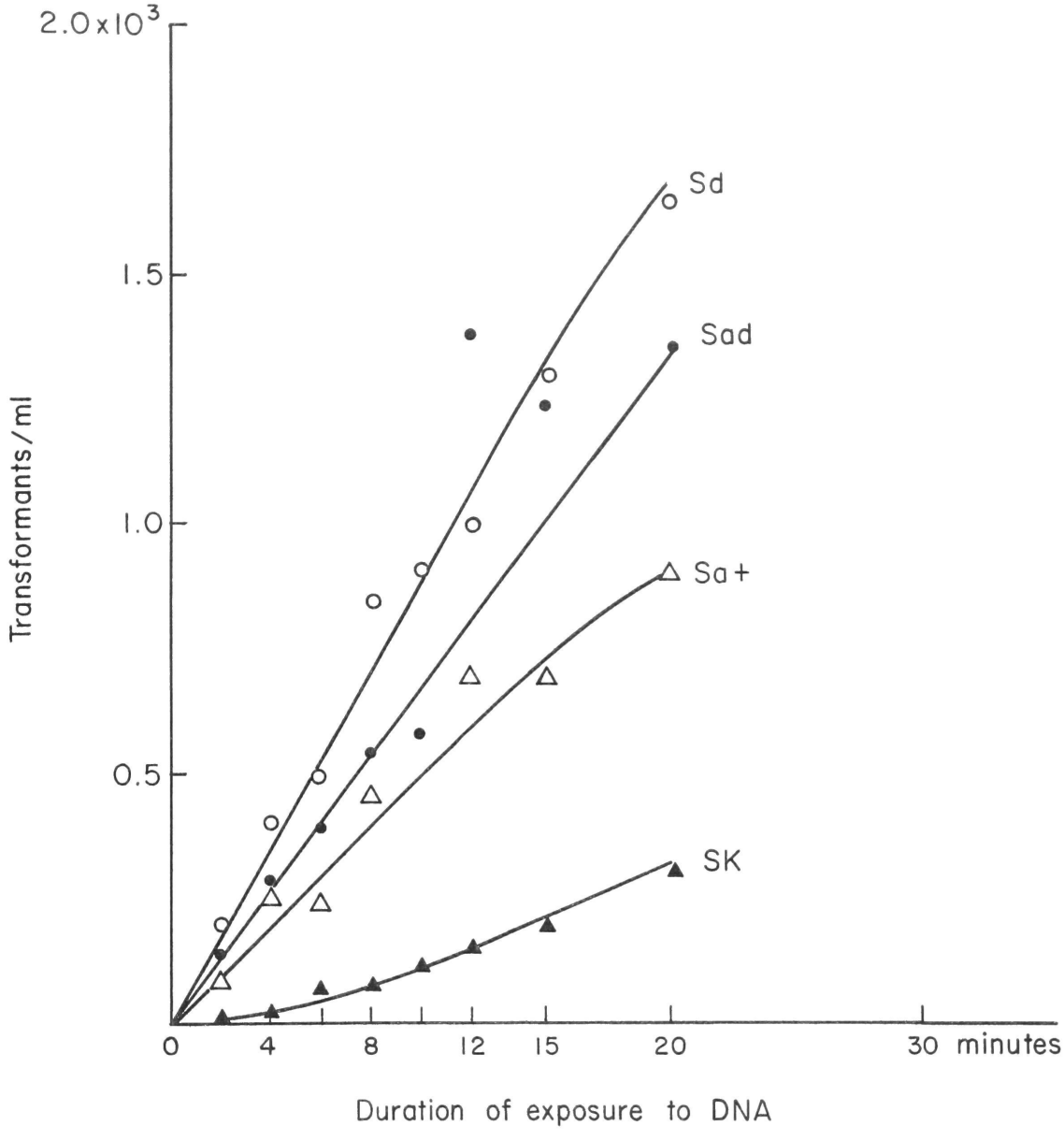


Figure 16. The linear rate of accumulation of multiply-marked transformants during exposure of a wild-type recipient strain to an Sad-DNA.

A concentrated competent culture (+++) at 4 x 10^7 viable count/ml was exposed to 0.2μg/ml DNA 264 at 30°C. At intervals after DNA addition, aliquots were removed to two volumes of expression medium, containing deoxyribonuclease, for 90 minutes growth at 37°C before selective scoring. The markers are: S - streptomycin resistance; K - micrococcin resistance; a+, d, ad - different and phenotypically distinguishable markers for sulfanilamide resistance.

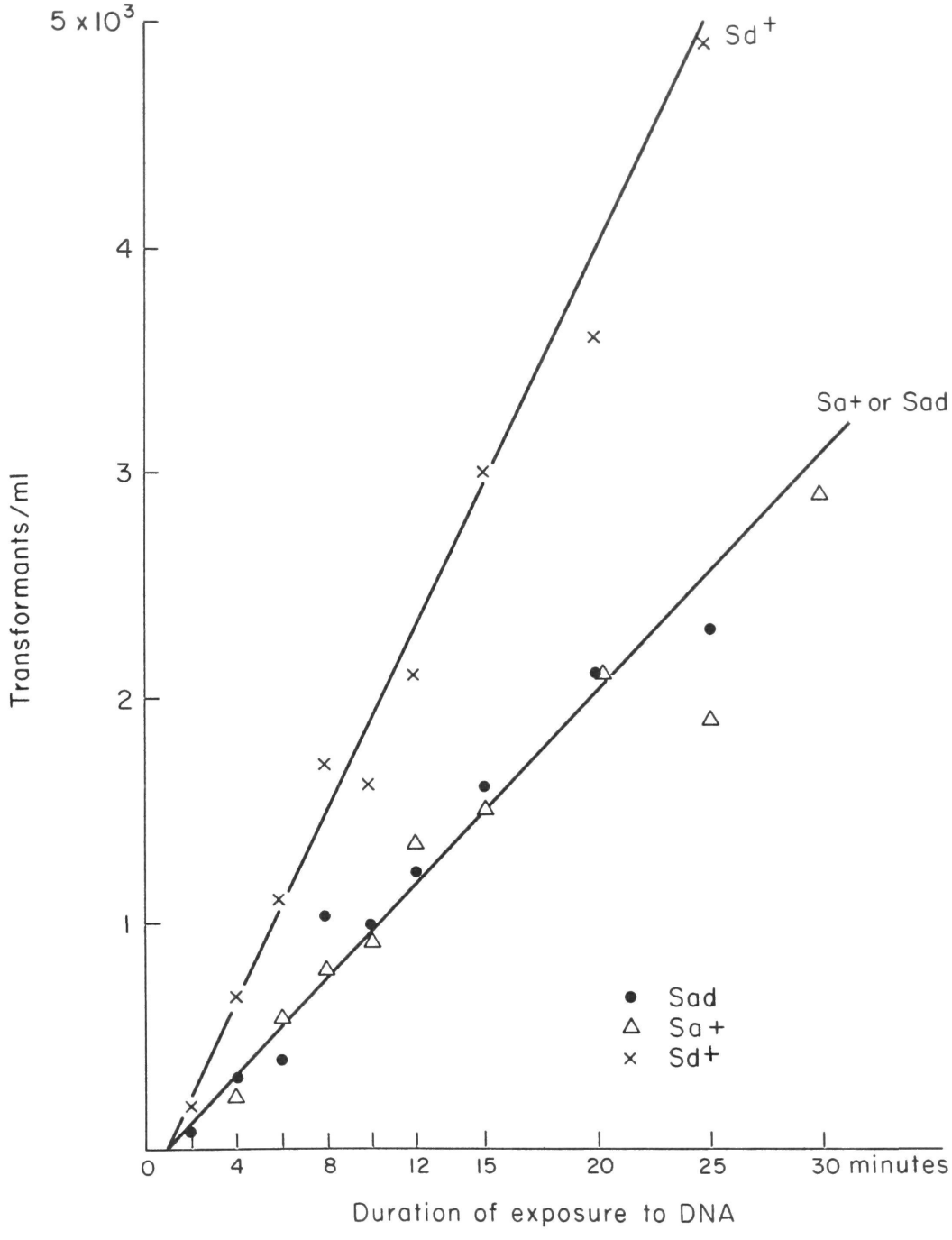


Figure 20. The linear rate of accumulation of multiply-marked transformants during exposure of (++d) recipient to Sa+-DNA.

The experiment was performed under conditions similar to those described for Figure 16. The markers are: S - streptomycin resistance; a+, ad - different and phenotypically distinguishable markers for resistance to sulfanilamide; d^+ - resistance to p-nitrobenzoic acid, an analog of sulfanilamide.

TABLE IV

Ratios of linear rates of accumulation of multiply-marked transformants.

Conditions of Experiment						Ratios of Observed Linear Rates				
DNA genotype	DNA preparation number	recipient genotype	temperature	genotype of multi-switch recombinant	selected phenotypes	Sd/S	ratio of selected phenotypes		frequency of "multi-switches" in Sd interval	
							with S	non-S	observed	calculated
Sad	237	(+++)	30°C	S+d	Sad;Sd	.05	0.75	0.5	0.25	0.35
			37°C			.045	~ 1	0.56	very small	0.35
	259		30°C				0.65	0.42	0.35	0.35
	264		30°C			.025	0.5-0.7	0.5	0.3-0.5	0.33
	281		30°C			.08	0.72	0.56	0.28	0.48
S+d	249	(+a+)	30°C	Sad	Sad;Sd	.02	0.65-0.80	0.5-0.7	0.65-0.80	0.29-0.49
S++	272	(+ad)	30°C	Sa+	Sa+;Sd^+	.03	0.53	0.36	0.53	0.36
Sa+	278	(++d)	30°C	S++	Sa+;Sd^+	.03	0.50	0.39	0.50	0.39

Ratios are formed from linear accumulation rates calculated as transformants/ml/minute. The absence of significant interference is shown by a comparison of the last two columns; the calculated fraction of multi-switch recombinations is computed on the basis of random, independent recombinations in each of the two intervals. Sd/S states the linkage frequency of Sd with respect to S. Each experiment was performed at a DNA concentration below that required to saturate the yield. The markers are: S - streptomycin resistance; a+, d, ad, d^+ - different and phenotypically distinguishable markers of a complex locus governing resistance to sulfanilamide.

switch recombinational event, Sad cells can be identified by selective scoring. In Figure 17 are shown the data for a typical experiment. The ratio of Sad:Sd ranges from 0.5 to 0.8 for many experiments utilizing different batches of transformable cells and both high and low DNA concentrations. By single colony analysis, it is shown that S+d transformants occur with a frequency of 20% to 30% among all transformants marked for both S and d (over 300 single colonies analysed). In addition, concentration kinetic experiments indicate that the recovery rates of Sad and Sd transformants are parallel throughout and both result from adsorption of single DNA particles (Table V; Figure 18).

For two allele configurations, therefore, it appears that multiple switches occur regularly and in high frequency relative to simpler recombinations within the interval spanned by the markers S and d. In all the other allele configurations possible for these three markers (with the exception that the streptomycin resistance marker is used in the donor strain for technical reasons), similar results are obtained (Table IV) for the pertinent types of transformants. The original data are graphically shown in Figures 19 and 20.

Although multi-switch recombinations seem to occur often in all allele configurations, nevertheless the frequency at which they occur is different for the various allele configurations, even though it is readily reproducible in replicate experiments of any one configuration. The value of the ratio Sad:Sd is the same for the first two experiments in Table IV, but the allele configurations change in such a way that Sad transformants represent single incorporations in one case and multi-switch recombinations in the other. Due to the reproducibility of data, experimental variables (such as state of a given DNA or cell preparation) cannot be invoked to explain this dilemma.

Suppose nascent recombinants in transformation to be heterozygous near regions involved in genetic recombination. If heterozygosis persisted or segregation to pure cell lines occurred during growth of the clone, then selection artifacts would grossly distort the outcome. Since all sulfanilamide drugs are bacteriostatic but not bactericidal, those cell types which arise -- by segregation several divisions later -- could still grow to form colonies in selective media. Alternatively, several rounds of transformation, separate in time, might be required to introduce discontinuous segments of

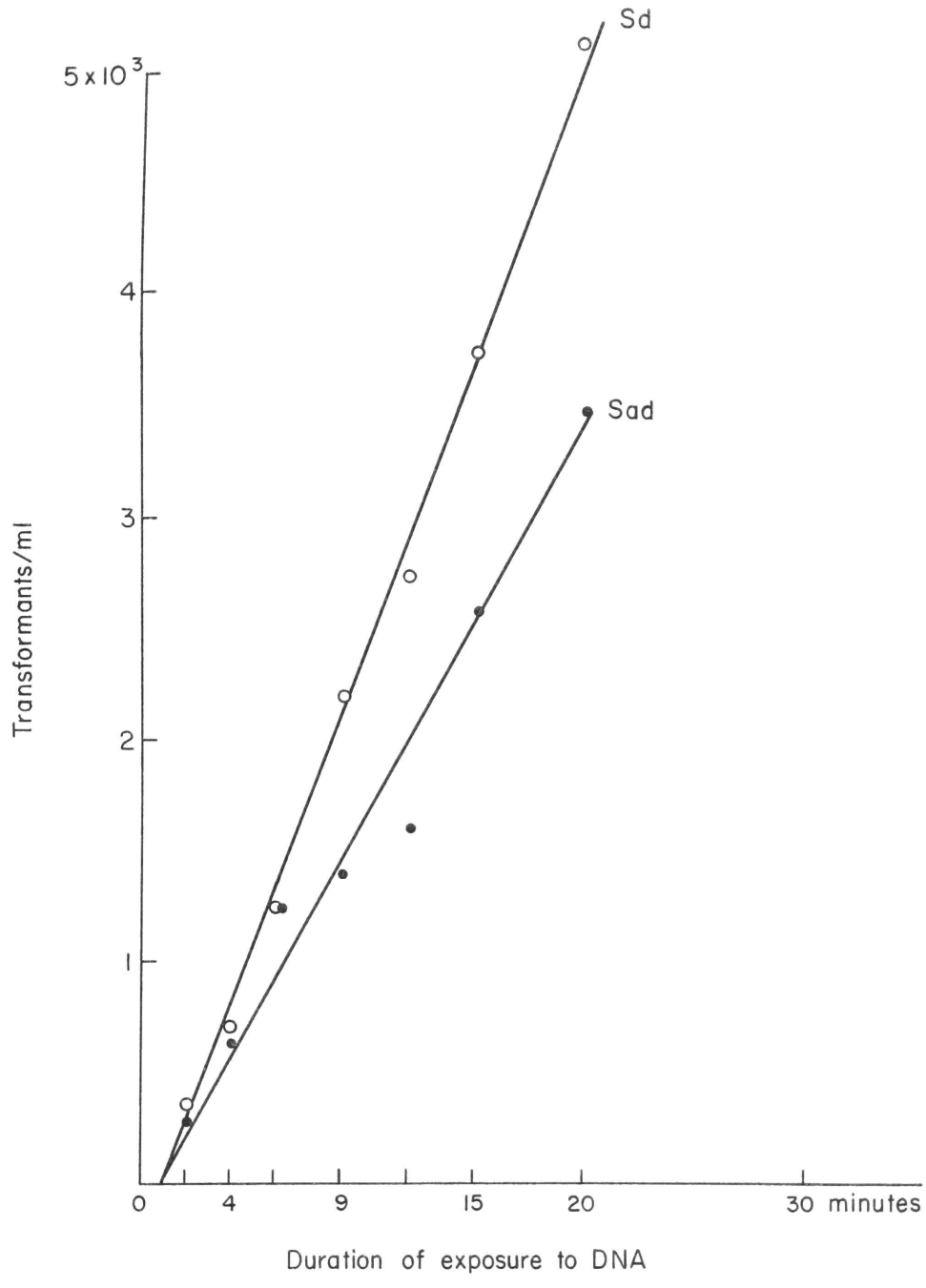


Figure 17. The linear rate of accumulation of multiply-marked transformants during exposure of (+a+) recipient to S+d-DNA.

The experiment was performed under conditions similar to those described for Figure 16, except that the (+a+) recipient strain was present at a viable count of 2 x 10^7/ml. The S+d-DNA, preparation 249, was added to a final concentration of 0.2μg/ml. The markers are: S - streptomycin resistance; d - low level sulfanilamide resistance; ad - a pair of markers controlling resistance to a high concentration of sulfanilamide.

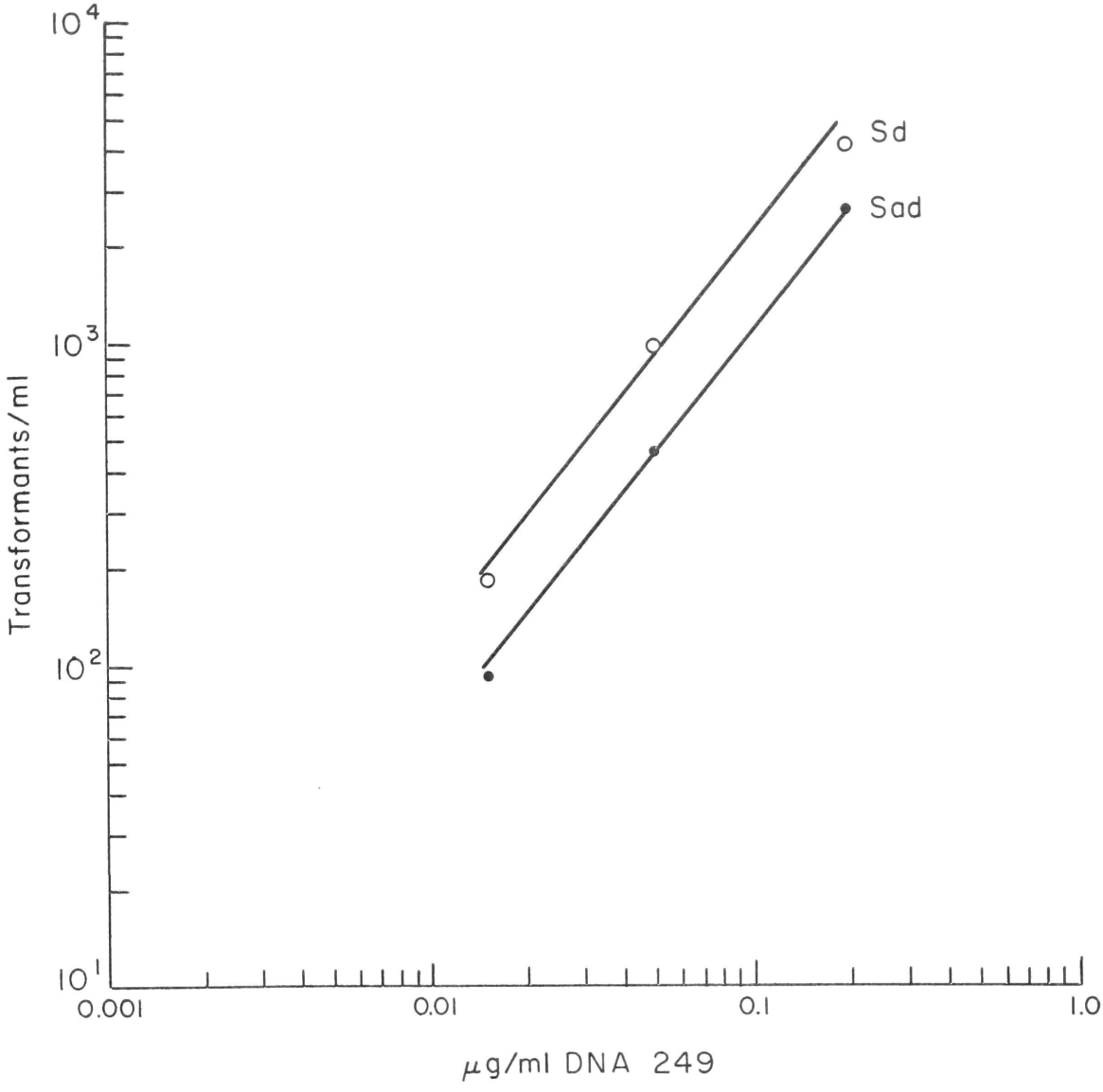


Figure 18. Titration curve for recovery of multiply-marked transformants at varying DNA concentration.

A competent culture of strain (+a+) at 2 x 10^7 viable count/ml was exposed to the indicated final concentration of S+d-DNA for 15 minutes at 30°C. The slope of each line is near one, and the two lines are approximately parallel. The markers are: S - streptomycin resistance; a and d - resistance to sulfanilamide.

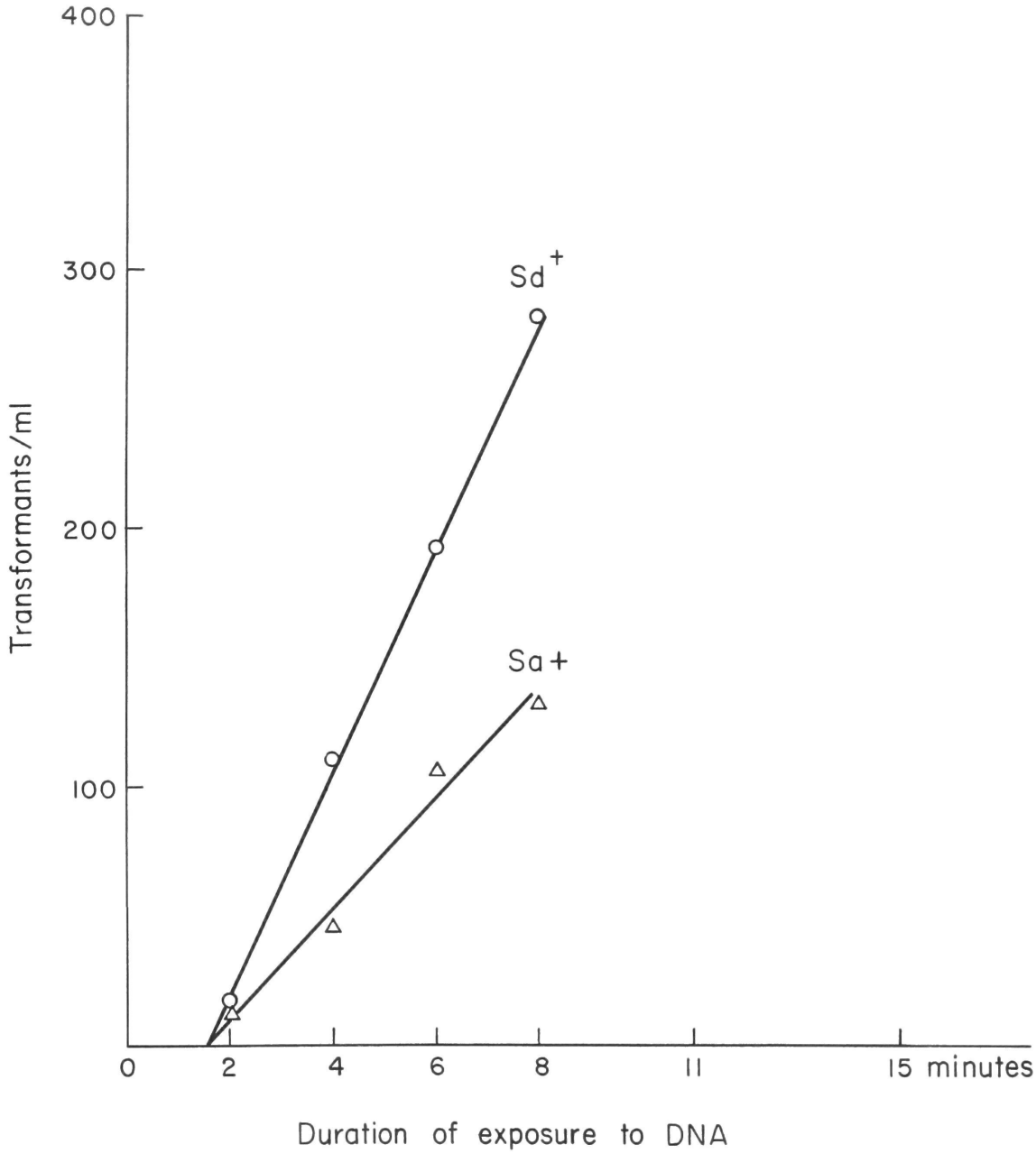


Figure 19. The linear rate of accumulation of multiply-marked transformants during exposure of (+ad) recipient to S++-DNA.

The experiment was performed under conditions similar to those described for Figure 16. The markers are: S - resistance to streptomycin; a+ - resistance to sulfanilamide; d^+ - resistance to p-nitrobenzoic acid, an analog of sulfanilamide.

TABLE V

Concentration dependence of recovery of transformant types after transformation of (+a+) recipient with S+d DNA.

DNA conc. μg/ml	S/ml	Sad/Sd
0.20	4.0×10^4	0.78
0.08	1.2×10^4	0.62
0.04	3.9×10^4	0.66
0.01	9.0×10^3	0.65
0.003	3.5×10^3	0.50

The culture, at a viable count of 2×10^7/ml, was exposed to DNA for 5 minutes at 30°C. The markers are: S - streptomycin resistance; d - resistance to low concentrations of sulfanilamide; ad - resistance to higher concentrations of sulfanilamide.

DNA particles into transformants. In either case, the data on rates of accumulation per transformant type would reflect not the events during a single genetic recombination but other subsequent phenomena. After bacteriophage infection, unstable partial heterozygotes are recovered, although infrequently for a given marker, and the length of the affected segment is similar to that over which high negative interference operates, as discussed in the introduction.

Clonal analysis of transformant progeny has been used to eliminate these alternative possibilities. The prediction was that segregation or delayed rounds of transformation would lead to mixed clones, whereas persistence of two alleles for one function would result in a clone of heterozygous cells. A heterozygous condition can be unequivocally demonstrated for the (ad/a+) or (a+/+d) genotypes since each component phenotype can be uniquely selected. If exposed to either type of selection, a clone of heterozygous cells would still grow equally well when inoculated into the other mutually exclusive medium. A mixed clone of several cell types, on the other hand, would be purified by a single selective passage.

Mixed clones were not found in an analogous experiment in pneumococcal transformation. Using DNA doubly-marked for the linked loci governing streptomycin resistance (S) and mannitol utilization (M), Hotchkiss (1956) examined individual unselected clones of transformants, which had been identified by the technique of replica plating. Since these clones were either pure M, pure S, or pure SM but never occurred as mixed clones of S cells and of M cells, Hotchkiss concluded that a single recombinational event had occurred to produce a multiply-marked transformant. Mixed clones, found after transformation to streptomycin resistance, consisted of resistant and sensitive types.

The question of early segregation of recipient cell types from a nascent transformant is complicated by the chain-like growth of pneumococci under the conditions used; the presence of recipient type cells in such a clone is readily attributable to growth of non-transformant partners in an original chain. After an acute, but brief selective challenge with streptomycin, only those cells having a resistant phenotype during the challenge survive to give rise to progeny. Hotchkiss (1956) has found that approximately 12% of the survivors give rise to colonies in which 25% to 50% of

the cells are streptomycin sensitive, indicating that the resident genome need not be eliminated from a transformant before the first division. Since the number of (haploid) nuclei in pneumococci could be greater than one, as is true for rod-shaped bacteria, e.g., for E. coli (Witkin, 1951), the recovery of mixed clones containing a recipient type cannot be used as unequivocal evidence regarding the fate of the two entities participating in genetic recombination. It should be noted that a mixed clone containing 50% of each of two cell types results from a segregation at the first division, whereas one with 25% of one of two cell types results from a segregation at the second division.

The first clonal analysis reported here was designed for the purpose of revealing either heterozygosity or segregation in clones of transformant progeny for the several genetic factors involved. After transformation of a (+a+) recipient with an S+d DNA under conditions of low DNA concentration and short duration of exposure to DNA (to ensure that the vast majority of multiply-marked transformants arise from single interactions between cells and DNA), the population is allowed to express its new genetic traits in non-selective medium. After a brief selective challenge with streptomycin (see below), single colonies are selected in medium permitting growth of +d and ad transformants but not of the (a+) background population. Each single colony is tested for type and purity of the clone by extensive plating in all the pertinent selective and non-selective media.

The efficiency of the selective challenge designed by Hotchkiss (personal communication) is based on the fact that low salt concentration increases the sensitivity of cells to streptomycin, presumably by altering the binding capacity of the cell surface for this very basic antibiotic. As seen in Table VI, essentially all the streptomycin sensitive population loses viability in a 10 minute exposure to appropriate levels of drug; the sample is immediately diluted a hundred-fold into fresh, high-salt medium to eliminate further drug effects, and the survivors are then selected in low concentrations of sulfanilamide. The results of two separate experiments, Table VII, show no evidence for mixedness in 159 clones analysed. With one exception, all clones are either pure S+d or pure Sad, and they contain streptomycin sensitive cells in undetectable frequency (less than about 30%). The single exceptional clone is a mixed clone and is puzzling

TABLE VI

Efficiency of acute selective challenge with streptomycin on a transformed population after expression.

Treatment	Viable count/ml	Transformants S/ml	% S	% S survival
Post expression (37°C)	7.0×10^5	1.2×10^3	0.2	100
Resuspension in low salt medium	5.5×10^5	1.2×10^3	0.2	100
Incubation 10 minutes				
37°C - no drug	6.2×10^5	1.4×10^3	0.2	100
37°C - 50μg/ml S	1.1×10^3	1.1×10^3	100	100

A competent population was exposed to S - DNA at 30°C and then allowed to express acquired resistance under standard conditions for 90 minutes at 37°C. The expressed culture was filtered on a millipore-filter and resuspended in an equal volume of low salt medium, which is the standard enzymatic casein hydrolysate medium diluted five-fold with distilled water. The sample was divided into two aliquots for incubation at 37°C in the presence or absence of streptomycin.

TABLE VII

Clonal analysis of sulfanilamide resistant survivors following acute streptomycin challenge, after transformation of (+a+) recipient with S+d DNA.

Experiment	Clones analysed	% pure S+d	% pure Sad	% mixed clones	Non - S clones (< 1% S)	Sad/Sd
1	20	30	70	0	6	0.70
2	139	30	70	.007*	10	0.70

A concentrated, competent culture at 4 x 10^7 viable count/ml was exposed to 0.02μg/ml DNA for 5 minutes at 30°C. The nascent transformants were allowed to complete expression at 37°C, before the acute selective challenge described in Table VI. Single colonies were selected on solid medium permitting growth of d and ad cells; all clones were subsequently tested for total cell count and then in media selective for S, d, ad, a+, Sd, Sad, Sa+ types (i.e., 7 media) with colony counts of several hundred for most fertile tests.

* This one clone was 10% S+d and 90% Sa+, after selection in medium which inhibited growth of a+ cells.

in that the predominant cell type, Sa+, is selected against in the medium used for isolation of the colony. The frequency of pure streptomycin sensitive clones is small enough to be neglected (2.7% in the larger experiment), and these clones are unmixed (less than 1% streptomycin resistant cells) so that there is no evidence for survival of a streptomycin resistance gene which could have contributed to a transiently resistant phenotype. In summary, the clones are neither grossly mixed with respect to S and a markers nor are their constituent cells heterozygous for the d marker to a significant extent under the conditions used. Clonal analysis of various types of sulfanilamide resistant transformants has indicated that less than 5% of the clones are mixed or otherwise aberrant (Hotchkiss and Evans, 1958).

Clones of heterozygous cells do occur after transformation, although rarely. In one experiment, after exposure of a wild-type recipient to very high concentrations of DNA at 37°C, sulfanilamide resistant transformants were selected in concentrations of drug so low as to inhibit growth only of background non-transformants. Clonal analysis of these transformants reveals rare heterozygous clones of the phenotype (a+/ad); these clones fall into two categories -- the first is stable to several selective passages in either medium yielding persistently equal cell counts in both drug-containing media, and the second is stable to selection in an a+ medium but unstable to selection in either d or ad medium, since after a single selective passage in either of these environments, the relative a+ titer is depressed more than a thousand-fold. These strains were not investigated further and are mentioned only to indicate that the detection methods are sufficient to reveal persistent heterozygosity at the d locus. The absence of persistent heterozygosity in the 159 single colonies analysed is an indication that multiple copies of the sulfanilamide resistance alleles are rarely, if ever, preserved after complex intramolecular recombinational events.

If one of the segregant types occurring after transformation were to have a selective growth advantage, that type could overgrow and obscure the presence of the other type in the fully-developed clone. To eliminate the possibility of segregation of nascent transformants during the 90 minute expression period at 37°C or during the first few divisions thereafter, a

premature plating experiment was performed. Aliquots of a transformed culture are plated in non-selective medium before complete expression has occurred and then allowed to complete expression in agar before addition of an overlayer of selective medium. The result is that the number and relative frequency of all the transformant types is independent of the time of transfer from liquid medium in which dividing cells separate. This is true both for a wild-type recipient population exposed to an Sad DNA (Figure 21) and for a (+a+) recipient population exposed to an S+d DNA (Figure 22), although the total population continues to increase exponentially in liquid medium during this time. Transformant progeny, therefore, neither segregate other types of cells nor increase in number during the expression period (Hotchkiss, 1957c). In addition, the time of onset of exponential growth and the rate of growth are approximately equal for all the types of transformant progeny in non-selective medium (Figures 21 and 22). This result is obtained by continued growth of the total population in liquid medium, without drug, after expression; in order to avoid exhaustion of the medium, the culture is diluted two-fold into fresh medium at intervals approximately equal to the doubling time. In addition, clonal analysis of 18 single colonies, plated 15 minutes after deoxyribonuclease treatment and selected, after expression, in streptomycin and very low concentration of sulfanilamide, revealed no mixed clones and no clones of cells heterozygous for sulfanilamide resistance markers.

It is, therefore, concluded that the ratios obtained by selective scoring of transformant progeny reflect the relative frequencies of genetic recombinational events which have occurred and not subsequent phenomena of heterozygosity or segregation among the nascent transformants. Furthermore, these complex recombinational events occur over a time interval which is less than one division time -- the latter being the lower limit of resolution of clonal analysis. Using a +d DNA as donor and an (a+) recipient, Fox and Hotchkiss (1960) examined the appearance of ad linkage in DNA extracted from the recipient strain after transformation. As measured by the biological activity of the DNA upon re-isolation from cell populations at increasing time intervals after deoxyribonuclease treatment, intramolecular recombination for a single marker has a half-time of about one-quarter the division period under similar conditions; the resolution by clonal analysis is thus of the same order of magnitude.

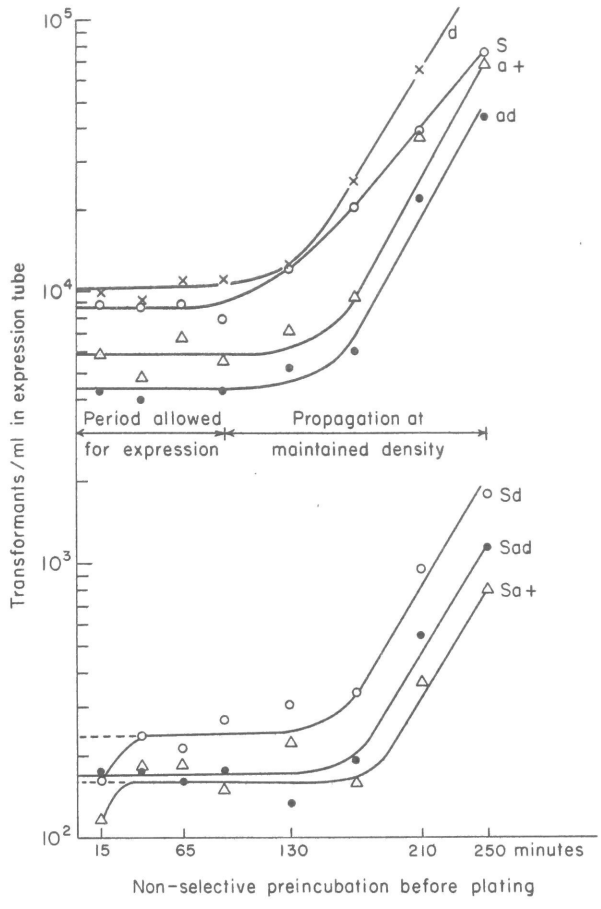


Figure 21. Increase in transformant titer during growth in non-selective medium following exposure to DNA.

A concentrated competent culture of a (+++) strain at 4 x 10^7 viable count/ml was exposed to 0.2μg/ml Sad-DNA for 5 minutes at 30°C. The entire sample was then diluted into two volumes of prewarmed expression medium (37°C), containing deoxyribonuclease; termination of DNA exposure is denoted as zero time. At intervals thereafter, appropriate aliquots of the expressing culture were plated in non-selective solid medium and allowed to complete expression. After a total elapsed time of 90 minutes in non-selective liquid and solid medium, an overlayer of drug-containing agar medium was added to each plate to allow selective scoring during overnight incubation. After a total elapsed time of 90 minutes in liquid medium, the remaining sample was diluted two-fold every 40 minutes in fresh growth medium. In this pseudo-chemostat, the transformants began to divide, and their replication during this period was measured by selective scoring in solid medium. The markers are: S - resistance to streptomycin; a+, d, ad - different and phenotypically distinguishable markers for sulfanilamide resistance.

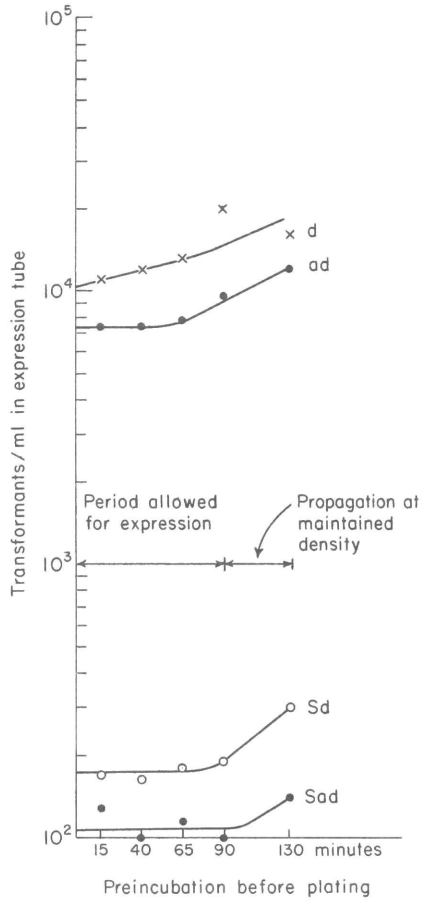


Figure 22. Increase in transformant titer during growth in non-selective medium following exposure to DNA.

A competent culture of a (+a+) strain was exposed to S+d-DNA. The experiment was otherwise identical to that described for Figure 21.

Variations observed in the ratio of frequencies of transformant progeny arising from multi-switch and simpler recombinations are perhaps attributable to the alleles themselves; each allele represents a specific structure in terms of purine and pyrimidine bases. Alteration in the degree of homology between recipient and donor genome over small regions or at specific sites could affect synapsis and/or recombination. In fact, "marker effects" -- defined as the modification of recombination frequencies by reshuffling of allele configurations -- have been reported in numerous experimental systems in microbial genetics. These include transduction analysis for fine structure mapping in Salmonella (Demerec, Goldman, and Lahr, 1958; Balbinder, 1962), and recombination in the tryptophan synthetase region of N. crassa (Kaplan, Suyama, and Bonner, 1962). In fine structure studies on the erythromycin resistance locus in a pneumococcal transformation system, Ravin and Iyer (1962) concluded that recombination frequencies in two-factor crosses are definitely altered by the presence of a third closely linked marker (also governing erythromycin resistance) in the genome of either recipient or donor cells. Direct evidence for a highly localised "marker effect" is found in the tryptophan synthetase system as studied by conjugation between E. coli strains (Helinski and Yanofsky, 1962). Two independent mutants recombine very rarely with one another to form wild-type progeny, and each mutation results in a single, though different, amino acid replacement at the same position within one protein. These two mutations are, therefore, very closely linked (within several nucleotide pairs perhaps), and each represents an alteration in a region near the size range of a single base pair. Nevertheless, each mutant gives distinctly different recombination frequencies with respect to neighboring loci.

In studies on inter-specific transformation, in Hemophilus, the recovery of streptomycin resistant transformants is substantially lower, if donor and recipient are of different species; if DNA is isolated from such a transformant and subsequently assayed for the same marker on the original recipient strain, the yield of streptomycin resistant transformants is restored to the value obtained for intra-specific systems. This result is explicable on the basis that 1) a greater degree of genetic and structural homology exists within strains of one species than between different species of the same

genus and 2) that the degree of homology, over a segment larger than the mutated region, influences the probability of recombination within the marked region (Schaeffer, 1958).

With the conclusion that the frequencies of transformant progeny recovered reflect the actual frequencies of genetic recombinational events, the data can be analysed for the occurrence of interference. Compare linkage frequencies between the two sulfanilamide resistance markers when the S marker is present and when it is absent (Table IV). These linkage frequencies are in the range of 50% for the sulfanilamide markers in all experiments but do vary with the allele configuration. The ratio of the appropriately marked transformants for streptomycin plus sulfanilamide resistance does not always vary in the same direction away from 50% within one experiment. Thus, the presence of a relatively distant S marker undergoing recombination with the bacterial genome may modify the probability of recombination between two sulfanilamide resistance markers located in the same molecule and involved in the same genetic recombination (or vice versa), but the effect is very small. If the frequency of multi-switch to simpler recombinations over the interval S to d is calculated on the basis of independent random events with no interference, the value agrees with the measured ratio within a factor of two; the excess or deficiency of multiple recombinations may be two-fold or less (Table IV). Since the effect is so small, interference is not significant in extent, and each of the two or more intramolecular recombinations happens essentially independently of the other. Even-numbered clusters of switches within the intervals would not register in this analysis. Neither interference in recombinational events nor heterozygosity among the transformant progeny are significant on the molecular and intramolecular level in this genetic system. On the basis of these experiments, it is also concluded that submolecular recombinational events, occurring within the cellular environment, determine the linkage frequencies between markers, rather than pre-existing variations in the length of DNA particles. The substitution of an exogenous DNA particle for its homolog within the resident genome cannot be invoked as the principal model for the mechanism of genetic recombination during transformation.

D-3. THE EFFECT OF EXPERIMENTALLY INDUCED MODIFICATIONS OF DNA STRUCTURE ON LINKAGE GROUP BEHAVIOR

Introduction.

With the development of a time kinetic procedure to determine and to measure linkage frequencies between markers located within a DNA particle (D-1), and with the discovery that complex, intramolecular recombinational events occur both frequently and regularly (D-2), it becomes possible to exploit certain unique features of the transformation system in studying the problem of genetic recombination. These items have already been delineated in Section B. It must be re-emphasized that genetic events (and frequency data) respond to such subtle factors as the physiological state of the competent cells or the allocation of alleles in the neighborhood of measured recombinational events, as well as to more classical features such as the (physical) distance between markers. Nevertheless, it is possible to standardize the "bio-assay" (i.e., conditions of transformation) to control most of these variables in order to examine effects created by the *in vitro* modification of pneumococcal DNA. Since inactivations by heat are interpretable on a molecular basis, the changes in recombination frequency data for linked markers and for three-factor crosses can be correlated with structural alterations in the pretreated DNA particles. Direct evidence on the response of recombination frequencies to the structure of genetic material derives from genetic studies performed on microbial systems after the DNA has been modified *in vivo* by physical or chemical means such as ultraviolet irradiation, P^{32} decay in labeled DNA, or 5-bromouracil incorporation.

Irradiation with ultraviolet light has been shown to increase recombination frequencies (i.e., decrease linkage) between linked markers in the conjugation system of *E. coli* (Jacob and Wollman, 1955a; 1961, p. 237) and in bacteriophage multiplication in *E. coli* (Jacob and Wollman, 1955b; 1961, p. 237). For the mating system, either the HFr (male donor) or F^- (female recipient) bacteria can be irradiated for an increase in recombination frequency (Jacob and Wollman, 1961, p. 238). This increase is shown by two different types of experiments. First, with increasing dosage of irradiation

of the HFr parent, the recovery of multiply-marked recombinant progeny decreases more rapidly than can be accounted for by the sum of the individual inactivation rates of the component selected markers or by decrease in the length of genome transferred to the female. Second, examination of recombinant progeny selected for late entering markers of the HFr genome reveals that the frequency with which unselected, early markers appear in these recombinants is decreased several-fold by the irradiation. The increase in recombination frequency occurs after irradiation of either parental strain, so that mere inactivation of markers does not explain the results adequately.

In recombinational events occurring after mixed infection of E. coli, with two genetically distinct strains of bacteriophage λ, ultraviolet irradiation can increase recombination frequencies three- to five-fold relative to control values for a pair of markers (Jacob and Wollman, 1955b). Not only is the increase proportional to the ultraviolet dose incident upon the phage before infection, but the rate of increase is a function of the distance between markers such that the more loosely linked (i.e., farther apart) the markers the greater the linear rate of increase in recombination frequency per unit dose. Furthermore, by examining the clonal distribution of recombinants released from individual bacteria after mixed infection with two phages differing by three markers, it is seen that recombinational events occur unusually early during exponential phage growth after irradiation of one parental type, so that the recombinants form an exceptionally high fraction of the bacteriophage particles recovered from infection of a single bacterium. Ultraviolet irradiation also introduces a "polarity" into the recombinational events because, after irradiation, most of the double recombinants contain only the central of three linked markers from the irradiated parental type.

Recently, impressive evidence has accumulated which suggests that one of the important effects of ultraviolet irradiation on living systems is the formation of thymine-thymine photo-dimers along polynucleotide strands (Beukers and Berends, 1960; Beukers et al, 1960; Johns, Rapaport and Debruck, 1962). These photo-dimers have been isolated from DNA after irradiation either in vivo or in vitro (though not from unirradiated controls). The dimer yield increases with dosage (Wacker et al, 1960; Wacker et al, 1961), and photoreactivation of DNA either by ultraviolet light of the ap-

propriate wave-length (Setlow and Setlow, 1962) or by enzymatic photocatalysis (Rupert et al, 1958; Rupert, 1962) partially restores biological activity of Hemophilus DNA and also results in the disappearance of the photo-dimers (Wulff and Rupert, 1962). The effects of ultraviolet irradiation on bacteriophage λ recombination are completely photoreversible under appropriate conditions (Jacob and Wollman, 1955b). It is, therefore, not unreasonable that the effects of ultraviolet irradiation on genetic recombination reflect alteration in DNA structure resulting from the formation of thymine-thymine dimers within polynucleotides.

Another method by which recombination frequencies can be increased in the E. coli mating system utilizes crosses in which the male parent has been grown with radioactive phosphorus (Jacob and Wollman, 1961, p. 238). Shortly after mating with an unlabeled recipient, the radioactive zygotes are frozen and aliquots stored for various periods of time to allow decay of P^{32}. When P^{32}-decay is allowed to occur in such frozen zygotes before selective plating, data are obtained similar to those found after ultraviolet irradiation of the parental strains, i.e., recombination frequencies between markers are increased. The rates at which various unselected markers begin to disappear from recombinant classes increases as a function of their distance from the selected markers. Radioactive disintegration of P^{32} within the sugar phosphate backbone substitutes a sulfur atom for the naturally-occurring phosphorus, but the interpretation of these experiments in terms of changes in DNA structure is somewhat complicated by the possibility that back-bone scission (Levinthal and Davison, 1961), concomitant with the decay, interrupts continuity of polynucleotide strands.

Again in the E. coli mating system, Folsome (1960) has found that incorporation of the thymine analog, 5-bromouracil, into the genome of HFr male strains prior to mating with normal females results, among other effects, in increased recombination frequencies. The increase is measured by clonal analysis of recombinants which have previously been selected for a distal marker. Although the effect is smaller than that after ultraviolet irradiation (i.e., approximately two-fold increase in recombination frequency), it is in the same direction and responsive to dose. The analog is specifically incorporated into DNA by growing cells to replace thymine (Dunn and Smith, 1954; Zamenhof and Griboff, 1954) and such DNA can still

act effectively as genetic donor in transformation (Ephrati-Elizur and Zamenhof, 1959). However, studies on the influence of bromouracil substitutions on replication of DNA *in vitro*, utilizing the Kornberg enzyme, suggest that the incorporation of this analog into primer DNA may affect not only base-pairing on opposite strands during replication but also nearest neighbor frequencies (Trautner, Swartz, and Kornberg, 1962; see also Shapiro and Chargaff, 1960). The effects of analog incorporation into DNA *in vivo* need not be precisely limited to substitution at the site of a thymine base residue but may spread -- upon biological replication -- to rather extensive alterations in base sequence in the vicinity of the initial substitution. Regardless of a diffuse response, however, it seems clear that a general increase in recombination frequency over the entire genome is correlated with the alteration of DNA structure. Treatments such as thymine starvation (or growth in 5-fluorouracil, which inhibits thymine synthesis) might yield similar results. The biological activity of transforming DNA extracted from bacteria undergoing thymine starvation seems to be unusually sensitive during standard purification procedures (Mennigmann and Szybalski, 1962) which may reflect pre-existing lesions.

The response of recombination frequency for linked marker systems to *in vitro* degradation of transforming DNA has been but little examined previously. Subcritical heat inactivation, the first method, has been discussed in Section D-1, where it is demonstrated that localized lesions are formed within DNA particles perhaps by the mechanism of depurination suggested previously by other workers. Single lesions inactivate single markers but not other loci within the same DNA particle, and therefore such heating does not degrade entire DNA particles. If the fate of two-and three-membered linkage groups is followed in the course of inactivation, then this experiment could indicate whether a small lesion along a polynucleotide strand (simulating a "hole" in the base array, if the mechanism is indeed depurination) can affect recombination frequencies on either side of the lesion.

The second method, critical heat denaturation or melting, is very different both in mechanism and effect, resulting in a gross disorganization of secondary structure within the molecule due to disruption of hydrogen-bonding. The loss of biological activity is rapid (occurring in minutes rather than in the hours required for subcritical inactivation under similar

conditions) and takes place abruptly at a critical temperature of about 88°C to 92°C under the conditions used -- over a range of 0.4°C for a given marker. The yield of transformants is thereby reduced to between 1% and 10% the initial value for single markers. The transition is analogous to a second-order phase transition and is hence referred to as "melting" (Doty et al, 1959; Marmur and Lane, 1960; Roger and Hotchkiss, 1961). It is influenced by ionic strength of the heated mixture and by the concentration at which the nucleic acid is heated (Roger and Hotchkiss, 1961; in contrast to Ginoza and Zimm, 1961), both typical polyelectrolyte effects. The following physico-chemical changes in preparations of DNA which cause the loss of biological activity upon heat denaturation may all be cited as evidence for a collapse of secondary structure: 1) strong hyperchromicity at 260 mμ (Gulland, Jordan and Taylor, 1947; Hotchkiss, 1957b; Thomas, 1954), 2) a decrease in the radius of gyration of the particle, but not in the molecular weight, as determined by light-scattering measurements (Rice and Doty, 1957), 3) a decrease in relative viscosity to about 10% the initial value (Zamenhof et al, 1953; Rice and Doty, 1957; Creeth, Gulland and Jordan, 1947), 4) an increase in buoyant density detected in CsCl density gradient centrifugation (Meselson et al, 1957; Meselson and Stahl, 1958).

No evidence against this hypothesis has been presented in the literature. Two important questions pertinent to genetic studies remain, however: the structure of the molecules which carry the residual activity of the denatured sample, and (somewhat of a corollary) the issue of complete separation of the two polynucleotide strands of the double helix as a result of melting out the hydrogen-bonded secondary structure.

The residual biological activity of a denatured sample of DNA appears to reside in those particles which have survived both melting at elevated temperature and rapid cooling to 0°C with enough structural integrity to interact with a cell population (Roger and Hotchkiss, 1961). The percent residual activity is not due to a small heat-resistant fraction of unchanged molecules, since it is unaffected by higher temperatures (e.g., boiling) which can be up to 30°C above the critical temperature under appropriate conditions of low ionic strength (Roger and Hotchkiss, 1961). Furthermore, the percent residual activity is similar for all single markers tested, including several unlinked markers each situated on a different particle

(Roger and Hotchkiss, 1961), and hence the residual yield is independent of particle type insofar as the latter reflects structure. Since the residual yield is concentration dependent (Roger and Hotchkiss, 1961; Schildkraut et al, 1961), the loss of biological activity cannot be attributed uniquely to chemical degradation during brief exposure to critical temperatures. On the basis of alkaline titration, Vinograd et al (1963) suggest that hydrogen-bonding is partially but not completely disrupted by heat denaturation at neutral pH, although they cannot determine the degree of specificity involved in this bonding. In addition, the corrected hyperchromicity at 260 mμ is much lower for denatured DNA if measured at room temperature than if measured at the ambient critical temperature, indicating that some (specific or non-specific) base bonding is re-established upon cooling after heat denaturation (Doty et al, 1960).

Lerman and Tolmach (1959) demonstrated that the decrease in biological activity of P^{32}-labeled pneumococcal DNA parallels the decline in isotope fixation by a competent cell population, after critical heating. In this series of experiments, moreover, the loss of biological activity was much more sensitive than isotope incorporation to chemical degradation by e.g., ultraviolet light or X-ray inactivation, deoxyribonuclease digestion, nitrogen mustard treatment, and subcritical-type heat inactivation. These experiments were interpreted to indicate that denatured DNA particles penetrate cells poorly, if at all, whereas degraded ones can penetrate relatively well but are inactive in biological transformation for the affected regions.

Lerman and Tolmach (1959) observed the changed adsorption of denatured DNA by comparing the relative changes in transformation yield and in P^{32}-fixation from labeled pneumococcal DNA after heat inactivation and clearly recognized that heat denaturation affects DNA penetration (as defined by irreversible isotope fixation) more radically than does heat degradation; however, these authors felt that DNA particles "largely or entirely" denatured cannot be adsorbed by cells. Therefore, they attributed the residual biological activity, which survived exposure to 95°C, to a small fraction of relatively intact, fully active particles within a population consisting largely of denatured, inactive particles. Having shown that the residual biological activity can survive autoclaving under conditions supra-critical even for pure guanine-cytosine regions and referring to the above decline

observed in isotope fixation after denaturation, Marmur and Lane (1960) decided that the low residual activity resided within those particles in which separated strands had specifically "reformed" during rapid cooling. They did not examine or discuss the structure of these particles relative to those in unheated preparations. The evidence offered by Roger and Hotchkiss (1961) was that the biological activity of denatured DNA was an intrinsic property of collapsed molecules, correlated with their loss of affinity for the cell surface, and therefore measured either by loss of transforming activity or of ability to compete with intact DNA for the cells.

To a discussion of the issue of strand separation during denaturation must be introduced the discovery made by Marmur and Lane (1960; Marmur and Doty, 1961), that the percent of biological activity which survives critical heat denaturation depends on the rate at which the sample is then cooled. For example, if rapid chilling of an aliquot of "melted" DNA in an ice-bath results in a residual yield of 5%, then slow cooling of an identical aliquot (e.g., 2 hours to reach room temperature) can yield 50% recovery of the initial activity to a single marker (Herriott, 1961). This recovery of yield, termed renaturation (Marmur and Lane, 1960), is accompanied by changes in the degree of "hyperchromicity" and in buoyant density in CsCl towards values observed for intact DNA (Doty et al, 1960; Schildkraut et al, 1961). In conjunction with the increased biological activity of the renatured samples, the above data are now interpreted to reflect an increase (but not necessarily a complete return) in the degree of secondary structure (helicity) in the population of particles. Renaturation should increase the affinity of denatured DNA for adsorption to the bacterial surface or significantly increase the number of fully active particles, depending on the source of biological activity in denatured samples.

On the basis that renaturation tends to restore structure to denatured polynucleotides (thereby increasing biological activity), it seems possible that renaturation of a mixed sample of two genetically distinct DNA preparations derived from the same biological species could result in the formation of hybrid helices in which each strand originates from a differently marked molecular species; this could occur if denaturation had caused a significant separation of strands in the original double helices. The principal physico-chemical data in favor of strand separation come from experi-

ments in which isotopically labeled hybrid E. coli DNA (purified, Schildkraut et al, 1961; or unpurified lysates, Melson and Stahl, 1958) is centrifuged in CsCl density gradients before and after denaturation by heat, formamide (Marmur and T'so, 1961) or acid-base titration (quoted in Schildkraut et al, 1961). This DNA is a hybrid structure bearing N^{14} and N^{15} in separate and distinct strands and resulting from a single semi-conservative replication after change of isotope in the growth medium. Since heat denatured DNA has a different buoyant density from intact DNA, the position of the DNA peak in the gradient shifts after denaturation (lysates, Meselson and Stahl, 1958; purified DNA, Schildkraut et al, 1961). The occurrence of strand separation is inferred from the appearance of two new bands after denaturation (one for N^{14} strands and one for N^{15} strands), each wider than the initial band which indicates a decrease in molecular weight (Meselson and Stahl, 1958). However, Meselson and Stahl also report (1958) that parallel experiments on unlabeled salmon sperm DNA result in an increase in density but no increase in band width; they conclude that this DNA collapses upon heat denaturation but does not decrease in molecular weight; i.e., perhaps the strands do not separate. In more recent experiments, Vinograd et al (1963) do not see definite evidence for strand separation after heat denaturation of non-labeled DNA. An additional observation which can be interpreted in favor of strand separation during denaturation is that the extent of renaturation is dependent on the concentration of DNA in the solution during slow cooling (Marmur and Lane, 1960). The issue of strand separation remains open, but a more useful form of the question might be: if strands have separated during denaturation, then will they rejoin during renaturation and will the pairing of strands be specific under these conditions.

A sample of a renatured mixture of DNA's might exhibit a unique biological activity, since genetic linkage between markers initially present in separate DNA molecules could be created if 1) strands separate during denaturation but rejoin during renaturation and if 2) both strands of the helix are active in genetic recombination. This experiment (suggested by Marmur and Lane, 1960) was attempted for the Hemophilus transformation system by Herriott (1961) who has reported that a slight genetic linkage between the closely linked genes for resistance to streptomycin and cathomycin (about 30%-50% linkage when present within one genome) arises after renaturation of

an equal mixture of singly-marked DNA's. Herriott concludes, from a titration experiment, that only one annealed unit is necessary to initiate the double transformation; the recovery of singly- and doubly-marked transformants depended in the same way on total DNA concentration (see Section D-1, Figure 1 vs. Figure 2). In view of the low yield reported and some doubt whether the doubly-marked entity was an annealed heteroduplex molecule or an aggregate, it seemed worthwhile to re-examine this question. A similar experiment is reported for the pneumococcal system, utilizing the Sad linkage group together with the unlinked marker K to examine the behavior of the renatured sample by the time kinetic technique.

Experimental Results.

Part 1: The response of recombination frequencies to modifications of the assay system.

Complex recombinational events, as seen in three-factor crosses, can be demonstrated for both freshly-grown and frozen batches of competent cell populations. In Figure 23 are shown data obtained in a time kinetic experiment on a freshly-grown pneumococcal population, synchronized by cold shock prior to exposure to Sad DNA; the two parallel aliquots were transferred to 30°C (Figure 23A) and to 37°C (Figure 23B), respectively. Although the linkage frequencies of Sd/S and of Sa/S are not significantly affected by the temperature shift (see Table IV, Section D-2), the complex recombinational events essentially disappear at 37°C as the recovery of Sad transformants approaches very close to the total Sd yield; under very similar experimental conditions, the ratio of ad/d is 1 (Hotchkiss and Evans, 1958). Nevertheless, at 30°C, the ratio of Sad:Sd is about 0.75 which is comparable to the data previously presented for frozen cell populations (Section D-2, Table IV). It seems, therefore, that the occurrence of complex recombinational events may be very sensitive to (unknown) physiological variables. Since these complex events can be eliminated by changing only the state of the cell population and not the DNA preparation, it becomes impossible to attribute their occurrence to the (poor) quality of a DNA preparation. It is necessary to emphasize this point in order to interpret data obtained with degraded DNA samples.

It should also be noted that the map order Sad for Pneumococcus -- which is unambiguously dictated by the data obtained at 37°C (Hotchkiss and Evans, 1958) -- cannot always be so determined in similar experiments performed at 30°C with the same DNA preparation, since the ratio Sa:Sd may approach unity (see, however, Table III).

Part 2: The effect of subcritical heat inactivation of DNA on the behavior of genetic linkage groups.

When a multiply-marked DNA is subcritically heated for progressively longer periods of time, transformation bio-assay of partially inactivated aliquots reveals multi-hit inactivation curves (Figures 24 and 25). The

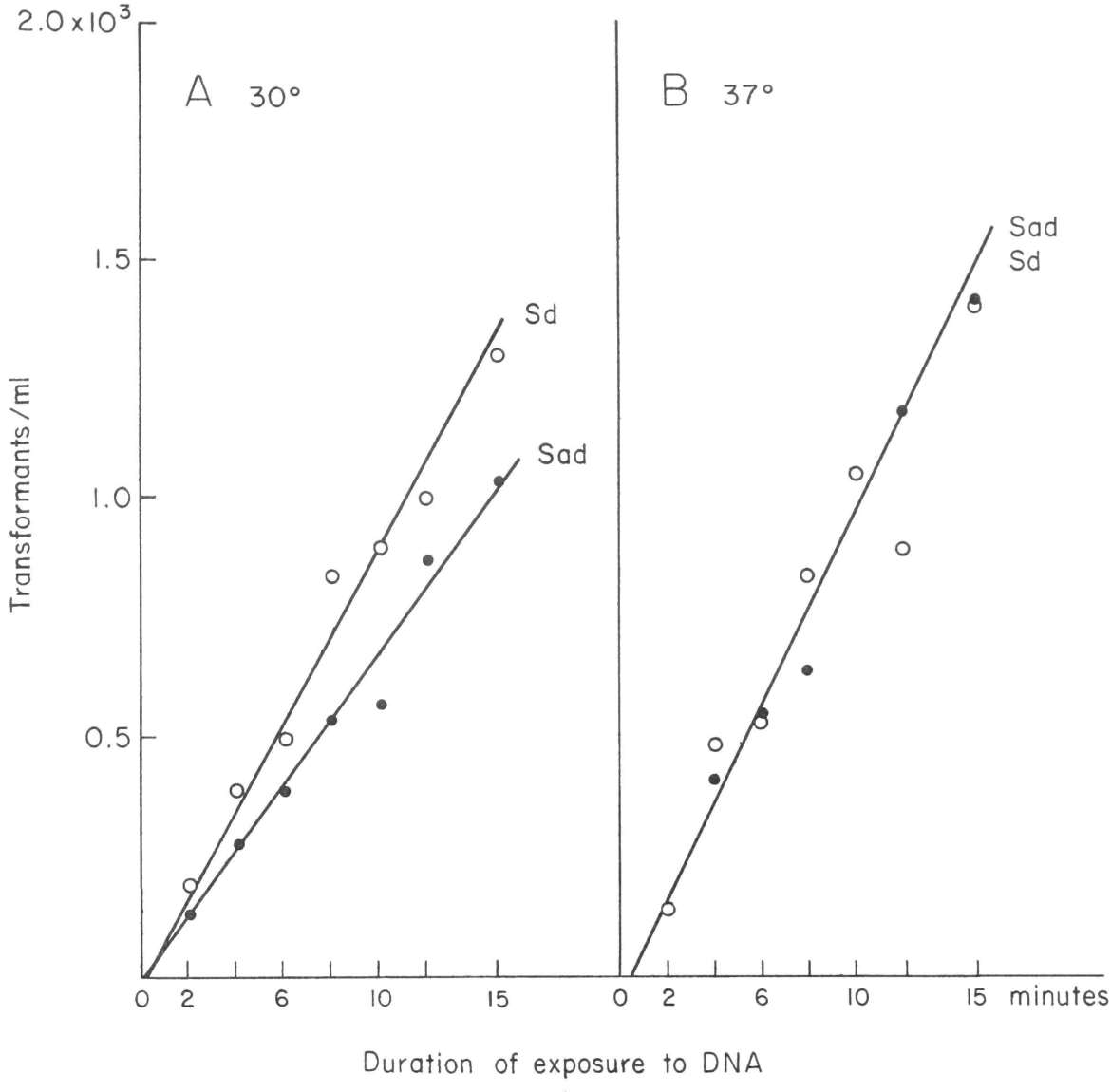


Figure 23. The effect of temperature on the relative frequency of complex and simpler intramolecular recombinational events.

A freshly grown and synchronized wild-type culture was diluted to 1 x 10^6 viable count/ml in prewarmed medium at 30°C (Part A) or 37°C (Part B). 0.5μg/ml Sad-DNA was present in the prewarmed transformation medium. The markers are: S - streptomycin resistance; d - resistance to low concentrations of sulfanilamide; ad - resistance to higher concentrations of sulfanilamide.

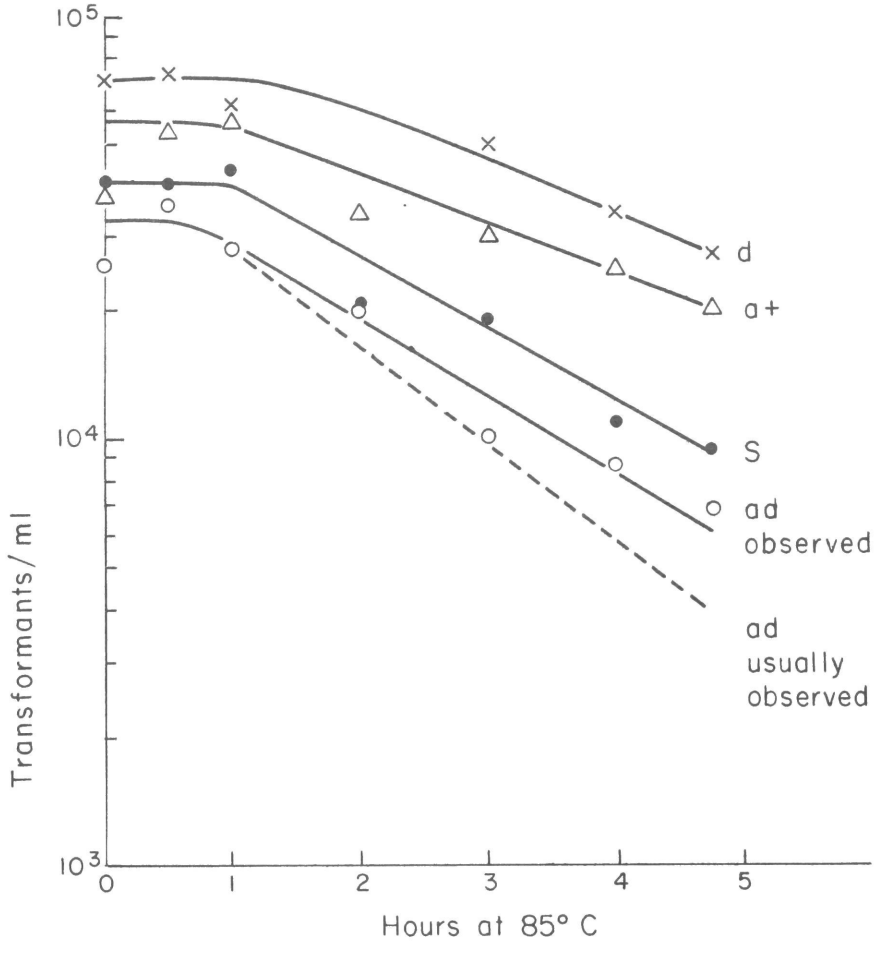


Figure 24. Subcritical heat inactivation of a multiply-marked SadK-DNA, I - singly-marked transformants.

An SadK-DNA was heated at 85°C for the interval denoted in the abscissa. The effects of thermal treatment were subsequently determined by bio-assay on a concentrated competent (+++) culture. In the transformation tube, the culture, at a viable count of 3 x 10^7/ml, was exposed to 0.02μg/ml DNA for 5 minutes at 30°C, which is in the linear response range. The markers are: S - streptomycin resistance; K - micrococcin resistance, a+, ad, d - different and phenotypically distinguishable markers for sulfanilamide resistance.

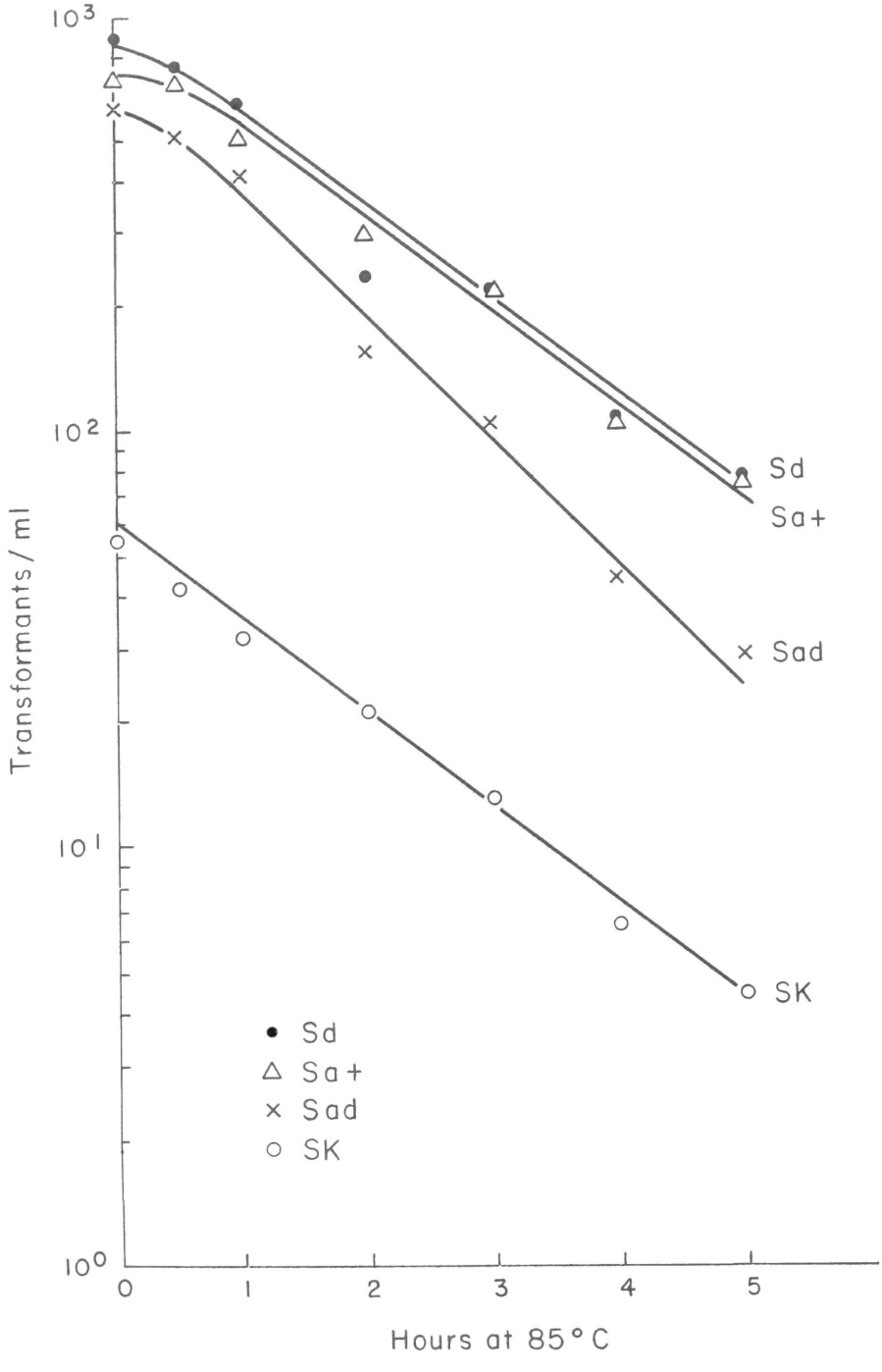


Figure 25. Subcritical heat-inactivation of a multiply-marked DNA, II - multiply-marked transformants.

The data are taken from the experiment described in the legend to Figure 24.

ultimate, exponential inactivation rates depend primarily on the number of markers introduced in a given category of transformants; in other words, when Sad DNA is heated, single markers tend to be inactivated less rapidly than pairs of markers, and pairs of markers are less sensitive than the triply-marked group Sad. In fact, Sd, Sa+, and the unlinked pair SK are inactivated at the same exponential rate, which is twice that of the exponential rate for the single marker a. The closely linked pair ad is here inactivated at about 1.5 times the rate of its component markers but is more usually twice the rate in other experiments. These data reinforce the statement that subcritical inactivation does not destroy entire DNA particles. They also clearly show that the only type of effective hit is directly on a marker site, but that a hit between two markers does not affect their individual or simultaneous incorporation into recombinant progeny. All the experiments are performed at low DNA concentration and for short exposure intervals in order to insure that most multiply-marked transformants derive from single particle (linked) transformation events.

Analysis of single colonies (Table VIII) confirms that the proportion of Sd clones in which a also appears decreases as heating is prolonged, but the ratio of Sa and Sd clones remains constant. Therefore, the S+d and Sa+ type recombinants are lost less rapidly, by inactivation, than the Sad type, although the latter constitutes at least 50% of all Sd transformants with undegraded DNA (see Section D-2). The frequency of impure clones does not increase upon heating. Since inactivation of the intermediate a region does not have a corresponding effect on S+d transformation, the genetic event seems to occur as if the cell were able in recombination to avoid or circumvent a damaged region within the DNA.

To determine more directly the fate of a central inactivated region in DNA, a similar degradation experiment was performed using a different allele configuration, namely S+d DNA on an (+a+) recipient. In this experiment, the central a region must derive from the cell genome, not from the DNA, when it appears in transformant progeny. First, the grouping of exponential inactivation rates according to the number of markers utilized is preserved in this experiment upon bio-assay after exposure to S+d DNA; the singly-marked transformants to S and ad (a from the recipient and d from the DNA) are inactivated at very nearly the same rate, and the number of doubly-marked

TABLE VIII

Single colony analysis of multiply-marked transformants recovered after exposure to subcritically inactivated samples of multiply-marked DNA.

Hours at 85°C for DNA	Number of clones analysed	Number of clones found				Sad/Sd	Sd/Sa
		S+d	Sad	Sa+	Mixed clones		
0	38	7	11	12	4	0.61	0.8
2	68	22	21	21	4	0.49	1.0
4	74	29	13	26	6	0.31	1.1

The transformations were carried out at 30°C for a 4 minute exposure of a (+++) recipient culture at 4 x 10^7 viable count/ml to 0.3μg/ml Sad-DNA (259). After expression, single colonies were selected in medium containing streptomycin and a concentration of sulfanilamide low enough to inhibit growth only of wild-type cells. Each single colony was then checked for growth in each of three media permitting growth of: 1) Sa+ types only, 2) S+d types and Sad types (Sd medium), 3) Sad types only. The markers are: S - streptomycin resistance; a+, d, ad - different and phenotypically distinguishable markers for resistance to sulfanilamide.

Sad (a from the recipient) transformants decays at approximately twice the exponential rate of the singles (Figures 26 and 27; Tables IX and X). However, the yield of S+d triple transformants (as determined by subtraction, Figure 27) decays logarithmically at 2.6 times the rate of the ad single transformant, in excellent agreement with the data obtained by direct measurement in the reciprocal system (Figure 25). Second, and more pertinent, is the observation that the progressive inactivation of the S+d DNA parallels an increase in the relative frequency with which the a marker (from the recipient cell's genome) appears in d transformant progeny, as demonstrated by the convergence of ad with d yields (Figure 26) and of Sad with Sd yields (Figure 27). Thus, after inactivation of a region within a DNA particle by subcritical heating, a recombinational event spanning the interval between S and d or one near d can still occur so as to by-pass the inactivated region, thereby retaining the homologous region of the host genome. The a region appears more frequently even in those transformants to which the bracketing markers (Sad transformant, i.e.) have been introduced in the same recombinational event (Table IX).

These data cannot be explained either by a mutagenic effect or loss of function due to heat. Both a and d alleles govern the structure of an enzyme involved in folic acid synthesis in Pneumococcus; mutation to either allele from the wild-type state does not determine synthesis or non-synthesis of a protein, but rather its catalytic properties (Hotchkiss and Evans, 1960; Wolf and Hotchkiss, 1963). Inactivation of the d^+ region by subcritical inactivation does not mimic mutation to the d state (Hotchkiss, personal communication). Using the Hemophilus transformation bio-assay, van Sluis and Stuy (1962) found no mutagenic effect of subcritical heat inactivation on six different markers tested. Nor may the data be attributed to secondary segregation phenomena in growing clones of transformant progeny because the relative frequency of impure clones is independent of the degree of inactivation of the DNA (Table VIII). Since exposure of cells to severely heated DNA causes no apparent loss of viability in competent pneumococcal populations, the irreversible fixation of such DNA is probably not lethal to a cell. Lerman and Tolmach (1959) showed that fixation of P^{32}-labeled DNA is much less affected by heat degradation than is the transformation yield, and therefore degraded DNA can still penetrate the cell.

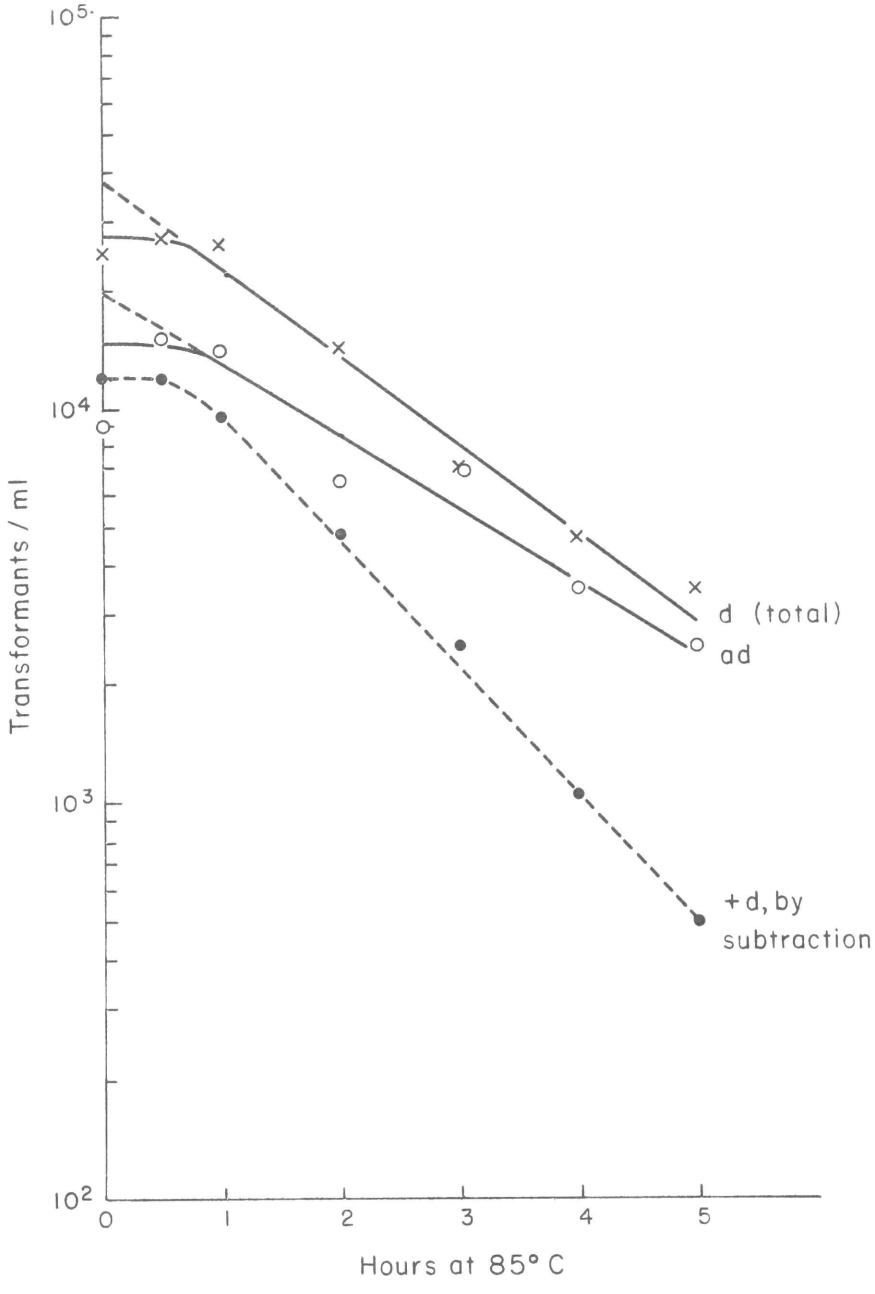


Figure 26. Subcritical heat inactivation of a multiply-marked S+d-DNA, I.

The experiment was similar to that described for Figure 24, but the bio-assay was performed on a (+a+) recipient strain. The exponential rate of inactivation is unusually rapid. The markers represent different levels of resistance to sulfanilamide with d being less resistant than ad. Note that ad represents a transformant to a single marker, since the a allele is present in the recipient.

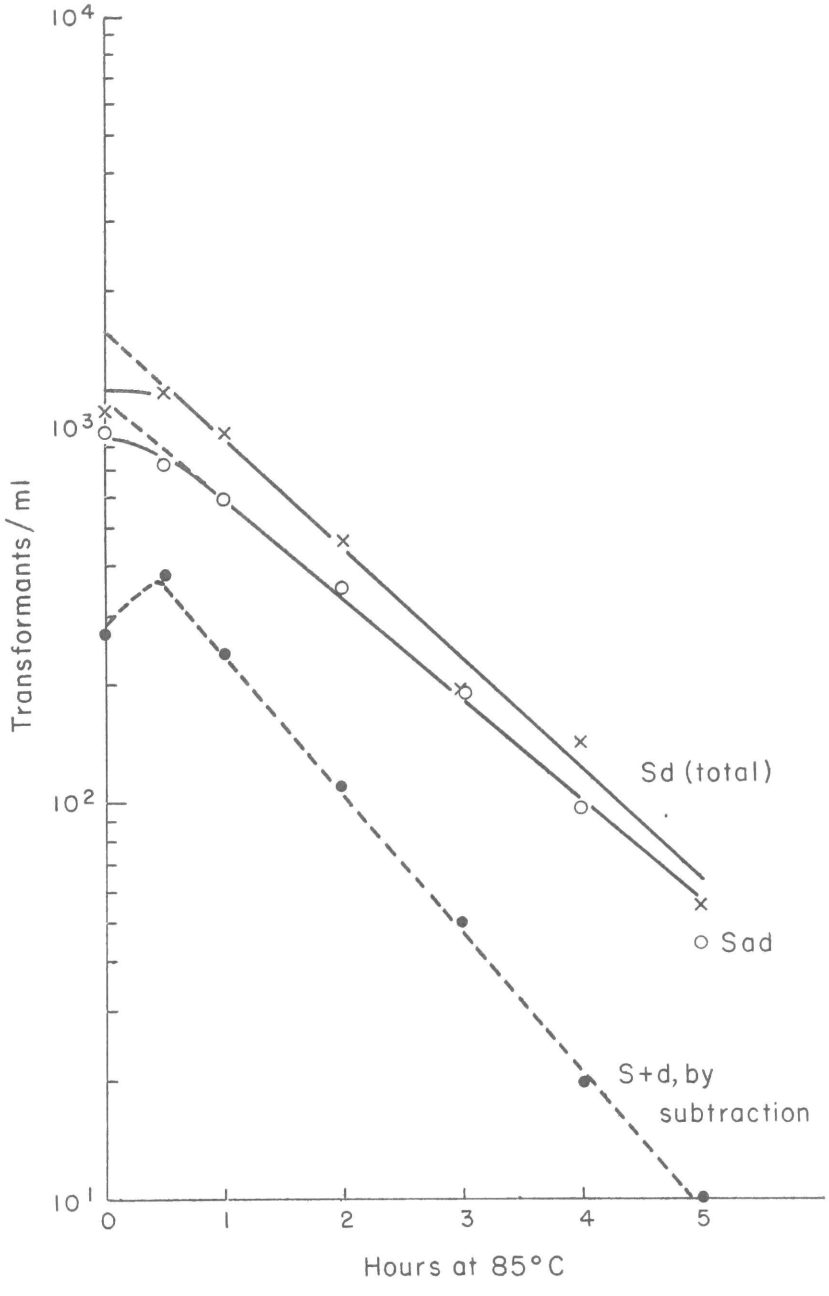


Figure 27. Subcritical heat inactivation of a multiply-marked S+d-DNA, II.

The experiment was performed under conditions similar to that described for Figure 24, but a (+a+) strain was used as the recipient culture. The DNA preparation was the same as in the experiment described for Figure 26, but this sample was heated separately.

TABLE IX

Changes in recovery ratios of transformant types after exposure of (+a+) recipient to subcritically inactivated aliquots of S+d DNA.

Hours at 85°C	Sad/Sd	Hours at 85°C	ad/d
0	0.64	0	0.58
0.5	0.66	0.5	0.56
1.0	0.75	1.0	0.56
2.0	0.79	2.0	0.62
	-	3.0	0.71
	-	4.0	0.82

The transformation data are reported for 2 separate heating experiments. Conditions of transformation: exposure at 30°C to 0.2μg/ml DNA 249 for 5 minutes at a viable count of 4×10^7/ml.

TABLE X

Relative rates of exponential inactivation of markers upon subcritical heat inactivation: S+d DNA on a (+a+) recipient

Marker	m	$m_{relative}$
Singles		
S	-.13	1.0
ad	-.13	1.0
Multiples		
d	-.15	1.1
Sd	-.32	2.4
Sad	-.28	2.1
S+d, by subtraction of measured values	-.35	2.6

Transformations were performed at $30^{o}C$, using 0.25μg/ml DNA 249 and a 5 minute exposure to 4×10^{7} viable count/ml. "m" represents the exponential inactivation rate per hour at $85^{o}C$ for the pertinent markers.

These experiments constitute independent confirmation of the frequent occurrence of multi-switch recombinational events on the intramolecular level. Otherwise, Sd and Sad transformant classes would be inactivated at the same rate while the Sa, Sd, ad, and (unlinked) SK categories would be inactivated at different rates. The observation that Sa (2% to 8% linkage) and ad (50% linkage) transformant classes respond identically to subcritical heating of DNA suggests that multi-switch events are very frequent, at least on exposure to partially heat degraded DNA.

The cell seems to be able to escape the consequences of exposure to damaged DNA. The fate of that class of DNA particles whose S and d loci are intact but which have sustained damage between these sites remains unclear. However, one would expect either a mutagenic or a lethal effect from incorporation of a damaged site into the genome; neither of these has been observed in the appropriate experiments. Secondary phenomena, which would compensate for prior incorporation of a damaged site during replication of a recombinant genome, can be disregarded insofar as these would result in impure clones (no conservation of two alleles). It is very possible that such damaged regions of DNA particles are not integrated into the genome at all or are eliminated, shortly after introduction by recombination, through an induced effect of the copy-error type, at that site (a "hole" in the template, perhaps) during an early subsequent replication. Either of these two latter alternatives would be equivalent to the formulation that the presence of a heat-induced lesion along a polynucleotide strand increases the probability of a recombinational event at or near that site above that likely for events initiated by a similar, but undegraded DNA particle (see also van Sluis and Stuy, 1962).

Part 3: The effect of critical heat denaturation and renaturation on genetic linkage groups.

For denatured or renatured DNA, titration of transformant yield against DNA concentration gives a response curve similar to the one already discussed for intact DNA (Figure 28; see also Section D-1, Figures 1 and 2). From these titration curves and from the linear accumulation rates of singly-

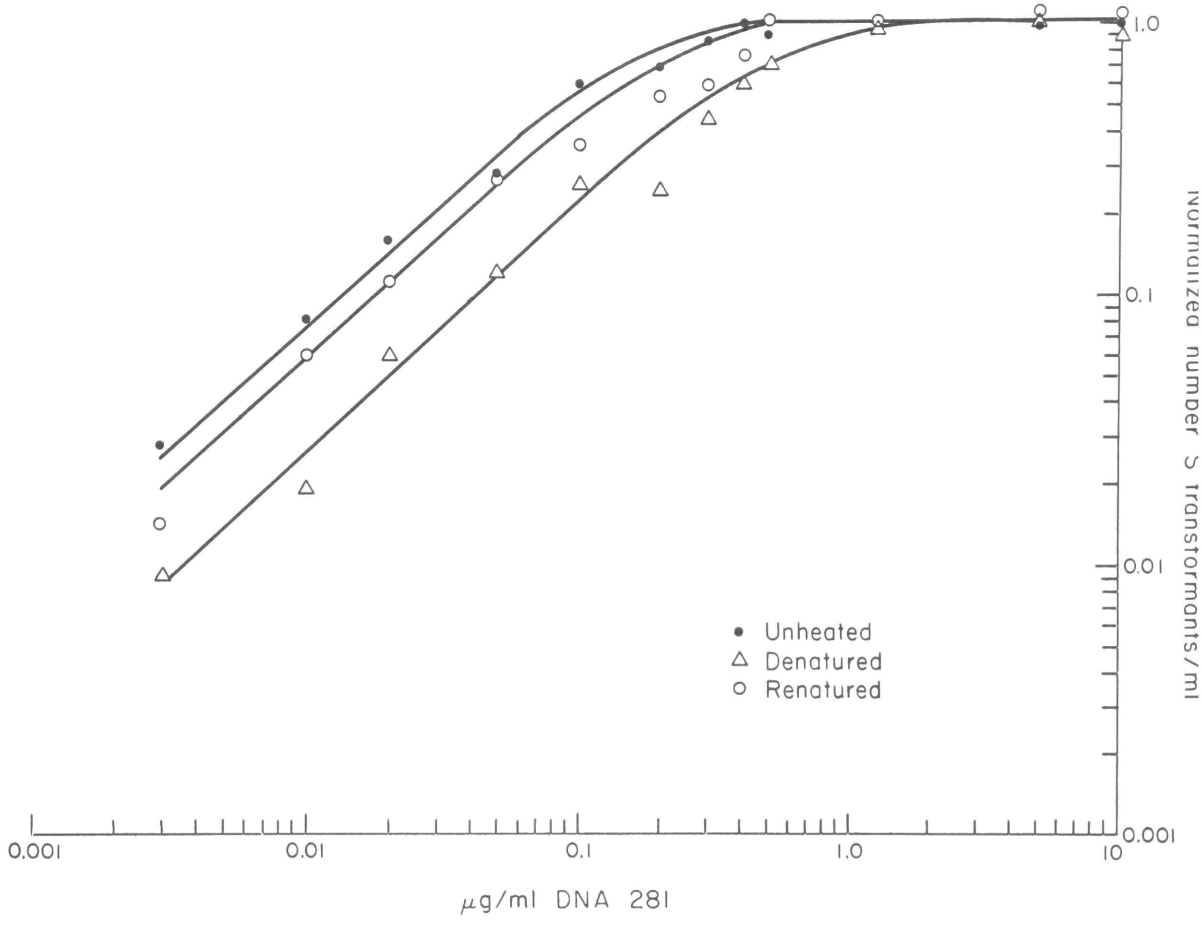


Figure 28. Titration curve for recovery of S transformants as a function of DNA concentration, using unheated, heat denatured, and renatured DNA, normalized to the same maximum yield.

The bioassay was carried out on a population of viable count 4×10^7/ml exposed to the indicated concentration of DNA for 30 minutes at $30^{\circ}C$. The transformant yield at saturating DNA concentrations was 3.6% for the denatured sample relative to control and 36% for the renatured sample relative to control. S - streptomycin resistance.

marked transformants, kinetic constants for interaction of cells with heated DNA can be determined by a model and calculations identical to those used by Fox and Hotchkiss (1957; see also pages 17-19 for discussion of this model). An apparent K_m (Michaelis-Menten dissociation constant) for the interaction of cells with the various DNA's was determined from a Lineweaver-Burk plot of the reciprocal of the linear rate of accumulation of singly-marked transformants against the reciprocal of the total DNA concentration present (Figure 29). The apparent K_m (Table XI) is equal to the slope of the graph in Figure 29 multiplied by the measured maximal velocity for accumulation of transformants at very high DNA concentrations; the data were taken from the experiment represented in Figure 28 in which the maximal yield of transformants was 3.6% of the control value for denatured DNA and 36% of the control value for renatured DNA. The increase in apparent K_m after denaturation is compatible with a lowered adsorption affinity of this material, as suggested by Roger and Hotchkiss (1961). Renaturation restores some but not all of the lost affinity, as manifested by a decrease in the apparent K_m to a value closer to that of the unheated DNA. As in the Michaelis-Menten formulation:

$$K_m = \frac{k_2 + k_3}{k_1} \qquad (4)$$

These individual constants have been defined on page 18.

Extrapolation of the linear accumulation curves of singly-marked or multiply-marked transformants to the time axis shows that the final rate is reached one minute after DNA addition, independent of any previous heating (e.g., see Figures 3 and 30). From this information, k_1 can be estimated:

$$t = \frac{1}{k_1 (K_m + DNA)} \qquad (5)$$

where DNA = total concentration in μg/ml.

t = 1 minute.

The rate of accumulation of transformants reaches a definite maximal plateau value for each DNA sample; this value represents the maximal velocity or V_{max}. Again, by analogy to the Michaelis-Menten analysis:

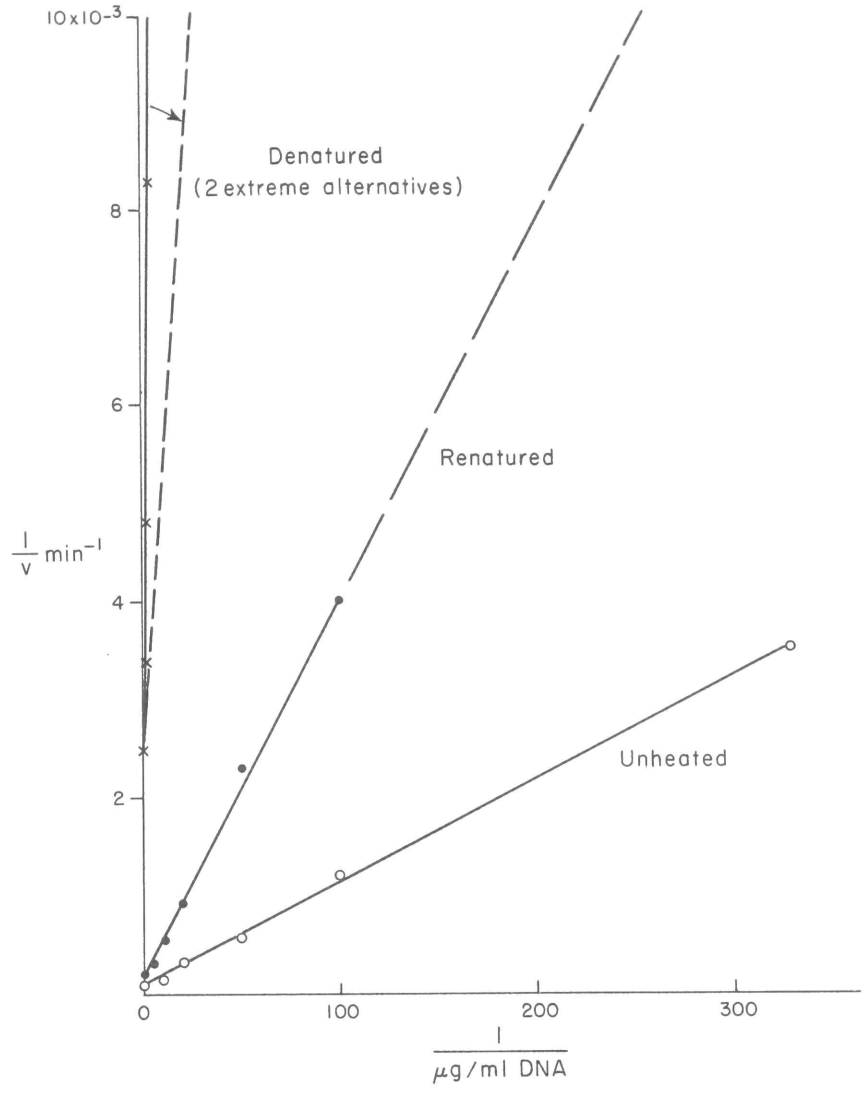


Figure 29. Graphical evaluation of the apparent K_m for cellular interaction with unheated, heat denatured, and renatured DNA.

The data are taken from the experiment described for Figure 28. The measured quantity is the transformant yield after a 30 minute exposure; since the rate is known to be constant with time, this figure was converted to a rate (v) per minute. The dashed line represents the true K_m for denatured DNA, assuming that the true concentration of active DNA particles is less than the total concentration. The slope of the line represents $\frac{K_m}{V_{max}}$. The y-intercept represents V_{max}^{-1}.

TABLE XI

The effect of heat denaturation and renaturation on the kinetics of interaction between cells and DNA.

DNA	Apparent K_m $\mu g\ ml^{-1}$	$\sim k_1$ $min^{-1}\ \mu g^{-1}\ ml^{-1}$	$\sim k_2$ min^{-1}	Relative k'_3 min^{-1}	V_{max} min^{-1}
unheated	0.12	3.1	0.37	100a	1.2×10^4
denatured	0.29	2.0	0.58	3.6a	460
renatured	0.19	2.6	0.49	36a	4.6×10^3
unheated DNA, from Fox and Hotchkiss (1957)	0.04	5	0.2	6×10^{-3}	--

The data are taken from the experiment represented and described in Figure 28. The method of calculation is described in the text and the graphical form is seen in Figure 29.

$$V_{max} = k_3 B(S) \quad (6)$$

Since B(S), the concentration of bacterial binding sites, is proportional to B, the concentration of bacteria/ml, the value of k_3 can be expressed in relative terms:

$$V_{max} = k_3'(B) \quad (7)$$

because a definite saturating yield is reached in each case. Since all the data represented in Figure 28 were obtained from replicate aliquots of one competent culture (i.e., B does not change), $V_{max} \propto k_3'$ for all three samples of DNA. Thus, both denaturation and renaturation lower the relative k_3' of the appropriate reaction. Since critical heating increases the apparent K_m but decreases both k_1 and k_3', k_2 should also increase relative to the control value for unheated DNA. The approximation that $k_2 \gg k_3$ for unheated DNA (Fox and Hotchkiss, 1957) can, therefore, safely be extended to each of the heated preparations to give:

$$K_m \cong \frac{k_2}{k_1} \quad (8)$$

from which k_2 is estimated. These constants are listed in Table XI in comparison to those initially calculated by Fox and Hotchkiss. The major change is in the value of k_3' which decreases 28-fold after denaturation; K_m increases only about 2.5-fold. In all cases, the values of the constants for renatured DNA are intermediate between those obtained for unheated and denatured samples.

Two experimental observations, discussed in the introduction, must be explained in analysing the kinetic data. First, upon critical heat denaturation, P^{32}-fixation from pneumococcal DNA declined sharply and to roughly the same extent as the biological activity (Lerman and Tolmach, 1959). This can be interpreted to reflect the abrupt decrease in k_3' which describes the rate of a penetration process. Second, when present in equimolar amounts with unheated DNA, denatured DNA competes very poorly if at all with marked, unheated DNA for cell adsorption sites (Roger and Hotchkiss, 1961), and can itself be excluded from initiating transformation by intact unmarked DNA; renatured DNA is intermediate between denatured and unheated

DNA in its ability to compete with unheated material (Roger, personal communication). This observation is more difficult to interpret.

Within the framework of the model, the increase in apparent K_m observed after denaturation is insufficient to explain the loss of competitive ability of denatured DNA. The component k_3', which has changed most extensively, should not be pertinent in this connection. It contributes only a small, and opposite, effect upon the changes in K_m, leaving the latter as essentially a measure of altered adsorption affinity. Regardless of whether a small or large fraction of the denatured DNA is biologically functional, the titration curve suggests that that portion has an affinity for cells (and K_m) near that of unheated DNA. The altered behavior of denatured DNA would be due to a loss of competitive ability explained either by a loss of affinity which is less than three-fold or dilution by inactive material, together with a much greater decrease in k_3' for collapsed denatured particles, reflected by the lowered maximal yield. If only 40% of the particles were active, K_m would be unchanged after heating; if very few of the particles were active, then K_m would be decreased by heating. Lacks (1962) has reported that during penetration of P^{32}-labeled pneumococcal DNA into competent cells, about half of the isotope is irreversibly bound in high molecular weight form while the remainder is converted into low molecular weight constituents. If penetration did involve an (enzymic) separation of DNA single strands, which is by no means clear, then the collapsed (denatured) molecule might be a grossly altered substrate for this step, which would be reflected in an altered k_3'.

Alternatively, the unusual nature of competitive ability of denatured DNA, relative to unheated DNA, tends to suggest that different classes of adsorption sites respond to the two kinds of particles. It is possible that adsorption sites on a cell surface are heterogeneous so that denatured DNA can interact with only a fraction of the sites available to the unheated component of the mixture. Such a hypothesis would explain all the data on competitive ability. The comparison of generalized K_m values, however, would then be meaningless.

In either case, collapsed (denatured) molecules are themselves responsible for the residual biological activity; whether these particles are single-stranded, partially double-stranded, or entangled single strands, is in-

determinate. Nor can the heterogeneity of molecular species in denatured or renatured samples be ascertained.

Data similar to those just discussed have been obtained repeatedly for other preparations of DNA with the single, notable exception that a greater shift in the DNA concentration required to saturate the yield for single markers appears upon denaturation of those DNA samples which also suffer greater decrease in linkage frequency between linked pairs of markers; such results are highly reproducible for each DNA preparation. All of the undegraded DNA preparations used in this study were of the same quality in having identical high biological activity for a single marker, but a difference in the apparent K_m which appears on denaturation suggests that more subtle criteria are necessary. The need for more rigorous standards of DNA quality becomes apparent upon examination of the behavior of linkage groups in multiply-marked, heat denatured DNA. Extensive kinetic experiments on many independently denatured aliquots of several DNA preparations are summarized in Tables XII, XIII and XIV, in which the reported linkage frequencies represent the relative linear accumulation rates of transformants with duration of exposure to DNA. The usual residual activity, at saturating DNA concentration, has been between 1% and 4% of the control activity for the d marker, but may be as high as 11% in other heated samples. Some of the experimental factors governing these effects are becoming clear (Roger, personal communication) but they can be considered empirical variables in this discussion.

Observed linkage frequencies in denatured DNA samples fluctuate relative to control values. The closely linked pair a and d can survive denaturation of DNA 281 at the initial value of 50% linkage (Table XIV), but in similar treatment of DNA 264, the level falls to 25% (Table XII), or one-half of the linkage present in either intact DNA. The more loosely linked pairs of markers, streptomycin and sulfanilamide resistance, survive denaturation imperfectly and with more variable results than the closely linked pair ad. For DNA 264, this linkage can disappear entirely (Table XII, heating 3 and 5) or remain quite close to the initial values (Table XII, heating 2 and 4). For DNA 281, the loose linkage decreases two- to four-fold, but always remains clearly demonstrable by the time kinetic technique (Figure 30). Therefore, even loose linkage groups can survive critical heat denaturation (see

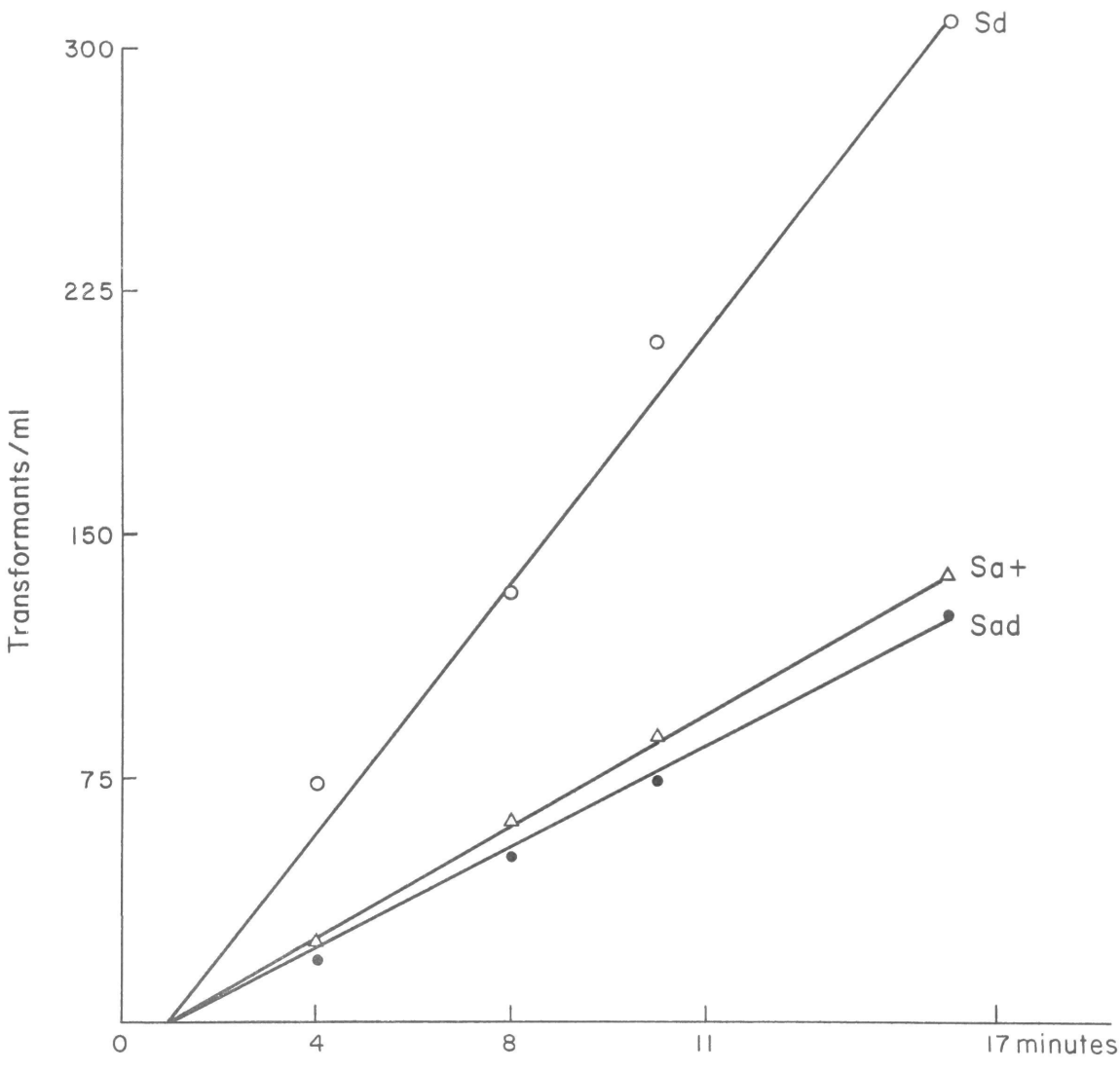


Figure 30. The linear accumulation rate of multiply-marked transformants from a heat denatured DNA preparation.

The heat denatured sample of DNA 281 (Sad) had 1.2% the activity of a control sample for the S marker, when measured at saturating DNA concentration; this experiment is summarized in Table XIV, heating 3. The assay was performed at 30°C using a concentrated competent (+++) culture at 4×10^7 viable count/ml and 0.2μg/ml denatured DNA. The markers are: S - streptomycin resistance; d, a+, ad - different and phenotypically distinguishable markers for resistance to sulfanilamide.

TABLE XII

Time kinetic study on linkage in denatured DNA SadK (264) on (+++) recipient and of the same preparations after renaturation: the linear rates of accumulation of several transformant types.

Heating	Residual Activity						Renatured Activity of Same Samples					
	% d survival	ad/d	Sd/S	Sad/S	Sa+/S	Sad/Sd	% d survival	ad/d	Sd/S	Sad/S	Sa+/S	Sad/Sd
0	100	0.5	.02-.03	.015-.020	.015-.020	0.5-0.7	Representing intact standard.					
	100	0.5	.035	.020	.018	0.6	Representing "annealed" without prior denaturation.					
1	3	-	.025	-	-	-	62	-	.025	.015	-	0.60
2	2.5	0.25	.017	.010	.010	0.6	38	-	.014	.004	.011	0.30
3	4	-	.003	.0004	.004	0.13	60	0.33	.006	.0025	.003	0.4
4	11	-	.060	.02	.02	0.33	42	0.25	.008	.005	.008	0.6
5	2	0.25	Exponential accumulation at yields consistent with random double events.				59	0.25	Exponential accumulation at yields consistent with random double events.			

Assays were performed on aliquots of frozen competent cultures, exposed to 0.2μg/ml DNA 264 at a viable count of 4×10^7/ml at 30°C. Each rate is calculated from 4 to 10 time points. Each experiment was performed after heating separate samples of the same DNA preparation; all five experiments were completed within a time interval of about two months. Heating 0 represents a control experiment in which the DNA has not been denatured.

TABLE XIII

Time kinetic study on linkage in denatured and renatured S+d DNA (249) assayed on (+a+) recipient: linear rates of accumulation of several transformant types.

Heating	Residual Activity					Renatured Activity of same Samples			
	% d survival	ad/d	Sd/S	Sad/S	Sad/Sd	% d survival	ad/d	Sd/S	Sad/S
0	100	0.5-0.7	.02	.013-.016	0.65-0.8	representing intact standard.			
5	6	0.60	.011	.006	0.6	26	1.0	< .001	< .001
6	5	0.8	-	-	-	38	0.7	-	-

Conditions as in Table XII but note that ad represents the single transformant and Sad the progeny of a multi-switch event in this experiment.

TABLE XIV

Time kinetic study on linkage in denatured and renatured SadK DNA (281) assayed on (+++) recipient: linear rates of accumulation of several transformant types.

Heating	Residual Activity						Renatured Activity of Same Samples					
	% d survival	ad/d	Sd/S	Sad/S	Sa+/S	Sad/Sd	% d survival	ad/d	Sd/S	Sad/S	Sa+/S	Sad/Sd
0	100	0.56	.079	.057	.028	0.72	Representing intact standard.					
1	3	0.41	.02	.01	.008	0.55	46	0.29	.02	.01	.012	0.5
2	4	0.55	average .024	average .012	average .012	0.50	33	0.23	average .015	average .006	average .009	average 0.5
3	1.2	0.56	.048	.020	.020	0.42	-	-	-	-	-	-

Conditions as described for Table XII, but a different DNA preparation was used.

Roger and Hotchkiss, 1961 on the ad pair; but contrary to the data on H. influenzae of Rownd et al, 1961), although the quantitative data can be variable as in the experiments reported here. Complex recombinational events, expressed by the ratio of the relevant transformant types, also persist (Tables XII, XIII and XIV). It is emphasized that in contrast to DNA 281, DNA 264 shows: 1) a greater apparent increase in K_m (decrease in adsorption affinity or dilution in the number of biologically effective particles) upon denaturation as well as 2) some loss of linkage in the closely linked pair ad and 3) a more pronounced tendency towards loss of linkage for the loosely linked markers.

The behavior of longer regions of DNA molecules is the more sensitive criterion of "intactness", but the behavior of single markers after critical heat denaturation of a DNA sample may also show the effect of pre-existing "molecular flaws" in a more obvious manner than the intact sample. For example, single-chain scissions in the back-bone of a double helix could be unimportant to the overall integrity of a molecule in the presence of a restraining hydrogen-bonded superstructure, but might become extremely significant after disruption of this secondary structure. Such single-chain breaks would obviously affect linkage more strongly than single marker yield. However, the greater decrease in yield of Sad transformants relative to all the Sd transformants (Table XIV-3, and Figure 30) suggests that some rapid, localized type of damage may also occur without induced chain scission during brief exposure to critical temperatures. The partial loss of linkage is thus influenced by the intrinsic quality of the particular DNA preparation (seen in the titration curves after denaturation), by currently undefined variations in the heating conditions (variability in data obtained from replicate heating experiments), and by possible inactivation reactions at elevated temperatures (greatest loss of Sad transformants). It is obvious, nevertheless, that even loose linkage groups can survive critical heat denaturation. Since the residual biological activity expresses the properties of DNA particles with grossly disrupted secondary structure, an intact hydrogen-bonded double helix may not be essential for a particle to initiate recombinational events in transformation once it has penetrated the cell. The partial or complete survival of linkage groups and the persistent occurrence of complex intramolecular recombinational events in denatured DNA is considered to be the

significant evidence for this hypothesis, because the longer segments of DNA particles required for such transformations are even less likely to retain intact secondary structure after denaturation than are the shorter regions required for transformation to singly-marked progeny.

If a heat denatured sample of DNA is cooled slowly to room temperature (one to two hours for equilibration) after brief exposure to critical temperatures, then the yield for the d marker is 30% to 60% of the control value (Tables XII, XIII and XIV). The relatively high yield recovered from samples of renatured DNA is interpreted to reflect an increase of secondary structure in previously denatured particles, as discussed above. If the increased biological activity for single markers were correlated with a return to control values of linkage frequencies, a requirement for secondary structure during genetic recombination might be indicated; however, renaturation either lowers these frequencies or at most maintains them at the existing level. Thus, the linkage of a and d remains below 50% for DNA 264 (Table XII), and drops to one-half of this value upon renaturation of DNA 281 (Table XIV) for which the linkage group was preserved intact in the denatured samples. Similarly, for DNA 249 (S+d), the same linkage groups can either be preserved intact through denaturation and renaturation (Table XIII, heating 6) or can be inactivated during renaturation (Table XIII, heating 5); in this allele configuration, the drop of linkage frequency is manifested by an increase in the ad:d ratio, since the a allele derives from the cell genome, and the net effect is in the direction of that observed after subcritical inactivation of DNA. The linkage for the loosely linked pairs of streptomycin and sulfanilamide resistance markers may remain at the level observed for the denatured sample (Table XII, heating 1) but is more likely to decrease on renaturation. Complex recombinational events also persist in relative frequencies near, but lower, than control values. These data are consistent with the hypothesis that intact secondary structure is not required for genetic recombination by those DNA particles which have penetrated into competent cells. An increase in k_3' value, a decrease in the true K_m, a return towards the original number of active particles, or the ability to use several classes of adsorption sites, could increase the transformation yield upon renaturation but not restore linkage frequencies because the genetic events occurring after penetration of DNA into the cell would be unaffected.

The additional decrease of linkage frequency in some renatured samples relative to denatured aliquots could be readily explained by subcritical inactivation during the prolonged exposure to elevated temperatures required for slow cooling. "Annealing" an intact sample of DNA 264 for one hour at 65°C, prior to slow equilibration to room temperature (Table XIV), does not significantly change single marker yield, linkage frequency, or the relative occurrence of the complex recombinations. A more pertinent control experiment reveals, however, that brief exposure to elevated temperatures near the critical value renders DNA particles more susceptible to inactivation by subsequent prolonged exposure to subcritical temperatures (Table XV). Two similar aliquots of DNA were briefly heated at 87°C (below the critical temperature of 90°C, under these conditions, for the relevant markers) and at 92.3°C before renaturation at 65°C for varying intervals. Exposure to 87°C causes very little or no loss of biological activity, as measured by the transformant yield of a rapidly cooled sample; subsequent exposure to 65°C does decrease the single marker yield to about 65% in 2.5 hours, and this number may be significant in that renaturation of previously denatured samples rarely surpasses a yield 60% of control values for single markers. Nevertheless, normal 50% linkage between a and d is retained. For the sample subjected to 92.3°C, which is above the critical value, the situation can be very different; rapid cooling of an aliquot of this DNA shows that denaturation has occurred (1.5% residual activity) but with complete retention of linkage between a and d. The yields for both singly- and doubly-marked transformants increase as the subsequent exposure to 65°C is prolonged, demonstrating that renaturation is taking place throughout the entire interval. The linkage frequency drops towards 25% during renaturation in this experiment; in parallel experiments on the same DNA, the ad linkage frequency varies between 25% and 50% for renatured samples. Loosening of secondary structure by brief exposure to temperatures very near the critical point can, therefore, increase the sensitivity of DNA particles to subsequent subcritical inactivation. This degradation need not result from depolymerization (Doty et al, 1960), since renaturation can affect the triply-marked Sad group more than the total Sd yield. Moreover, Rice and Doty (1957) found that ten hours of heating at 84°C changed neither the size nor configuration of calf thymus DNA, as determined by the pertinent and sensitive criteria of light-scattering measurements.

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TABLE XV

Effect of prolonged annealing on recovery of biological activity in renatured SadK DNA (281).

DNA	Originally heated 15 minutes at 87°C			Originally heated 15 minutes at 92.3°C		
	d/ml	ad/ml	ad/d	d/ml	ad/ml	ad/d
intact, unheated	1.0×10^5	5.8×10^4	0.58	1.2×10^5	7.2×10^4	0.60
heated and iced	9.4×10^4	4.5×10^4	0.48	1.8×10^3	1.0×10^3	0.56
renatured, 65°C 0.5 hours	7.0×10^4	3.9×10^4	0.56	9.1×10^3	3.1×10^3	0.34
1.5 hours	6.8×10^4	3.2×10^4	0.47	2.1×10^4	5.0×10^3	0.24
2.5 hours	6.4×10^4	3.2×10^4	0.50	2.8×10^4	7.7×10^3	0.27
DNA 264 annealed 1.0 hour only	3.4×10^4	1.8×10^4	0.53			

Assays were performed by exposure of 6×10^7 viable count/ml to 0.84μg DNA/ml at 30°C for 30 minutes, except for DNA 264. DNA 264 was "annealed" at 65°C without prior exposure to elevated temperature; the transformation was performed on an aliquot of a different preparation of competent cells at 4×10^7 viable count/ml exposed to 0.2μg DNA/ml, and the yields are quoted for 15 minute exposure. The d yield for DNA 264 is $\geq$ 90% of the intact standard.

All the above data are consistent with the hypothesis that a DNA particle will tend to participate in recombinational events during transformation independent of the extent of secondary structure it possesses in solution. The structural requirement would pertain rather more to its ability to penetrate into a cell.

Part 4: The behavior of annealed mixtures of genetically distinct DNA's.

After denaturation and renaturation of mixtures of differently marked DNA's, the time kinetic technique can be utilized to follow the accumulation of multiply-marked transformants in order to determine whether the thermal treatment has created new species of biologically active DNA particles. An S+d and Ka DNA were used in equal proportions; for the control, each DNA was denatured and renatured in a separate tube prior to mixing for transformation, whereas in the experimental system the final mixture was denatured and renatured in one tube. In this experiment, the pattern of appearance with time of SK transformants can be compared with that of Sa+, ad, S+d, and Sad types. Note that Sd is a pre-existing linked pair in this experiment.

Since S and K are unlinked, the appearance of SK transformants should be second-order in time and at a yield of approximately 0.06 the calculated value, which is the actual ratio observed after transformation with a 1:1 mixture of intact DNA's, each individually marked for S or K. An increase in the yield above this level or a linear pattern of accumulation with time, following transformation with an annealed mixture of DNA's, would suggest the formation of biologically active, non-specific hybrid helices; this does not occur (Table XVI). Similar analysis of the pattern of accumulation and of the yield to Sa+ and to ad transformants clearly and quantitatively shows that no detectable formation of biologically active, specific hybrid particles occurred (Table XVI and Figure 31). Sad transformants are not detectable. ad transformants should be present in much higher yield if the biologically active hybrids had formed in significant frequency, since ad linkage has not been observed to drop below 25% after renaturation of a multiply-marked DNA. The drop in S+d linkage from the initial 3% level to 0.05% following renaturation can be invoked, as an internal control, to explain the independent behavior of S and a markers.

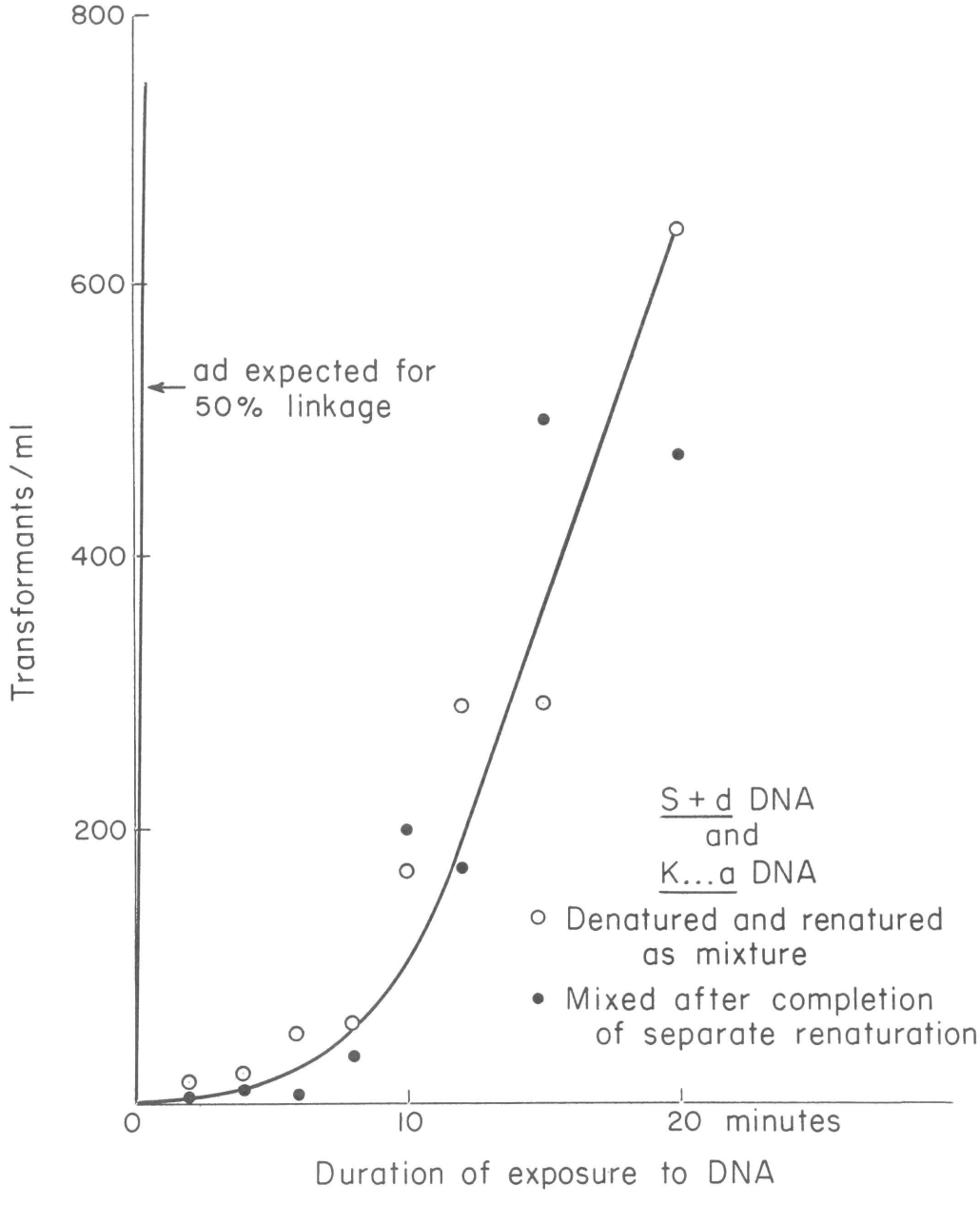

Figure 31. Time course of appearance of ad transformants after exposure to annealed mixtures of DNA (S+d and K ··· a).

The experiment, from which the data are taken, is described in the legend to Table XVI. The +d transformant accumulated at a linear time rate of 4.5×10^3/minute in this experiment.

TABLE XVI

Recovery of multiply-marked transformant progeny after transformation with an annealed mixture of DNA's (S+d with K···a): Ratio of number transformants observed to number calculated for random double events.

Transformant type	Genetic association when within a single genome	DNA mixture	Ratio: observed to calculated after varying exposure to DNA (minutes)								
			2	4	6	8	10	12	15	20	30
SK	unlinked	experimental	0.1	0.04	0.05	0.06	0.06	0.05	0.05	0.04	0.04
		control	0.1	0.04	0.04	0.03	0.04	0.04	0.05	0.04	0.03
Sa+	loosely linked (~ 2%)	experimental	0.27	0.13	0.13	0.12	0.11	0.12	0.06	0.07	0.05
		control	0.08	0.07	0.07	0.07	0.07	0.06	0.08	0.08	0.09
ad	tightly linked (~50%)	experimental	0.20	0.07	0.07	0.05	0.08	0.10	0.06	0.08	0.03
		control	0.08	0.04	0.01	0.04	0.20	0.10	0.18	0.10	0.03

Experiment was performed at 30°C, using a concentrated competent cell population of viable count 4×10^7/ml and DNA at a total final concentration of 0.4μg/ml (i.e., 0.2μg/ml/DNA). The experimental mixture was denatured and annealed together, but the control mixture was made up only after separate denaturation and renaturation of each DNA. The renaturation yield was 45% for the S marker relative to the intact control; the ratio of K:S was 0.7 for both renatured mixtures, in accord with control values on intact multiply-marked DNA. The linkage of Sd/S dropped to 0.05% after renaturation in comparison with 3.0% in the undenatured sample. For a mixture of unheated DNA's, the ratio of the number SK transformants observed to the number calculated is 0.06.

If active hybrid helices are formed, the trans juxtaposition of the a and d alleles on opposite strands of a double helix might result in transformation to mixed clones of a+ and +d cell types, rather than to ad colonies. Clonal analysis of 123 single colonies selected in very low concentrations of sulfanilamide (which inhibit only the background growth of wild-type cells) gave no indication of mixed clones of this type (Table XVII). About 2% of the clones were mixed and these include cells of all three selected types, i.e., a+, +d, and ad, in very unequal ratios and with type +d predominating. Clones of this particular type have not been observed in other clonal analyses, but unusual or mixed clones appear with about 5% frequencies in most such analyses for unknown reasons.

It is concluded that new biologically active hybrid DNA particles are not formed in significant frequency by annealing mixtures of genetically distinct DNA's.

TABLE XVII

Clonal analysis of sulfanilamide resistant progeny recovered after transformation with an annealed mixture of DNA's, DNA S+d and DNA K···a.

Clonal type	Number recovered	Number expected by random doubles
pure a+ or +d	121	-
pure ad	0	0.6
mixed clones, which contained ad cells	2	?

The aliquot analysed for clonal composition was taken from the experimental tube after 4 minute exposure to DNA in the experiment described in Table XVI. The aliquot was diluted ten-fold into fresh medium containing deoxyribonuclease and allowed to express acquired resistance for 15 minutes at 37°C, which is sufficient for scoring of sulfanilamide resistance. Samples were selectively plated in blood agar plus 20μg/ml of sulfanilamide to yield about 200 colonies/plate. These were picked into fresh medium and tested. At 20μg/ml sulfanilamide, all three categories of transformants a+, +d, ad can grow, but the wild-type background is inhibited. The mixed clones were predominantly +d with both a+ and ad cells as minor constituents ($< 10\%$).

E. GENERAL DISCUSSION

In the pneumococcal transformation system, kinetic analysis is the preferred means to determine the multiplicity of DNA particles involved in transforming a competent cell for one or more genetic markers. Since only one DNA particle is required to initiate transformation to groups of linked markers, the direct study of intramolecular recombination is accessible. With the development of a time kinetic technique to assess linkage, the existence of complex intramolecular recombinations in three-factor crosses could be demonstrated. Clonal analysis has shown that the entire multi-switch recombination occurs within one division cycle of the cell. The resultant clones of transformant progeny are pure, so that neither mixed clones nor clones of heterozygous cells are found in significant frequency. Within a factor of about two, there is no interference between two genetic recombinational events occurring over the interval between S and d in one DNA particle.

Multi-switch recombinations can occur regularly on the intramolecular level both in DNA of good quality and also in DNA bearing localised damages. Studies on heat degraded DNA offer independent evidence for the occurrence of multi-switch recombinations from the differential rates of inactivation obtained for the triply-marked and doubly-marked transformants, if three linked markers are followed relative to two linked markers bracketing the third. The data also give some suggestion that the damaged region is not integrated into the bacterial genome; in this case, the presence of a lesion would increase the probability of occurrence of a recombinational event in its vicinity. Denaturation, on the other hand, has a relatively small effect on linkage group behavior compared to the drastic effect on total yield. Collapse of secondary structure affects rather the ability of active DNA particles to penetrate the cell and is also manifested by a loss of the ability of the preparation to compete for cell adsorption sites with unheated material. Since renaturation improves total yield but does not restore any linkage previously lost to denatured aliquots, it is concluded that intact secondary structure is not necessary for intracellular recombinations.

The diversity of physiological, genetic, and structural factors which affect the subtler aspects of intramolecular recombinations render detailed model building unpalatable; indeed, it is evident that some basic principles remain unenunciated. The intactness of DNA structure in the preparation seems to be much more important than formerly suspected. Although different aspects of the structure probably affect penetration into the cell and recombination with the genome, the lowered biological activity of some DNA preparations is, nevertheless, an obvious symptom of DNA quality and reflects deviations away from a structural standard.

With reference to pneumococcal transformation, the quality of the DNA preparation clearly has a significant influence on its biological activity. Implicit throughout is the assumption that the DNA preparation with highest biological activity is the most "native". Thus, behavior of linkage groups is a more sensitive criterion than that of single markers when otherwise comparable DNA preparations are assayed either after prolonged storage of stock solutions or even shortly after purification. Upon subcritical heating, the differences between individual DNA preparations show up in the duration of the lag phase before inactivation becomes exponential with time. After critical heat denaturation, similar distinctions are revealed by the tendencies of treated aliquots to yield lower linkage values and by the higher DNA concentrations required to reach a saturating yield. As reported by Roger and Hotchkiss (1961), the sharpness of the critical inactivation per marker as a function of temperature can also reflect the intrinsic quality of a DNA preparation. Some of these subtler distinctions between otherwise equivalent preparations may result from localized damage within molecules incurred in vivo, or, more probably, during purification. Others might reflect local lesions which interrupt the continuity of polynucleotide strands (single-strand scissions or double-strand breaks, i.e.).

The structure of DNA particles is significant in at least two independent stages of transformation: penetration and recombination. Heterologous DNA from a wide variety of sources competes very effectively with transforming DNA for cell absorption sites; this fact is readily rationalized on the basis that double helices of DNA in solution are very much alike from the outside, in their size, shape, and charge distribution. Upon collapse of this secondary structure (critical heat denaturation), the DNA loses

much of its biological activity, ability to penetrate the cell, and competitive value. Its affinity for adsorption by cell populations may be but slightly changed, although the ability to penetrate is markedly decreased. Perhaps some of the particles become biologically inert as a result of collapse; alternatively, heterogeneity among cell adsorption sites could limit the number of sites available to collapsed particles. Either of these two hypotheses would explain the data and transformation yields obtained with heated and unheated material. The secondary structure of the DNA is, therefore, probably of greater importance in penetration than in intracellular recombination.

Once having penetrated the cell, DNA particles may participate in events leading to genetic recombination. Heterologous DNA may also penetrate the cell, as evidenced by fixation of P^{32} from labeled DNA, but does not participate in recombination on an equivalent basis (Lerman and Tolmach, 1957; Shaeffer, 1958). By analogy to chromosomes, the pairing of homologous genetic regions precedes, in all probability, the recombinational event; the forces governing synapsis and regulating its specificity are unknown, but the concept of mutual recognition between specific base sequences is difficult to avoid, especially in contrast to the general lack of specificity at the stage of cell penetration, as shown by the competitive ability of heterologous DNA. As discussed in the introduction, the data of Fox and Hotchkiss (1960) and of Fox (1962a) show that P^{32} from labeled pneumococcal transforming DNA is incorporated into the cell genome in amounts which could involve large segments of DNA molecules. Fox and Hotchkiss's (1960) estimate that less than 5% net DNA synthesis need occur during genetic recombination suggests a separation of recombination from replication of genetic material, although synthetic activity in the vicinity of the recombinational event itself cannot be evaluated. Complex, intramolecular recombinations would require the operation of active and accurate switching mechanisms between two homologous DNA particles probably with concomitant breakage and reunion at phosphodiester bonds in one or both of the homologous particles. Multiple recombinational events occur frequently on the intramolecular level.

Activity of the switching mechanisms on the molecular level appears to be governed by several variables, including the structural homologies (or, as a corollary, integrity) of the interacting genetic entities and the physio-

logical milieu of the cell in which the interaction occurs. The degree of linkage between pneumococcal sulfanilamide resistance markers is dependent upon the biochemical environment of the cells, e.g., the composition of the medium, and factors such as the stage of the culture in the bacterial growth curve and in the cell division cycle after synchronization (Hotchkiss and Evans, 1958; Iyer and Ravin, 1962). Thus, the presence of loose linkage between markers is more evident in transformations performed at 30°C than at 37°C (Hotchkiss and Marmur, 1954; Hotchkiss and Evans, 1958), which was interpreted to reflect an increased probability for integration of longer segments of DNA particles at the lower temperature. The "higher linkage" would mean that there was a longer region over which the "switch back" could occur to terminate the event. The relative frequency of complex intramolecular recombinations is even more sensitive to the temperature at which transformation occurs than the total linkage frequency. This would be in keeping with a longer total region of "effective pairing" or a greater number of such regions over the interval at the lower temperature but rather suggests that even some of the apparently simple, linked incorporations involve multiple events. Switching mechanisms are probably temperature dependent and, therefore, perhaps metabolic in nature; this would be in apposition to the "torsion" or mechanical stress theories, as proposed by Darlington (1937, chapter VII) for chromosomes. However, chromosomal recombinations respond sensitively to physiological variables similar to those encountered on the molecular level (reviewed in Morgan et al, 1925).

Interference may be defined as a "surprise coefficient", since it is a number which indicates excess or deficiency of multiple cross-over events relative to the value calculated from the probability of their simultaneous, independent occurrence (Haldane, 1919). However, the terminology was initially set up to describe recombination on the chromosomal level, in which one cross-over is sufficient to produce a recombinant chromosome (see page 1). In the type of molecular non-reciprocal recombination under discussion, the minimal complete "switch" event resulting in a viable transformant (complete genome) probably requires two exchanges, as diagrammed in Figure 15, so that interference may be a phenomenon undissociable from the fact of recombination. In this connection, it is significant to note that the doubly-marked H. influenzae transformant for streptomycin and kanamycin resistance can occur more frequently than either singly-marked recombinant (Stuy, 1961; van Sluis

and Stuy, 1962); the two markers are so closely linked (apparently 90% linkage) that it becomes more difficult to incorporate the smaller singly-marked segment from the donor DNA particle than the larger doubly-marked piece of the same particle. As confirmatory data, the marker pair is inactivated, on subcritical heating, at an initial exponential rate only slightly greater than either single marker; for streptomycin and cathomycin resistance markers which are about 50% linked, the results were similar to those reported in Section D-2 in that the exponential inactivation rate is about equal to the sum of the single marker rates (van Sluis and Stuy, 1962). The data on pneumococcal three-factor crosses should be interpreted to show that there is no additional interference over the interval between S and d when these two markers are integrated, by recombination from a single DNA particle, into the genome.

After a complex intramolecular recombinational event in pneumococcal transformation, there is no indication of persistence of two alleles for the same function within one cell (heterozygosis). The event initiated by one particle of DNA produces a pure clone of recombinant progeny. The fate of the resident genetic segment is still unknown, although the recovery of mixed or heterozygous clones of other types of transformants indicates that multiple copies of one marker can persist for varying periods of time within a cell before segregation of pure types occurs (Hotchkiss, 1956). The fate of transforming DNA which has penetrated the cell but does not recombine with its homolog in the recipient cell is also unclear; Lacks (1962) has presented evidence, from studies with P^{32}-labeled pneumococcal DNA, that substantial amounts are broken down into small pieces which are subsequently re-utilized by the cell in biosynthetic processes. Whether one or two DNA strands participate in synapsis and recombination within the cell remains an open question, and one with very direct bearing on the fate of both external DNA and of resident genome during and after genetic recombination in Pneumococcus. The absence of new linkage properties in annealed mixtures of DNA is not relevant, at this time, because the structure and source of the biologically active particles is ambiguous. If it could be demonstrated that the annealed mixtures contain many biologically hybrid molecules, then this experiment could suggest that only one strand is active in recombination.

The importance of structural homology between interacting entities in genetic recombination has already been discussed is some detail in Section D-3

with evidence from interspecific transformations, "marker effects" and the response of recombination frequencies in microbial systems to modification of DNA structure *in vivo* or *in vitro*. All the effective structural alterations -- ultraviolet irradiation, P^{32}-decay, incorporation of 5-bromouracil *in vivo* and perhaps subcritical heat inactivation *in vitro* -- can be categorized as altering the "profile" along or within a polynucleotide strand surface, and their effect can be at least partially rationalized to result from a local change in homology between the interacting entities. Whether this structural homology is more significant for synapsis ("effective pairing") or for the actual recombinational event cannot be clearly distinguished, but the switching mechanisms responsible for genetic recombination on the molecular level appear to respond sensitively to changes in DNA structure at one or both of these two stages.

From the point of view that accurate preservation of genetic material is beneficial to survival of a bacterial cell and of its progeny, the response of switching mechanisms to localized inhomologies in genetic material could constitute a distinct advantage. Perhaps mutability as well as recombination, both attributes unique to genetic material, respond to similar types of localised changes in molecular structure of genetic material. For example, ultraviolet light is mutagenic to cells which have been exposed to it, and the resultant mutation generally represents a loss of some synthetic capacity in the cell. Ultraviolet irradiation also increases recombination frequencies (Jacob and Wollman, 1955a,b; Jacob and Wollman, 1961, p. 237), but it is not mutagenic when pneumococcal DNA is irradiated *in vitro* (Litman and Ephrussi-Taylor, 1959) before bio-assay on cell populations. A parallel set of observations has been reported for heat treatments, whose effects may be highly mutagenic *in vivo* (Zamenhof and Greer, 1958; Ephrati-Elizur et al, 1961) but not *in vitro* (Hotchkiss, personal communication; van Sluis and Stuy, 1962); it is possible that these lesions increase the probability of recombination in their vicinity when subcritically heated DNA is used as donor (Section D-3; van Sluis and Stuy, 1962). For other agents that are mutagenic both *in vivo* and *in vitro*, no data have been published that relate to recombination frequencies. It is tempting to speculate that a cell could circumvent a potential mutation (the ultra-violet or heat-induced lesion) through an increased probability of recombination at that site, there-

by preventing incorporation of the lesion into the genome. If the lesion is introduced *in vivo*, on the other hand, then the alternative of recombination would not be available, and the potential mutation could be established more efficiently. Those agents which are mutagenic to DNA both *in vivo* and *in vitro* would be expected to have a different effect on recombination frequencies according to this speculation (nitrous acid, Zamenhof, 1961; Litman and Ephrussi-Taylor, 1959; Litman, 1961; but see also Tessman, 1962 on induction of large deletions by this mutagen; hydroxylamine, Freese and Strack, 1962; alkylating agents, Loveless, 1959; Bautz and Freese, 1960). Similarly, one might predict that 5-bromouracil substituted transforming DNA, if it has not replicated extensively in the presence of the analog (Terzaghi et al, 1962), would exhibit no mutated properties, although the incorporated base analog is mutagenic *in vivo* (Zamenhof, deGiovanni and Greer, 1958) and has been demonstrated to increase recombination frequencies (Folsome, 1960).

Studies on *in vivo* systems can also be invoked to suggest similarities of control mechanisms for mutation and recombination in microbial systems, which have rather limited opportunity for genetic exchange because of the absence of a requisite sexual cycle. In fine structure mapping of the tryptophan locus by three-point transduction tests, Balbinder (1962) obtained independent spontaneous mutants of *S. typhimurium* which show exceptional recombinational behavior. The striking observation, however, was that the presence of a loosely linked *mutator* gene in the *recipient* cells restores normal recombination frequencies in these crosses. Since the mutator gene must be present in the recipient, it may act by influencing the recombinational event; this gene is known to increase spontaneous reversion frequencies at many sites in the genome (Miyake, 1960) but its effect on the exceptional alleles is not reported. Also intriguing is the observation of Dewitt and Adelberg (1962) on genetic transposition of a segment equivalent to 8% of the *E. coli* genome. Transposition of the histidine locus apparently occurred during phage transduction and was revealed by the altered order and time of appearance of male (HFr) markers in recombinant progeny. If male (HFr) strains containing the transposed segment are used as genetic donors in conjugation, then this segment appears in recombinant progeny in either the standard *or* in the transposed position; all these recombinants are unusual in being mutationally unstable for six markers tested, but the histidine locus + mutationally unstable only in the transposed position.

The arrangement of genetic loci in a linear array, or map, has been a triumph of genetics, and extensive maps have been elaborated for several genetic systems. Nevertheless, anomalies have appeared as geneticists sought to map progressively smaller regions so that it is no longer always possible to devise a consistent and unique map for genetic loci. These phenomena have been of interest because of their promise for increased understanding of the mechanism(s) of genetic recombination. Under these circumstances, the bacterial transformation systems are uniquely suited for a precise experimental approach to the problem; in these systems, biological studies on cells and chemical studies on DNA can be made independently, but compared directly.

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