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PHYSICOCHEMICAL PROPERTIES OF THE *E. coli* F'Lac EPISOME

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Dorothy Louise Read Freifelder

(Ph. D. thesis)

August 1966

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Physicochemical Properties of the *E. coli* F'Lac Episome

Abstract

Dorothy Louise Read Freifelder

A technique for the specific labeling of the F'Lac particle of the bacterium *E. coli* has been developed using a system in which only a transferred particle can replicate and thereby incorporate labeled thymine. The system consists of a male cell which contains a genetic block such that it cannot incorporate exogenous thymine and a female which cannot replicate its own DNA as a consequence of its inability to repair lesions produced by a heavy dose of ultraviolet light. These cells are mated in the presence of radioactive thymine. In this system, the particle is transferred to a killed female with near-normal efficiency and can replicate and be transcribed in the irradiated cell. It was demonstrated by agar-gel chromatography that the labeled material is primarily F'Lac DNA. A rough measurement of the sedimentation coefficient of the labeled DNA has yielded a value of 39 ± 2. From this value the molecular weight has been calculated to be $55 \pm 8 \times 10^6$. A discussion of the possible conformation of the molecule is given. A melting curve of the labeled material has been obtained by agar-gel chromatography; the T_m at an ionic strength of 0.04 is 71 C. These experiments have been repeated in less complete form with the F factor, giving a tentative molecular weight of $35 \pm 7 \times 10^6$.

Chapter 1-- Introduction

F'Lac is a DNA-containing genetic element found in the bacterium, Escherichia coli, which carries the genes for lactose fermentation and which can be transferred from male donor cells to female recipients by bacterial conjugation. It is one of a number of factors found in E. coli which can also promote transfer of genes other than those which it carries itself. The present state of knowledge of the genetic properties of these factors is derived in large part from the course of events in bacterial conjugation, an important example of the more general phenomenon of genetic exchange.

In bacteria, genetic exchange is accomplished by transformation (1), transduction (2), and conjugation (3,4). In each process DNA derived from one cell is introduced into another cell and genetic recombinants, i.e., clones of cells having characteristics of both, may arise. Transformation (direct uptake of free DNA from the external medium) and transduction (DNA transported by a phage and injected into the bacterium) both involve the transfer of relatively small amounts of genetic material. In bacterial conjugation, however, large amounts of genetic material, occasionally a complete genome, may be transferred. The study of each of these processes, and in particular bacterial conjugation, has yielded a great deal of information concerning the organization of genetic material in bacteria. Some of these studies will be reviewed in the present work.

Whereas great effort has gone into genetic and physiological studies, little work has been reported concerning the molecular structure and physicochemical properties of the various elements which promote conjugation. It is only just recently that many workers have addressed themselves to the problem of the physical mechanism of transfer and of bacterial recombination. In the present work a beginning has been made in the characterization of one of these particles: a technique for specific radioactive labeling of the F'Lac particle has been developed. Preliminary investigations of some of the physical properties of its DNA will be described.

1. Conjugation in E. coli

A. Determination of mating type

In 1946 Lederberg and Tatum (5) found that genetic recombinants had arisen in mixtures of two strains of E. coli K12, each of which carried different genetic markers. These recombinants were found only when cells were permitted to come into contact-- they did not result from addition of supernatants or filtrates of one culture to the other (5,6) nor even from mixing of the culture fluids through a sintered glass filter during growth (7).

Later the formation of recombinants was shown to be an asymmetric process in that lethal streptomycin treatment or ultraviolet irradiation of one parent strain prevented recombinant formation while such treatment of the other parent did

not (8,9). Hence a simple fusion process such as that found in Protozoa seemed unlikely. The insensitive parent was considered to be a donor of genetic material, the other a recipient. A large number of strains of *E. coli* were found to fall into two groups or mating types, each strain infertile within the group but fertile with members of the other group. The distinction was however not absolute since recipient cultures were totally sterile with each other, while donor-donor matings showed slight fertility (10,11,12). Also, donor strains were occasionally observed to lose fertility with recipients, apparently spontaneously, and simultaneously to acquire fertility with other donor strains (10,11,12). The reverse was not observed to occur. This led to the idea that donor strains contain some element which can be lost but which does not arise spontaneously.

The discovery that recipient cells were converted to donors by cell contact, or bacterial conjugation, with donors (10,11,12) led to the concept that a sex or fertility factor, "F"(10), determined donor ability and was frequently transferred from donor (F+) strains to recipients (F-). This transfer of donor ability occurred with high frequency (approaching 100%) as opposed to transfer of chromosomal markers (ca. 10^{-7} per donor cell) (5,6).

When chromosomal markers were transferred, the recombinants in general retained most of the markers of the F- parent (10,11,12) suggesting that the donor transferred only part of its genome to the recipient. The nature of this transfer

process and the precise role of the donor was an object of consideration for some time. The first step forward was the discovery of a third mating type, a highly fertile donor which was derived from an F+ cell line and for which the recombination frequency approached 100% for some markers (although not for others). In contrast with F+ strains, donor ability was only rarely transferred. These strains were called Hfr (High frequency of recombination) (13,14). Several Hfr strains were isolated and it was found that for a given Hfr the recombination frequency depended upon the marker considered and that these values differed for different Hfr's. The properties of these various Hfr strains and the information gained from a study of the kinetics of transfer will be discussed in detail in Section 1.C.

A fourth mating type, Vhf (Very high frequency of recombination), has also been isolated (15,16). Such strains have the property that not only is mating very efficient, as in Hfr strains, but also the recombination frequency is very high for almost all markers. This type has proved to be probably a variant of an Hfr with an increased capacity to maintain the stability of a mating pair, and will not be discussed further.

B. The mating process

Conjugation may be divided into several successive steps: specific pairing of donors to recipients, effective contact, cellular union, and transfer of the conjugation factors and/or

the chromosome (3,4).

Specific pairing refers to that interaction between male and female cells which accounts for the male-female pairs observed microscopically (17,18,19,20); such pairs are not observed in all-male or all-female cultures. The molecular basis of this pairing is not known but it is likely to be related to the differences in surface properties of the male and female cells. Several examples of these differences are that there are male- and female-specific antigens (21); males and females can be separated by countercurrent distribution (22); there are male- and female-specific phages (17,23,24,25a,25b); and males show a higher affinity for acidic dyes, a higher negative charge, and a greater tendency to autoagglutinate than do female cells (26,27,28). Evidence that specificity of pairing involves these surface properties comes from the fact that mating can be inhibited by altering some of them. For example, treatment of the males with periodate (29) or by starvation by aerobic growth to maximum cell density (10,30) results simultaneously in loss of mating ability (10), loss of sensitivity to male-specific phages (24), and increased ability to act as recipients (10,16,29). It is possible then that a single male-specific surface component is responsible for all of them. Periodate sensitivity suggests that this may be a polysaccharide. It has not been possible up to the present to alter the female cell in such a way that mating is inhibited, although females which are resistant to

female-specific phages have not been investigated. A first step in the characterization of the specific substance has been the isolation of a substance from the cell wall which competitively inhibits mating (31).

Effective contact is defined as that interaction within a specific pair which is strong enough to withstand dilution of the mating mixture. Specific pairing and effective contact are not operationally distinguishable.

Cellular union refers to the alteration of the cell walls and membranes necessary to permit transfer of the genetic material without exposure to the external medium. Completion of cellular union is operationally defined by detection of the initiation of transfer-- that is, measurement of the shortest mating time at which mating can be interrupted and recombinants still observed. If the spread in time of formation of effective contact is reduced by dilution of the mating mixture after a short time, there is still a variable time delay before the initiation of transfer; whether this represents the time for the physiological processes of cellular union to occur or the time of transfer of an initial portion of the chromosome containing no known genetic marker is not known.

The effects of cellular union on the cell are variable, depending both on mating conditions and the strains used (32). In general, both male and female cells remain viable (33) although in one situation, the F- exconjugate may be growth-inhibited or even killed; this latter effect occurs only at

high ratios of Hfr to F- cells (32), in which case mating with several Hfr cells is possible, and is probably analogous to lysis-from-without with bacteriophage (34).

Chromosomal transfer will be discussed in detail in the next section.

The mating process is dependent upon energy and is inhibited by anaerobiosis or the addition of dinitrophenol (35, 36,37). However, apparently only the male requires energy since (a) prestarvation for an energy source of the male but not of the female reduces the yield of recombinants and (b) mating proceeds normally with an adenine-requiring female previously starved of adenine, a procedure which should deplete the reserves of ATP (38,39). In addition the female can be starved for amino acids (D. Freifelder, pers. commun.) or be irradiated with ultraviolet light (38) and still serve as a recipient, whereas such treatment of the male markedly reduces transfer. In one experiment it has been suggested that DNA synthesis in the female is prerequisite for transfer (F. Bonhoeffer, pers. commun.); this controversial result will be discussed at length in Section 3, which is devoted to the role of DNA synthesis in the transfer process.

C. Hfr transfer and the genetic map

When an Hfr cell is mated with an F- cell, the recombination frequency observed is marker-dependent, showing as much as a 10^4-fold range between the maximum and minimum values (13). Although these values are relatively constant for a given Hfr, for each Hfr the marker dependence is

different. By arranging all known markers of the Hfr in the order of frequency of recombination, it is possible to define a genetic map.

Another type of map can also be defined using the technique of interrupted mating. Mating pairs can be physically separated by violent agitation (often called blending since it was first accomplished with a Waring kitchen blendor)(40). Mating can also be terminated by treating the mating pairs with a high multiplicity of a bacteriophage which can adsorb to the males but not to the females (41). If it is asked whether the genetic map defined above (recombination map) is affected by the duration of the mating period using either of these interruption techniques, it is found that the recombination frequency for each marker is time dependent in the following way. Each marker begins to be transferred at a given time in a given Hfr strain (40,41). Partly because of the spread in time of initiation of transfer and possibly because of variation in rate of transfer by individual cells in the population, there is a spread in the time of transfer for each marker; i.e., there is a difference between the time at which the marker is first detectable in recombinants (the time of entry) and the time at which the maximum recombination frequency is reached (20). During this period recombinants accumulate linearly with time and ultimately reach a maximum, plateau value. In general markers which appear at early times reach higher recombination frequencies. Donor ability is not

usually a property of the recombinants, appearing only as a very late (possibly the last) marker and at the lowest frequency. From these experiments a temporal map can be constructed in which markers are arranged in order of their time of entry. From the correlation between the recombination and temporal maps a model for transfer (42) has evolved which is generally accepted today: In Hfr mating, the Hfr chromosome is transferred to the female sequentially from a point called Origin until the final determinant, that of donor ability, is transferred. However, mating pairs are unstable and break apart so that transfer is interrupted at random times. Therefore, more females receive early markers than late markers. The transferred DNA is then integrated by some unknown recombinational process into the female chromosome to form a recombinant.

This model is supported strongly by an analysis of zygotic induction (42). When a prophage which is integrated into an Hfr chromosome is transferred to a non-lysogenic recipient, phage production is derepressed, the phage multiplies, and the recipient cell is thereby lysed. Thus, the presence of transferred prophage can be detected in the female cell without the necessity of considering complexities of the integration process. In this case, again the time of entry and the final frequency of transfer correlate highly with each other and with the position of the prophage attachment site on the chromosome, as determined as an unselected marker in other

matings.

When different Hfr strains are examined, it is found that whereas the gradient of recombination frequencies and the temporal map are constant for a given Hfr, they differ for different Hfr's. The pattern that early markers achieve higher recombination frequencies is not violated though. If the maps for different Hfr's are compared, they are found to be cyclic permutations of one another, except for the position of the marker for donor ability; in some cases the sense of the cycle is reversed. A peculiarity of this permutation is that the marker for donor ability is always transferred last and with the lowest frequency. Hence, formally all of these maps can be expressed as one circular map with each Hfr characterized by having an origin and the marker for donor ability at a particular point and either a clockwise or counterclockwise sense. The map derived in this way is shown in Figure 1.

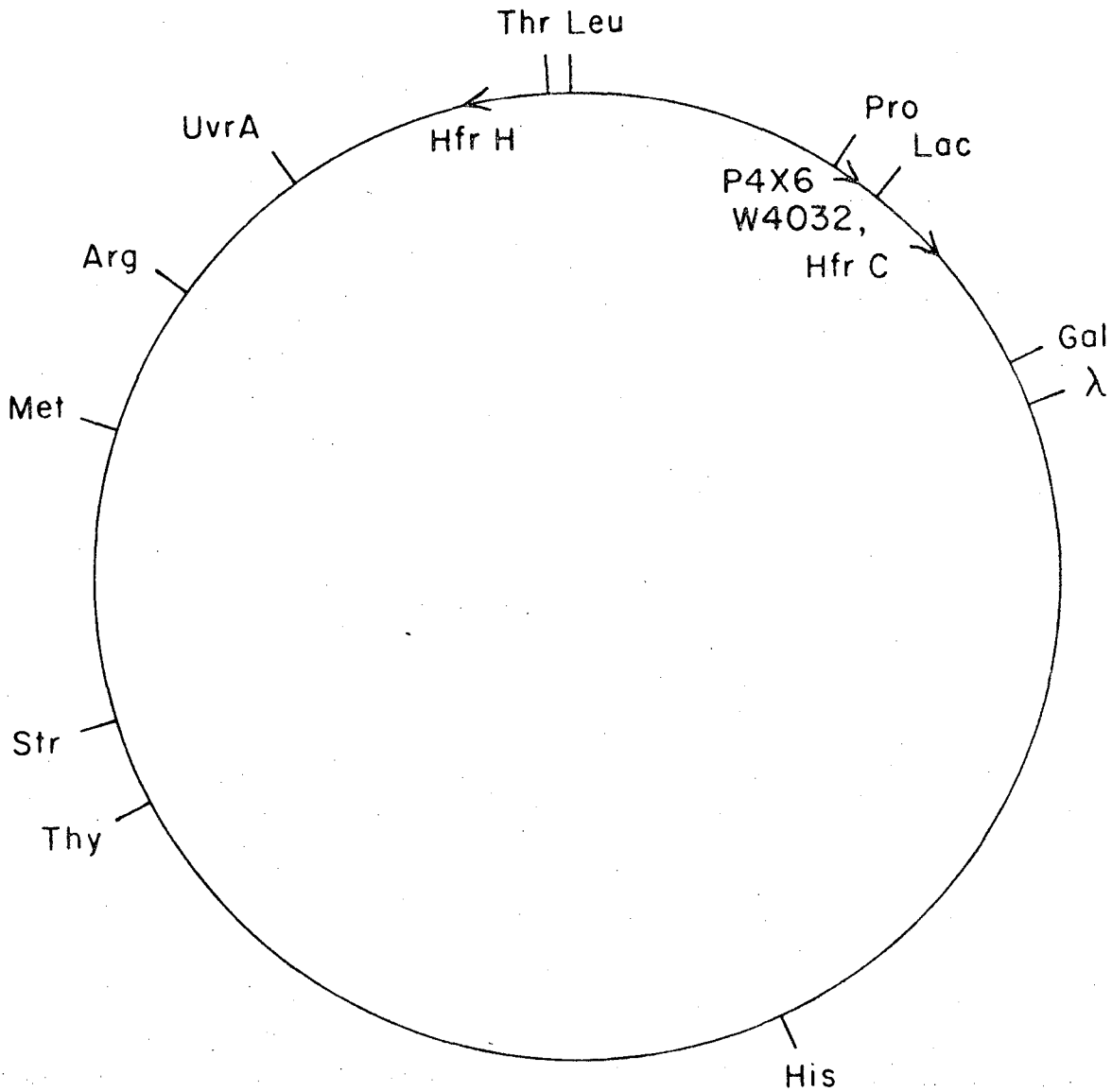
One might imagine that the female map, if it could be constructed, would also be circular and that the Hfr map is a formal consequence of breaking of the circle and attachment of the gene for donor ability or fertility to one of the free ends, the choice of ends determining the polarity of the Hfr. A female map can be constructed from linkage and it is found that in an Hfr mating, the first and last markers tend to be linked, thus implying a formally circular structure (13,43,44).

It might be asked whether the circular map indicates that the bacterial DNA is a giant circular molecule. Whereas

Figure 1-- Genetic map of *E. coli* K12

The symbols correspond to: requirement for arginine (Arg), histidine (His), leucine (Leu), methionine (Met), proline (Pro), and threonine (Thr); ability to ferment galactose (Gal), and lactose (Lac); resistance to streptomycin (Sm) and ultraviolet irradiation (UvrA); and prophage λ.

Arrows: origin and direction of transfer by corresponding Hfr strains. W4032 and Hfr C have the same origin.


Thr Leu
Hfr H
UvrA
Arg
Met
Str
Thy
His
Pro
Lac
P4X6
W4032,
Hfr C
Gal
λ

this need not be the case since the DNA of the female population might consist of randomly permuted linear molecules, there is strong evidence in favor of molecular circularity from the autoradiographic representation of the E. coli chromosome, as shown by Cairns (45). In these autoradiograms grain patterns were seen in the form both of closed structures having a length of 1100-1400 μ and of partially replicated forms.

Since Hfr strains were derived from an F+ cell line, it is likely that the gene for donor ability referred to above is F itself. Other evidence for this is that (i) Hfr populations segregate F+ cells (some "unstable" Hfr lines do this at very high frequency) (10,13,47), (ii) female recipients of Hfr chromosomal material never segregate F unless they have received the marker for donor ability, (iii) F+ and Hfr cells, but not females, are sensitive to certain phages which are called male-specific (17,23,24,25a,25b), and (iv) donor ability can be co-transduced with the final chromosomal marker by the generalized transducing phage, P1, to give Hfr cells (46).

D. Chromosomal transfer by F+ cells and the origin of Hfr's

In view of the existence of Hfr cells and the fact that they are derived from F+ cell lines, it might be hypothesized that the chromosome donating ability of F+ populations is entirely a result of the existence of a small number of stable Hfr cells in the population. Fluctuation tests show that stable Hfr cells do arise in F+ populations (48) but other studies (13, 49,50) show that transfer cannot be completely accounted for by this means. It is likely that occasionally in an F+ popula-

tion the circular chromosome is broken and F is attached to form what may be called a transient Hfr, which could remain for one or more generations. If this transient attachment can occur at a large number of positions (say, 20) which are scattered more or less uniformly around the map, the recombination data for F+ crosses can be explained.

2. Other systems of conjugation

All systems of bacterial conjugation studied appear to be controlled by autonomous cytoplasmic elements, many of which carry determinants of properties other than conjugation, such as drug resistance (the Resistance Transfer Factor, RTF) (51), or bacteriocin production (colicinogenic factor) (52). Some but not all of these elements promote transfer of chromosomal markers.

Fertility in crosses with E. coli strain K12 has been examined for several hundred other strains of E. coli and found in 2-20% of them (53,54); in general, fertility is better between related strains (10,11,55). We know now that in many cases, lack of fertility is a result of host restriction such as that found in phage λ (56,57). For example, strains B and K show very low inter-strain fertility (58). However, clones derived from the rare cells of strain B which have accepted F from K possess the ability to transfer F efficiently to strain B but only rarely to K (59,60,61,62). An understanding of the host restriction phenomenon has recently been used

to develop a receptive strain of E. coli B.(63)

Crosses between donor strains of E. coli and other species of Enterobacteriaceae and even other genera yield low numbers of recombinants. For example, certain strains of Salmonella (64,65,66,67), Shigella (68), Serratia (69), Proteus (70), and Pseudomonas (C. Willson, pers. commun.) can serve as recipients. Strains which have received the sex factor are in some cases able to transfer back to E. coli or to other cells of their own species (68,70,71,72).

Conjugation factors indigenous to other species than E. coli have also been found in Salmonella (73,74), Pseudomonas (75), Vibrio (76,77,78), Proteus (79), and possibly Serratia (80).

3. The F' elements: in particular, F'Lac

The discovery of the transmissible factors (sex factors) regulating genetic transfer in conjugation and their special properties led to the definition of a new class of genetic elements, termed episomes (81). These elements are defined, first, by their accessory nature, i.e., they are not required for growth by wild type cells under normal conditions, and, secondly, by the ability to exist in two distinct, possibly mutually exclusive, states. In one state the particle is autonomous in that it is apparently not associated with the chromosome and replicates independently (although replication may use the machinery and precursor supply provided by the host cell). In the second state

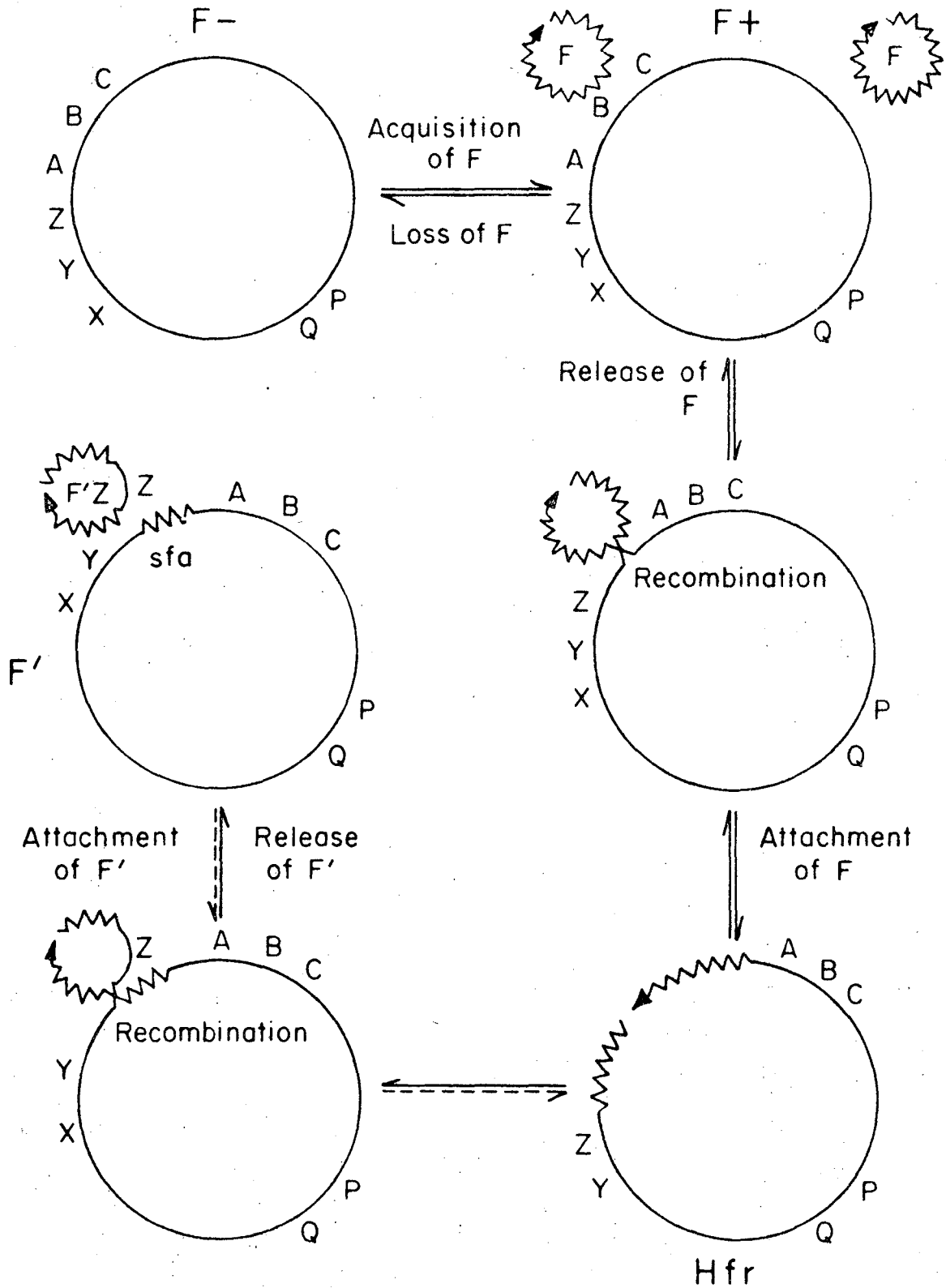
the episome is attached to or integrated into the chromosome. Examples of episomes other than sex factors include temperate bacteriophages, colicinogenic factors, and drug resistance transfer factors (82,83). These elements share many other properties of sex factors but will not otherwise be discussed here.

A. A model for association with and dissociation from the chromosome

On the basis of the fact that the genetic map of the prophage of the E. coli phage λ is a cyclic permutation of the vegetative map, Campbell (82) proposed a simple model in which lysogenization and induction are each the result of a single recombinational event. This model has four essential assumptions: (i) When integrated, the prophage is inserted linearly into the bacterial chromosome. This assumption has recently been proven by Franklin (84). (ii) Prior to insertion into the chromosome, the free phage DNA molecule circularizes. That this can occur for λ DNA has been demonstrated by Hershey and Burgi (85,86). (iii) Insertion is the result of a single cross-over of the circular DNA with the chromosome. (iv) Induction is the result of formation of a loop in the chromosome followed by a single cross-over of the type proposed in (iii). This model, as applied to F, is present diagrammatically in Figure 2. At the present time there is a good deal of circumstantial evidence in favor of these ideas although the model is not rigorously proved. An interesting

Figure 2-- A model for the alternation of states of F: the Campbell model

Zig-zag lines refer to F material; smooth lines to chromosomal material. The arrowheads are the Origins and "breakpoints" of F, F', and Hfr. The letters ABC..PQ..XYZ are hypothetical chromosomal markers. A dashed connecting line means that it is not known whether the transition occurs in the indicated direction. sfa is the sex factor affinity locus, which may not arise in all cases (see text). F-: cell lacking the sex factor; F+:cell containing an autonomous sex factor; Hfr: cell containing an attached sex factor; F': cell containing an autonomous sex factor which carries one or more chromosomal genetic markers


F−
C
B
A
Z
Y
X
P
Q
Acquisition of F
Loss of F
F+
F
F
Release of F
F′Z
Z
sfa
F′
Recombination
Attachment of F′
Release of F′
Attachment of F
Recombination
Hfr

feature of the model is that when the prophage is excised from the chromosome, if the loop were made at the wrong place, a DNA molecule would result which had lost phage markers and gained bacterial loci. This is presumably the origin of λ and other specialized transducing particles. As we shall see shortly, a related situation exists for the F system.

This model has recently been invoked to explain the alternation of states of episomes (4). However, whereas it is likely that F is linearly inserted into the chromosome and therefore satisfies assumption (i), there is no physical evidence, such as is known for λ DNA (85,86) that F can circularize. Nonetheless it is an attractive explanation for the formation of stable Hfr strains from F+ cell lines, the segregation of F+ cells from Hfr strains, and the transfer of chromosomal markers by F+ strains. The best evidence that the essential features of the model are correct is the existence of a new class of particles, F' or F-primes, which arise from stable Hfr cell lines and contain chromosomal determinants. These elements will be described in detail in the following sections.

B. Origin of F' cell lines: F'Lac

F'Lac is one of a number of variants of F in which a portion of the bacterial chromosome has become attached to the free sex factor. A partial list of other such variants is given in Table I. In the case of F'Lac, the incorporated segment includes the locus for fermentation of lactose. It is probable that many other loci are also present in F'Lac

Table I -- Partial List of Known F' Factors

Designation	Genetic Markers	Reference
F_1	F	10
F_2	F; others unknown	88
F_8	F; Gal	47
F_{13}	F; Lac; Pho; Mb; T6; Pur	47
F_{14}	F; Ilva; Met; Arg	116
F_{15}	F; Thy; Arg	86a
F'Lac	F; Lac	87
F'Pro	F; Pro	87

Abbreviations: Lac, lactose; Pro, proline; Gal, galactose; Pho, alkaline phosphatase; Mb, methylene blue; T6, coliphage T6 receptor; Pur, purine; Ilva, isoleucine-valine; Met, methionine; Arg, arginine; Thy, thymine

but these have not yet been detected. Cells containing F'Lac are identified by the fact that they show a high frequency of transfer of the Lac character with concomitant acquisition of maleness by the recipient; chromosomal markers (e.g., proline) are transferred also but with much lower frequency (at most ca. 5% of that for Lac transfer) and without associated transfer of maleness (87). The frequency of transfer of chromosome by F'Lac (ca. 10^{-2}) is however considerably higher than that of normal F+ donors (10^{-7} - 10^{-5} (5,6)). This property, i.e., the capacity to transfer one or a small number of markers with high frequency accompanied by transfer of donor ability and the transfer of chromosomal markers at frequencies intermediate between that of Hfr and F+ is the defining characteristic of the F-variants known as F' or F-primes.

The first F' was isolated by Adelberg and Burns (88) and was called F_2 (F_1 = F). This strain arose from an Hfr strain (in which F is associated with the chromosome) and possessed the property that it transferred chromosomal markers with the same orientation and origin as the Hfr from which it was derived, although at 10% the frequency. Also even though chromosomal markers were transferred at 10% the Hfr frequency, the ability to be such an <u>intermediate donor</u> was transferred with nearly 100% <u>efficiency</u> and thereby resembled transfer of F alone. To explain the origin of this strain, it was postulated that F, in reverting to the autonomous state, had

incorporated into itself some adjacent chromosomal material. Hence because of the homology of the material now present in the episome with the corresponding chromosomal region, there resulted a higher affinity not only for the chromosome but also for a particular region of the chromosome. In fact, F' factors have been shown to recombine with the homologous region of the chromosome in conjunction with F'-promoted transfer of the chromosome (91,92,93). (It is clear that F_2 could not have arisen by a strict application of the Campbell model because, since the same chromosomal material was present in both episome and chromosome, the recombinational event must have been a kind of unequal crossing-over).

F_2 apparently incorporated chromosomal segments which did not include known genetic markers and therefore was difficult to investigate. Thus, other F' particles were sought and indeed when, in Hfr populations in which F was attached very near a known marker, F' strains were looked for by selecting for rapid transfer of a terminal, or F-linked marker, F' factors carrying these terminal markers were found (87). For example, F' strains carrying the Lac locus on the episome can be isolated from the Hfr, P4X6 (see map, Figure 1). In general, in F' strains it is assumed that the incorporated chromosomal marker(s) is added to the sex factor rather than replacing any significant portion of it since the F' appears to retain all <u>known</u> capacities of F-- e.g., autonomous self-replication, ability to transfer, male surface properties of

the cell, and acridine curability (47). Also, if F_2, for example, is in a cell in which the homologous region is absent from the chromosome because of a deletion, the F' behaves much like F (89). (We shall see later that this is also the case for F'Lac.) In addition, from the greater rate of inactivation by P^{32}- decay it can be concluded that F_2 is physically larger (90). One possible exception to the identity of the F region of an F' and F itself is that most F' strains have not yet been demonstrated to become stably attached to the chromosome; in only one case has an Hfr strain been derived from an F' strain (90a). (For this reason, F' particles do not satisfy the strict definition of an episome and should perhaps more properly be termed plasmids, cf. Clark and Adelberg (3)).

In some cases when an F' arises from an Hfr, not only has F acquired chromosomal markers, but also the chromosome appears to have acquired some sex factor material. For example, if the strain harboring F_2 is cured by treatment with acridine orange and the resulting F- strain is re-infected with F, the F+ male is also an intermediate donor showing a high frequency of chromosomal transfer of the F_2 type, i.e., having the same origin and direction. Hence, F, which normally transfers chromosomal markers at low frequency without any preferred direction, is influenced in some way by the chromosome (88). The locus for this chromosomal property of the F- strain maps at the site at which F was attached in the Hfr from which F_2 arose;

this locus is called sfa-2 (sex factor affinity). The interpretation of this finding is that the chromosome has acquired a site of homology with the sex factor, thus favoring attachment at that point. The recombinational event which gave rise to sfa-2 and F_2 is not well understood and certainly involved more than that proposed by the simple Campbell model. Since F_2 remains homologous to the chromosome, if this homology is a matter of identity of base sequence in the DNA, then a net increase in the amount of genetic material must have occurred. The alternative is that when the F_2-containing cell was isolated, F_2 was no longer in a cell having the same genotype as that from which it was originally excised but that it had infected another cell of the population by bacterial conjugation. However, this seems unlikely since its host cell contains sfa-2. A multiple event (either simultaneous or separated in time) or some exchange between replicas seems to be the answer (83).

C. Condition of F'Lac in the male cell

F' strains are generally heterogenotic; that is, they carry two alleles of the marker(s) found on the merogenote, one allele presumably being on the cell chromosome and the other allele carried by the episome. Therefore, from an F' population, numerous cell types can arise. For example, by occasional loss of the episome, F' strains segregate female cells with the recessive female genotype at a low rate, about 10^3 per cell division (87). Recombination can also occur

between the F'merogenote and the homologous chromosomal region in the absence of transfer, producing either haploid segregants (no longer F+ but now Hfr or F-), homogenotes, or heterogenotes of different type from the original heterogenote (94,95). In a few cases recombination has also been observed between the merogenotes of two different F' elements (47).

The properties of F' cells can also be altered by mutation of either the sex factor or the associated merogenote. However, mutations in F are difficult to detect and in fact the detection of defective mutants of the sex factor awaited the isolation of F' particles carrying recognizable chromosomal markers so that the presence of the particle could be established despite the inability of the mutant factor to transfer itself or promote chromosomal transfer. For instance, a mutant of F'Lac (F_{D5}-Lac) which is presumably altered in F has been isolated which confers on the bacterium resistance to male-specific phages (25a), reduced donor ability (by a factor of 10^4), and increased ability to serve as a recipient (96). Temperature-sensitive mutants of F in F'Lac have also been found which have impaired ability to replicate and be transferred (97). The identification of additional F mutations should aid in understanding sex factor function.

F'-merogenotes exist which include prophage attachment sites. These retain the ability to be both lysogenized (98)

and induced (99). Such F' factors obviously can be useful in the study of phage function, as well as function of bacterial markers on the merogenote.

Evidence concerning the number of F' particles per male cell and the mechanism by which this number is controlled is scanty. In several cases the amount of enzyme produced by cells carrying the genetic determinants for that enzyme on the F'-merogenote has been measured and found to be two to three times the enzyme level of a haploid cell (100,101,102, 103). If it is assumed that the amount of enzyme produced is directly proportional to the number of copies of the corresponding genetic locus, one can conclude that the number of sex factors per chromosome in these situations is approximately two to three. However, the validity of this assumption is not known since it has not been possible to do a control experiment with cells having a <u>known</u> number of genetic copies greater than two.

During conjugation, in general only one particle is transferred to a recipient (90,122). However, F' particles appear to reach the normal number per cell soon after transfer (in much less than one generation time) and F can spread throughout an F- population exposed to a few F+ donors more rapidly than can be accounted for by the small number of F particles presumably present in the original donor cells (10,104,106) so

that the sex factor must be capable of replicating more rapidly than the host cell chromosome. Since it is probable that only a relatively small number of copies of F exist per cell, some control of F replication should exist. Sex factor replication is influenced by both the factor itself and/or the host cell. For example, E. coli F'Gal, when introduced into Proteus mirabilis PM-1, multiplies in an uncontrolled fashion until many copies (20-50) are accumulated, as measured by the amount of DNA with characteristic E. coli banding density (S. Brenner, pers. commun.). However, if the E. coli F'Lac is introduced into the same Proteus strain, a smaller amount of E. coli density DNA is found which is consistent with a smaller number of copies per cell. Both F' particles apparently exist in small numbers when in E. coli, judging from segregation data (122; D. Freifelder, pers. commun.).

Another type of control of F' replication exists in that multiplication of a newly-introduced F' is inhibited in male cells carrying either attached F (i.e., in a Hfr) (107) or a previously established sex factor (47,96). However, this inhibition can be overcome since strains have been isolated in which F'Lac coexists with attached F (104) and F'Gal (97, 105) and there is one instance of two attached F factors at different chromosomal sites (108). Some system of repression and immunity similar to that of lysogenic phage is suggested, with the possibility of occasional escape or breakthrough of the repression.

The possibility that the sex factor is located at a

particular site in the cell, possibly on the cell membrane, has been suggested as an explanation for the differential sensitivity to such curing agents as acridine orange (109), which inhibits replication of F and F' particles at dye concentrations which do not affect replication of chromosomal DNA, and for the apparent orderly assortment of the small number of copies of the sex factor at cell division. That the acridine orange is acting at the level of the cell membrane is also suggested by the fact that it is effective in curing only in a very narrow range of pH of the growth medium. The evidence given for this orderly assortment is that daughter cells receiving no sex factor, i.e., F- segregants, are produced less frequently than would be predicted from a random assortment of a small number of particles (e.g., less than ten per dividing cell) between two daughter cells. The observed value is 0.1% F- cells, whereas the theoretical value for nine particles per dividing cell would be 0.5%. However, if it is remembered that in broth-grown cells there are approximately three nuclei per cell, then if the number of particles per cell averages two per nucleus, which is not inconsistent with the gene dosage studies (100,101,102,103), and if F replication usually occurs shortly after cell division, then <u>random</u> assortment of these twelve copies would give the observed number of F- segregants. Definitive experiments could probably be carried out with uninucleate cells, e.g., those grown in a poor, minimal medium (110), but these have not been reported. Therefore, the 0.1% segregation need not compel us

to assume the existence of a special assortment mechanism.

It has also been hypothesized (97) that the chromosome too is associated with the cell membrane-- possibly at the site of the integrated F in an Hfr-- and that this association is involved in the control of replication (vegetative and/or during transfer) and assortment of the daughter chromosomes at cell division. If this is the case, an explanation for the susceptibility of episomes to acridine orange is still needed. Although much of this model is hypothetical, there is some physical evidence which suggests that there may be some form of membrane association. For example, Ganesan and Lederberg (111) have demonstrated that in extracts of Bacillus subtilis the growing point (the most recently replicated part) of the chromosome is associated with a cell membrane fraction. Similar results exist for E. coli (112,113). However, if the site of attachment is that of F attachment, this point cannot be at a unique position in all cells since electron micrographs of mating pairs have revealed a connection or bridge between male and female cells, thought to be the conjugation tube, and this bridge is not confined to any particular area of the male cell (20).

D. Transfer of F' factors

Like F, F' particles can be transferred with high efficiency (up to 80% in 30 minutes). Interrupted mating studies show that the F factor can be transferred in four to five minutes (29,114) and F' particles in a somewhat longer time depending on the size of the merogenote (47,105). For example,

F_{13} is transferred in slightly more than 10 minutes after initiation of mating (47). The fraction of this time spent in preparation for transfer and that for actual material transfer is not known. Since F amounts to ca. 1% of the genome (115) and in Hfr matings, 120 minutes is required for complete chromosomal transfer (42), it is likely that actual transfer of F takes only about one minute.

Transfer of an F' is oriented with the merogenote markers entering in succession, followed by the determinant of maleness (F) usually as the last marker (47). In the case of F_{13} and F_{14}, whose merogenotes are quite large (ca. 5% and 10% of the bacterial chromosome, respectively (47,116)), early interruption of transfer can produce F- recombinants (47,116). Thus, F' transfer appears to be analogous to Hfr transfer except for the number of markers transferred. After transfer the F' particle multiplies rapidly in the recipient, as mentioned previously (10,104,105,106). It is also subject to the same possibilities of recombination and integration with the female chromosome as discussed above in relation to its state in the male cell (94,95).

E. Physical and chemical nature of F'Lac

F and several derivatives (F_2, F'Lac, and F'Gal) were first shown to contain phosphorus by P^{32} inactivation of these factors after transfer from P^{32}-labeled males (117,118). That this phosphorus is contained in DNA has been demonstrated in several ways. Driskell and Adelberg (90) found that if net DNA synthesis is inhibited by the antibiotic, Mitomycin C, at a concentration not affecting RNA or protein synthesis, P^{32}

incorporation into F or F_2 and into the bacterial chromosome are reduced by the same amount, as measured by survival of both maleness and viability. These results also support the idea that RNA is not a major constituent of F. Several workers showed that episome transfer is associated with transfer of H^3-thymine or -thymidine, and therefore of DNA (115,119). Also, if a male strain of E. coli is mated with Serratia marcescens (a female), a small fraction of DNA whose density is that of E. coli can be found in extracts of the Serratia cells (120). Similar experiments have been done by transferring the E. coli F'Lac to Proteus mirabilis (70), and Pseudomonas fluorescens (C. Willson, pers. commun.). Transfer of C^{14}-thymidine has also been observed with transfer of a colicinogenic factor (121).

Silver (119) was able to determine upper limits for RNA and protein transfer in an Hfr mating. The limits obtained per recombinant are 1.5% and 1% of the total cellular RNA and protein, respectively. However, since in the donor cells there is about ten times as much RNA and protein as there is DNA, this limit amounts to more than the DNA transferred, on a weight-to-weight basis. Therefore, the upper limit is still very high and whether the sex factor transfers associated RNA or protein remains unanswered.

There is some evidence that small amounts of cytoplasmic material may be transferred during conjugation. Such substances include lactose (123); a product of UV irradiation which induces λ prophage (124); and a substance which represses λ

phage multiplication (125); transfer of this latter substance was detected only in F+ and not Hfr matings. However, when the repressor of beta-galactosidase synthesis was investigated, no transfer was detected (123,125). In no case has transfer of material from the female to the male been detected. These results suggest that transfer in conjugation is unidirectional but not necessarily restricted to DNA. Transfer of some of these substances may of course be just leakage through the conjugation tube although it is possible that some of them are carried by the DNA.

Estimates of the molecular weight of the DNA of various sex factors were first obtained by analysis of P^{32} inactivation curves. Values obtained include: for F, 120 x 10^6 (118) and 25-75 x 10^6 (90); for F_2, 57-160 x 10^6 (90). Numerous complications involved in P^{32} inactivation, e.g., whether the percentage of decays which are lethal is a constant for all DNA (126,127,128), make these results difficult to interpret. Herman and Forro (115) found transfer of an amount of H^3-DNA equal to 0.5 - 0.9% of the chromosome associated with F transfer. Since it is probable that only one strand of the transferred DNA was synthesized prior to transfer and therefore labeled (see Section 4), the molecular weight of the double-stranded F DNA is 1.0 - 1.8% of the chromosome. Using Cairns' value of 2.2 - 2.8 x 10^9 for the molecular weight of the *E. coli* chromosome (45), a molecular weight of 22-50 x 10^6 is obtained. This value is probably more reliable than those

obtained from the P^{32} inactivation studies. Silver (119) found 3.2 % of the total cellular C^{14}-thymidine transferred per observed recombinant. To determine the amount of F_{13} DNA transferred, this value must be doubled since only one strand is transferred, but also the contribution due to chromosomal transfer must be subtracted from this. Since the mating time was long (90 minutes), transfer of chromosomal material promoted by F_{13} (47) presumably occurred to an extent involving an amount of DNA at least comparable to that of F_{13}. Since data on this point are not available from the experiment, a molecular weight cannot be calculated for F_{13} DNA from this measurement.

Falkow and Citarella (129) have estimated the size of F and of F'Lac (a segregant of F_{13} differing from the F'Lac used in the present work) by measuring the amount of DNA, isolated from strains of Proteus mirabilis harboring this particle, required to saturate agar gel columns containing DNA from various Hfr and F- strains of E. coli. They obtained values of 1.9 and 2.5% of the E. coli genome for F and F'Lac, respectively. Using the Cairns value the molecular weights would be 42-53 x 10^6 and 55-70 x 10^6, in fair agreement with the value of Herman and Forro for F. Since F may have partial homology to many small areas of the E. coli genome (in view of the fact that F integrates to form Hfr's at many sites), these numbers are probably slightly overestimated.

4. Mechanism of transfer and the relation to DNA replication

The physical mechanism for the transfer of DNA from the male to the female is not clearly understood. It is known that it is an energy-requiring process (36,37) and the results of many experiments strongly suggest that DNA replication is somehow involved. In the following some of the more significant experiments and theories concerning the role of DNA synthesis in this process will be described.

There are several well-established facts concerning the replicative process which should be remembered. DNA replication in *E. coli* is semi-conservative, i.e., the parental double-stranded molecule serves as a template for synthesis of two double-stranded molecules and in so doing is divided so that each of the two new "daughter" molecules receives one parental strand (130); hence the conserved unit is the single strand.

Autoradiographic studies by Cairns (45,131) show that the chromosome in *E. coli* is a closed structure and that replication proceeds along this circular structure with only one growing point. Evidence confirming the existence of a single growing point has also been provided by both physical and genetic experiments in E. coli (132,133) and *B. subtilis* (134). As discussed below , it is not yet clear whether the initiation point is unique.

Under normal growth conditions replication continues throughout most of a generation (135,136). Reinitiation of replication requires protein and/or RNA synthesis although, once initiated, DNA synthesis can continue without such

synthesis (133,137).

On the basis of these facts about DNA replication and from genetic data concerning chromosome transfer, Jacob, Brenner, and Cuzin (97) have proposed a simple model for DNA replication in which positive control of DNA synthesis is exerted by determinants found on the chromosome and on F and by the physical relation of F to the chromosome. In a corollary to their model the mechanism of chromosomal transfer in bacterial conjugation was also proposed. Whether or not this theory is correct, it is important in that it has served as a stimulus for a large number of significant experiments. These workers suggested that each independent unit of replication, which they have termed a "replicon", has a unique site, the replicator, at which DNA replication is triggered by an interaction with a hypothetical diffusible gene product, the initiator, which is specific for the particular replicator, and is possibly a protein (hence the requirement for protein synthesis for initiation). Thus, the bacterial chromosome constitutes a replicon, as does an autonomous replicating particle such as F or any F'. (It is actually not clear how independent F is). A second part of the hypothesis is that the replicator is attached to the cell membrane, an idea for which there is some evidence, as was described in Section 3.

The relation between F and the chromosome stated in the replicon hypothesis is that F can be in two states: one in which autonomous F controls its own replication and is therefore an entire replicon and the other, the integrated state, in

which F is replicated as part of the bacterial chromosome. In the latter state its replication is controlled by the chromosomal replicator and is therefore part of the chromosomal replicon. This idea is based upon the one striking difference in the response to acridine orange of free F, integrated F, and the chromosome: F and F' particles are strongly inhibited by concentrations of acridine orange which leave replication of an F- or Hfr chromosome unaffected (109). Because of this inhibition a population of F+ or F' cells can be cured by growth in medium containing acridine orange; however, Hfr cells retain the Hfr character following such treatment (i.e., F is being replicated by the acridine orange-insensitive chromosomal replicon.) . Some role of F in chromosomal replication has however been suggested by Nagata (138) and Messer (pers. comm.), who have shown in several strains of E. coli that the Hfr chromosome starts replicating at or very near the integrated F and with a polarity apparently determined by F. In contrast, the F- chromosome does not seem to have a fixed starting point for replication; if there were such a fixed point in F- cells, it would have to change with respect to the genetic map at least every tenth generation to give the results obtained. However, the interpretation of these results may be questioned in view of the finding of Newman and Wolf (pers. commun.) that in several strains of E. coli (one of them being the same F- strain used by Messer), there is a starting point between the hypoxanthine and xylose loci. Furthermore they found that this point and the polarity of replication are independent of

the presence or absence and the polarity of integrated F. However, these experiments employed amino acid starvation to align the chromosome (in contrast with the logarithmic cells used by Messer and the cells "synchronized" by the filter pile method used by Nagata) and it is always possible that following such treatment, there is a special starting point not normally used. Using the method of chromosome alignment by treatment with phenylethyl alcohol, Tomizawa (pers. commun.) has obtained results similar to those of Newman and Wolf. It is clear that this question is not yet settled.

To account for the apparent control by F in chromosomal transfer, Jacob, Brenner, and Cuzin proposed that when transfer is initiated, the F factor takes control of chromosomal replication. Then under the control of the F replicator, DNA synthesis is re-initiated at the origin (the end ofthe chromosome which is transferred first) and the newly synthesized DNA is transferred to the female. If the mating is not interrupted, the last piece of DNA to be synthesized is F itself which is then transferred. While there exists considerable evidence for DNA replication concomitant with transfer (see below), the fact that Nagata and Messer found the sequence of replication of Hfr markers in normal growth to be opposite to the sequence of transfer (in contrast with the prediction of the theory) poses certain conceptual difficulties for the transition between normal replication and transfer replication-- for example, how to traverse the replication fork. Some of these difficulties are avoided in the model of Bouck and Adelberg (139) according to which

chromosome replication must be completed before transfer and one of the completed replicas is then transferred without further replication. Other models are, for example: that replication of the chromosome occurs simultaneously with transfer but in the recipient rather than the donor cell; that transfer occurs without regard to replication or the state of completion of the chromosome (in which case replication might be necessary only to move the growing point so that transfer could occur across it); that transfer is normally accompanied by replication but can proceed without it; or that transfer requires some replication for efficient and complete transfer but can proceed slowly or partially without it. This seeming proliferation of alternatives (which is by no means exhaustive) will be seen to arise from the unresolved state of many of the experimental findings. Each of these models makes certain predictions about the kinetics of transfer, effect of inhibition of DNA replication, and the nature of the transferred DNA, and experiments supporting or refuting one or more of the predictions have been reported.

In evaluating these experiments, some of which shall be discussed below, the following questions must be considered:

Is F transfer normally by the same mechanism as Hfr transfer?

How many chromosomes does the donor cell contain under the experimental conditions, how many are involved in transfer, and how might this affect the results?

Does the DNA synthesis which is observed during transfer

mean that DNA synthesis is necessary for transfer?

When inhibitors of DNA synthesis are used, is DNA synthesis actually measured during a mating, can the observed level of transfer be accounted for by residual synthesis, and is DNA synthesis the only process affected by the inhibitor?

When DNA synthesis is measured under inhibitory conditions is the newly synthesized or transferred DNA biologically active?

Could there be non-lethal mutations and hence strain differences with respect to the mechanisms of DNA replication and transfer?

A. Experiments concerning the kinetics of transfer

The Bouck-Adelberg (BA) model predicts that only those Hfr cells which have just finished and not reinitiated DNA replication will be able to start transfer immediately upon contact with a female; the other Hfr cells will require variable periods of time, up to one generation, to initiate transfer. The Jacob-Brenner-Cuzin (JBC) and other models predict no such delay, although a delay might occur for other reasons. If the period of contact is restricted, Hfr transfer of early markers and hence initiation of transfer reach a maximum in considerably less than one generation time after contact; furthermore the time at which this maximum is reached does not depend markedly upon the generation time of the bacteria (140,141). In other experiments (38) in which contact formation was not restricted, but in which the appearance of male DNA in the female could be detected autoradiographically, most cells started to transfer within less than one generation time

after mixing. These results are not in agreement with the BA model.

B. Experiments involving inhibitors of DNA synthesis

The BA and JBC models both predict that stopping DNA synthesis in the donor cell will affect transfer whereas the other models mentioned above do not. In the model in which replication is obligatory in the recipient cell for transfer to occur (Bonhoeffer), inhibition of DNA synthesis in the female should stop transfer; this is not predicted by any of the other models. The BA model also predicts that cessation of DNA replication in the male should prevent initiation of transfer only in those cells which have not yet started to transfer, but should have no effect on transfer already in progress. In contrast, the JBC model predicts that blocking DNA synthesis in the male should both prevent initiation of transfer and halt transfer in progress.

(i) Suit et al (143) have reported that early marker transfer by cells immobilized on a membrane filter was unaffected by (a) prestarvation of the Hfr for a required amino acid for a period prior to mating which allowed a 10-15% increase in the total DNA, most of the increase occurring in the starvation period; and (b) by UV-irradiation of the Hfr cells to 45% survival, which should give a lag of from a few minutes to an hour during which net DNA synthesis would proceed at 0-10% of the normal rate, depending on the extent of repair, followed by approximately the normal rate of DNA synthesis, if protein synthesis were permitted. These results

would tend to disagree with the JBC model. However, net DNA synthesis was measured during amino acid starvation but not during mating. Also, and probably most important, for early markers the amount of DNA synthesis required by the JBC theory is certainly below the limit of resolution of this measurement.

(ii) In contrast, Gross et al (cited in ref. 143) measured transfer in liquid medium by Hfr cells which had been UV-irradiated to 10-90% survival and found greatly reduced transfer by the survivors and then only after a long lag (presumably due to repair). This tends to support the JBC model. However, DNA synthesis in the irradiated cells was not measured. In general, the effect of liquid versus solid media (used by Suit et al) on the requirements for transfer is not known although it is unlikely that there would be a difference in the transfer mechanism; in view of these differences in experimental results in what appears to be the same system, no conclusions can be drawn from these data.

(iii) Pritchard (141) has described experiments using thymine-requiring Hfr strains mated in the absence of thymine, in which varying results with different strains were obtained. With Hfr R4(thy-) transfer was reduced only about 25% and this varied somewhat with the marker and the time after initiation of mating; with Hfr H(thy-) the reduction was about a factor of three. A short period of pre-starvation for thymine prior to mixing the cells reduced transfer to a greater extent in both cases with a slightly greater effect for Hfr H. Net DNA synthesis in the presence and absence of thymine was measured

although not during the actual mating. In the absence of thymine the reduction of DNA synthesis was about 8-fold. These experiments suggest that there is some role of DNA synthesis but are not unambiguous because of the lack of correlation between the extent of transfer and the amount of synthesis. Furthermore these results are complicated by the poorly understood effects of thymineless death (144), possibly large pools of thymine-containing compounds present during the starvation period, and the leakiness of the particular thymine marker. Also, since mating is usually performed in growth media in which each cell contains several chromosomes (presumably three in these experiments), in experiments involving inhibition of DNA synthesis by deprivation of precursors, the possibility of precursor diversion and therefore preferential replication of the DNA in particular cells or chromosomes (for instance, those engaged in transfer) must be considered. In fact, such a diversion effect can occur, since in slow growth media replication of the several chromosomes proceeds in succession rather than simultaneously (145).

(iv) In experiments in which DNA synthesis in a thymine-requiring Hfr is reduced to a predetermined low level by thymine deprivation and by addition of the DNA synthesis inhibitor, cytosine arabinoside (145,146), DeWitt and Freifelder have found a reduction in the rate of chromosomal transfer (to be published). The effect is again not as great as the reduction in the rate of DNA synthesis measured during the mating. Here

again precursor pools and possible preferential synthesis of the transferred material must be considered. In preliminary experiments the latter seems to be an important factor: when uninucleate cells (prepared by growth in aspartate medium (136)) were used, the effect was considerably greater.

(v) Phenylethyl alcohol has also been used by several workers to inhibit DNA synthesis, with varying effects on genetic transfer (139). In various hands this agent has been reported to inhibit only DNA synthesis (149), to inhibit only the reinitiation of DNA replication (150), to inhibit RNA and protein synthesis (142), to cause extensive cell death (142), and to increase greatly cell membrane permeability (Silver, in press). Results with this agent are not readily interpretable.

(vi) Experiments using nalidixic acid, an agent which blocks DNA synthesis rapidly (151) have shown that in mating cultures transfer is inhibited whether nalidixate is added prior to or during mating (152). These results have been considered to be unambiguous support for the JBC theory. However, it has recently been shown (153) that nalidixic acid, in a manner strongly affected by physiological conditions, causes rapid degradation of DNA to soluble fragments so that transfer of usable DNA would presumably be stopped by this agent whether or not DNA synthesis is involved in mating.

(vii) Jacob, Brenner, and Cuzin (97) reported isolation of temperature-sensitive mutants of F'Lac which are unable to replicate at 42° and which show impaired mating ability at <u>both</u>

high and low (30°) temperature. The host bacterium itself grows normally at 42°. The relation between mating ability and replication in these mutants is not clear especially since, in addition to inhibition of replication, growth at 42° probably led to actual inactivation of some or all of the functions of the particle since the rate of loss of the Lac function was greater than that expected from simple dilution by cell growth. In the same paper it was reported that there is greater than 99% inhibition of both Hfr and F'Lac transfer when mating was carried out in the presence of acridine orange. These experiments suggested to Jacob et al that the F replicator is responsible for Hfr transfer and F' transfer. However, Freifelder (pers. commun.) was able to observe only a small and variable inhibition (0-40%) using the same conditions. Furthermore, the kinetics of loss of F'Gal and F'Lac from an acridine orange-treated culture are such that the inhibition of F' replication must be only partial (122; D. Freifelder, pers. commun.) In addition there is a strong dependence on the growth medium and the concentration of the dye, in agreement with the results of Hirota (109). However, at concentrations at which inhibition of F replication becomes very great, inhibition of chromosomal replication also sets in, thus complicating the interpretation of experiments of this type.

(viii) Recent experiments by Bonhoeffer (pers. commun) suggest, in contrast with all work found in the literature, that it is DNA synthesis in the female and not the male which is important. Hfr and female strains were isolated which were

unable to synthesize DNA at 42°, although synthesis was normal at 37°. At the higher temperature the kinases and polymerase in extracts behaved normally; the step which is blocked is not known. To determine the extent to which residual DNA synthesis occurs in these strains at 42°, the uptake of H^3-5-bromouracil into material with hybrid density (which distinguishes new replication from small areas of repair) was measured; unfortunately, even though this measurement was carried out under the same conditions used in mating except for cell concentration, it was never done during an actual mating so that it is possible that other effects were occurring. Nevertheless, under these conditions less than 0.01% of the DNA was replicated. When these two temperature-sensitive male and female cells were mated with normal females or males, respectively, at both 37° and 42°, it was found, contrary to expectations, that inhibition of DNA synthesis in the recipient reduced the appearance of recombinants (transfer, as measured by zygotic induction, was not determined) essentially to zero (less than 10^{-4} the control value) while thermal inhibition of the donor was without any significant effect (there may have actually been a small increase). The inhibition of transfer by heating the temperature-sensitive female was shown to be reversible in that late markers were transferred, if after a temperature shift-up during mating, the temperature was returned to the lower value before the mating couples were separated by blending. Bonhoeffer considered the possibility that the difference between the effect of high temperature in the

male and in the female might be a result of different genetic defects or that synthesis in the Hfr was somehow reactivated by contact with the females, possibly by some exchange of small molecules through the conjugation tube. This possibility was eliminated by mating a normal Hfr with the temperature-sensitive Hfr which had been converted to a female phenocopy. Again it was found that at elevated temperatures recombination was eliminated. This experiments also rules out the possibility that at high temperature DNA synthesis and recombination are prevented by production of a substance which inhibits both processes.

These experiments, which contrast with several sets of experiments in which inhibition of DNA synthesis in the female by adenine starvation and by UV irradiation did not inhibit transfer (38,154), should be carefully considered; an unambiguous interpretation of them must await and understanding of the nature of the high temperature block.

C. Experiments on the nature of the transferred DNA

The JBC model and the Bonhoeffer model predict that one strand of the transferred DNA will be "old", i.e. existing before the initiation of transfer, and that the other strand will be "new", i.e., synthesized during transfer. The BA model and any other model in which DNA replication is in no way involved in transfer predict that both strands transferred <u>may</u> be old. Several experiments have been reported which bear on this question. In each the donor DNA is labeled before or during transfer and the state of the label is determined after mating is terminated.

A difficulty in the interpretation of some of these experiments is that of ascertaining the extent to which the initiation of labeling and transfer are simultaneous; that there is a time-spread in each, particularly in the initiation of transfer, is well-known (140).

(i) Quantitative autoradiography has been employed by two groups of workers. Herman and Forro (115) investigated transfer of the F particle alone as follows. Streptomycin-sensitive (Sm^s) donor cells were grown in H^3-thymidine until uniformly labeled, then mated with Sm^r F- cells in the absence of H^3, blended, plated on Sm agar, and allowed to grow into microcolonies of about 50 cells. The amount and distribution of label among the cells of the microcolony was determined auto-radiographically. The number of colonies containing any label at all was approximately equal to the number of Sm^r colonies which possessed the F character, when tested independently, so that the label could be assumed to represent transferred F. Usually all of the grains were loaclized in only one cell of the microcolony; when label was found in more than one cell, the total number of grains in the colony was no higher than when all of the grains were in one cell. Such dispersion of label was sufficiently rare that it could be concluded that the label was contained in a single, conserved unit. The interpretation of this result is that pre-labeled F replicated in the unlabeled mating mixture either before or during transfer, so that only one of the transferred strands was labeled; the rare dispersion of label could be explained by fragmentation

of the single strand. Hence, transfer of F without replication probably does not occur.

(ii) In another autoradiographic experiment, in this case with Hfr transfer, Gross and Caro (38) studied the kinetics of appearance of label into transferred chromosomal material. DNA synthesis by the F- cell was prevented by adenine starvation or UV irradiation. Residual incorporation by the F- cells alone was measured and found to be on the order of 10-20% as much as the label appearing during the Hfr transfer and the results were corrected for this background when appropriate. Transferred material was recognized by the appearance of label in the F- cells, which were morphologically distinguishable. From experiments in which label (H^3-thymidine) was added either continuously at various times before or during mating, or in pulses, it was concluded that DNA synthesis occurs simultaneously with transfer. Furthermore, from the fact that labeling of material transferred from a thymine-requiring Hfr was heavier than from an Hfr not requiring thymine, it was argued that this synthesis uses thymine primarily from the Hfr precursor pool rather than from the F- pool. These experiments probably provide the best evidence in support of the JBC model.

(iii) The nature of the transferred DNA has also been characterized by use of other physical labeling techniques, such as density labeling, P^{32}- decay, and 5-bromouracil sensitization to visible light. Ptashne (99) examined the state of λ prophage DNA contained in an F'Gal(λ) after transfer using a system in which prophage DNA could be released from the

merogenote and incorporated into a mature phage particle without replication. Heavy density-labeled F'Gal(λ) cells were mated with light F- cells in light medium. Immediately after mating the transferred cells were treated so that phage which contained unreplicated prophage DNA were obtained from female cells. No phage particles could be found which contained two heavy strands although many had one light and one heavy strand. From this result Ptashne concluded that the F' particle carrying the prophage replicated prior to or during transfer. However it was also found that if the prophage-carrying F' is transferred from a donor which carried a host-modified λ to a recipient with different modifying properties, the parental modification of the transferred DNA is carried primarily by only one of the DNA strands, rather than by both as would be expected if DNA synthesis occurred in the male. This fact could be used to argue that the replication occurred in the female and not in the male. To avoid this conclusion, Ptashne proposed that modification might normally occur a short time after replication and that in this case transfer to the female occurred before the new strand was modified. In this way, this experiment is taken as support for the JBC model although it is clear that if the latter (unproven) assumption were incorrect, the results might then support the Bonhoeffer model.

(iv) Freifelder (154) labeled F'Lac with 5-bromouracil during transfer by mating a thymine-requiring male with an adenine-requiring female previously starved for adenine, in the presence of 5-bromouracil and in the absence of adenine. Under

these conditions the female synthesized virtually no DNA. After mating and blending, the cells were irradiated with either UV or "visible light", and plated on medium on which the male parent could not grow and on which the ability to ferment lactose could be scored. It is known that cells containing 5-BU in their DNA are more sensitive to UV than normal cells and can be inactivated by wavelengths of visible light to which normal cells are resistant (155). With UV irradiation the Lac+ character was inactivated in a 5-BU mating at a rate much greater than that of female cells alone or than that of the Lac+ character in a thymine mating. This inactivation was exponential without the appearance of a resistant fraction to a survival level of less than 1% indicating that more than 99% of the transferred F'Lac DNA contained 5-BU, although replication in the female did not occur. Hence, fewer than 1% of the male cells transferred F'Lac without any replication and replication must have occurred before or during transfer. With visible light irradiation of the 5-BU mating the Lac+ character was inactivated to the extent of 70%, the remaining 30% being completely resistant. Fox and Meselson (156) have found that only 50% of a λ phage population whose DNA is labeled with 5-BU in only one strand can be inactivated (presumably because damage in one of the strands is not lethal). If it can be assumed that the 5-BU-containing F'Lac would behave analogously, one may conclude that 40% [100 - (2 x 30%)] must be labeled in both strands and must have replicated at least once in the male before the final transfer-associated replication. This is not

unexpected because in the 30 minute mating period with conditions of continued contact formation, replication could have occurred prior to the completion of a later cellular union. The conclusion from these experiments is that DNA synthesis occurs prior to or during transfer since the adenine starvation of the female should prevent synthesis in the female (i.e., consistent with both JBC and BA models and in conflict with the Bonhoeffer model). However, it conceivable that sufficient adenine could leak from the male through the conjugation bridge to serve to replicate this small particle in the female. An appropriate control would have been to mate the combinations, Thy+ male x Thy- female and Thy- male x Thy+ female, since Thy+ cells will not utilize 5-BU efficiently.

It is clear that the experiments of Ptashne and of Freifelder, while suggestive that replication occurs prior to or during transfer, were not rigorously controlled since replication in the female cannot be ruled out. However, the experiments of Herman and Forro, in which only one conserved unit is transferred, state more strongly that the DNA synthesis must have occurred in the male.

(v) Blinkova, Bresler, and Lanzov (157) used P^{32} to label DNA synthesized during Hfr mating and examined the inactivation of the transferred markers by P^{32} decay during subsequent storage of the zygotes. It was found that markers transferred during the time P^{32} was present in the mating medium showed rapid inactivation compared to a marker transferred 15 minutes after the removal of P^{32}, which was inactivated only at the slower rate

of the whole female population. In order to decrease DNA synthesis and therefore P^{32} incorporation in the female, tryptophan (required by the female) was omitted from the mating mixture. Indeed this reduced the killing of the female population but since DNA synthesis was not eliminated, it is possible that transferred DNA was replicated in the female. Admittedly there is no precedent for the replication of the DNA immediately after transfer, but until the possibility is eliminated, it must be considered. If this complication may be ignored, one may conclude that replication occurs during or not more than 15 minutes before transfer. Clearly this experiment cannot at present be considered unambiguous. In a second experiment in which Hfr cells were "synchronized" by filtration, it was shown that the time of entry of early markers is independent of the portion of the life cycle from which the Hfr's were taken (which disagrees with the predictions of the BA model). However, quantitative aspects of the rate of change of cell number with time (e.g., the increase in cell number in equal steps and the lack of the expected loss of "synchrony" after two or three steps due to the distribution of generation times) makes one question whether the cell population was indeed synchronized with respect to DNA synthesis.

(vi) Jacob, Brenner, and Cuzin (97) examined the DNA obtained from unlabeled females which had been mated for 25 minutes in light density medium with Hfr's prelabeled uniformly with heavy isotopes and H^3. DNA from the males was removed by lysis-from-without of the males followed by DNA-ase treatment; the

efficiency of this removal was such that all label isolated from the female could be assumed to be transferred DNA. The density of the transferred DNA was found to be predominately hybrid, i.e., half heavy and half light, although there was a large heavy density shoulder. A control culture of heavy males incubated under the same conditions but without females yielded DNA primarily of the heavy density with a shoulder in the hybrid region. This is unexplained since in 25 minutes it would be expected that most of the DNA would have replicated and therefore been hybrid. From these two results it was concluded that the heavy DNA in the mated system was contaminating non-transferred DNA from the male and therefore that all transferred DNA replicated in the male during transfer. However, this result, i.e., hybrid plus heavy DNA, is what also would be expected if the BA model were correct, since the first DNA to be transferred would consist of DNA replicated at the end of the replication cycle and this would be followed by heavy DNA which had replicated before mating. A kinetic analysis should yield some information on this point.

D. Conclusion

It is evident that no definitive model can be derived from all of the experiments concerning the mechanism of DNA transfer in conjugation. It is clear that DNA synthesis is somehow involved. There is a great deal of suggestive, though not rigorous, evidence that replication normally accompanies transfer and that it is not necessary to complete a round of replication of the chromosome before initiation of transfer. However, at the present

time it is not known with any certainty (a) if DNA synthesis is a <u>necessary</u> condition for transfer, (b) how DNA synthesis and transfer are related, and (c) whether Hfr, F, and F' transfer occur by the same mechanism.

Chapter 2-- Materials and methods

A. Bacterial strains

The bacterial strains of *E. coli* K12 are listed in Table 2. For growth of bacteriophage, *E. coli* B(B) or B/r(Thy-) was used. *Proteus mirabilis* PM-I/F'Lac was a gift of S. Falkow. *Pseudomonas aeroginosa* 1C was originally obtained from B. W. Holloway. All strains were maintained on Difco Nutrient Agar slants supplemented with 10 μg/ml thymine.

B. Bacteriophage strains

E. coli phage T4 O^r (osmotic shock resistant) is a laboratory stock originally obtained from P. F. Davison. T6r+ was obtained from S. Luria. Phage T7M is described in Davison and Freifelder (128).

C. Liquid media

(i) Minimal medium

A stock called 10X salts contained per liter

NaCl	46.76 gm
KCl	14.91
NH_4Cl	10.7
$MgCl_2 \cdot 6H_2O$	2.03
$CaCl_2$	2.22
Na_2SO_4	28.41
Tris	145.32
HCl, 12 N	80 ml, to make pH 7.5

This solution was stored over $CHCl_3$. The minimal salt solution, 1X121, was prepared by diluting 10X salts ten-fold. 1X121 + P was prepared by adding 0.1 M KH_2PO_4 to make 10^{-3} M.

Table 2-- Strains of bacteria used

Strain Designation	Mating Type	Lac(C)	Thy	TL	Bl	Met	Pro	Arg His	Sm	$\emptyset^r$	Other	History
200P	F'Lac	-	+	-	-	+	+	+	s			From F. Jacob
AB1885	F+	-	+	+	+	+	+	+	s			From A. J. Clark
AB1886	F-	-	+	-	-	+	-	-	r		UV^s	From P. Howard-Flanders
W4032	Hfr	del	+	+	+	-	-	+	s			From E. Lederberg
W4132	F-	del	+	+	+	-	-	+	r	T1,5		From N. Franklin
Hfr C	Hfr	+	+	+	+	-	+	+	s			From B. Wolf
P4X6	Hfr	+	+	+	+	+	+	+	s			From A. J. Clark
DF1	F-	y-	-	-	-	(-)	+	+	s			Strain CR34, M. Meselson
DF3	F'Lac	y-	-	-	-	(-)	+	+	r			200P x DF4 [Sm]
DF4	F-	y-	-	-	-	(-)	+	+	r			UV-irradiation of DF1
DF6	F-	y-	-	-	-	(-)	+	+	s	T6		UV-irradiation of DF1
DF18	F'Lac	y-	-	-	-	(-)	+	+	s	T6		200P x DF6 [T6]
DF19	F-	y-	-	-	-	(-)	+	+	s	T6	Az^r	From DF6,nitrosoguanidine

Strain Designation	Mating Type	Lac(C)	Thy	TL	B1	Met	Pro	Arg His	Sm	$Ø^r$	Other	History
DF20	F-	del	+	+	-	(-)	-	+	s	T6		W4032 x DF6 [Thy]
DF47	Hfr	+	-	+	+	+	+	+	s		Az^r	P4X6 x DF19 [Az,Lac]
DF69	F-	-	-	+	+	+	+	+	r		UV^s	DF47 x AB1886 [His,Sm]
DF85	F-	del	+	+	-	(-)	-	+	s		BU^r	UV-irradiation of DF20
DF87	F'Lac	del	+	+	-	(-)	-	+	s		BU^r	DF18 x DF85 [BU]
DF89	F+	del	+	+	-	(-)	-	+	s		BU^r	AB1885 x DF85 [BU]

Notation: F = fertility factor; F'Lac = F' containing marker for lactose fermentation; Lac(C) = chromosomal marker for ability to ferment lactose; Thy, thymine; TL, threonine and leucine; B1, vitamin B1 (thiamine); Met, methionine; Pro, proline; Arg, arginine; His, histidine; Sm^r, resistance to 500 µg/ml streptomycin; $Ø^r$, resistance to bacteriophages; Az^r, resistance to 5×10^{-4} M sodium azide; BU^r, resistance to 100 µg/ml 5-bromouracil; UV^s, extreme sensitivity to ultraviolet, the A gene; +, not requiring; Lac+, ability to ferment lactose; -, requires; (-), not strictly required but growth enhanced if present; del, deletion; y, Lac permease gene; [ ] selection and counter-selection in matings.

Minimal growth medium was made up as needed, usually 10 ml at a time. To 10 ml 1X121 + P was added 0.25 ml of 20% glucose (0.5% final concentration) and supplements when required: amino acids, 20 μg/ml; vitamins, 2 μg/ml; thymine, 10 μg/ml. All stock solutions of these supplements were autoclaved except for threonine, thiamine, and streptomycin which seemed to be degraded by high temperature; these were dissolved in sterile water and sterilized by passage through a membrane filter with 0.45 μ pore size.

(ii) Glucose casamino acids medium

This was 1X121 + P + glucose containing 0.2% Difco casamino acids, purified.

(iii) Tryptone broth

Per liter of water,

10 gm Bacto-Tryptone

5 gm NaCl

This solution normally had a pH of 6.8-7.2. When used for acridine curing, the pH was adjusted to 7.8 with 1 M NaOH, and called pH 7.8 Tryptone.

(iv) Nutrient broth

Per liter of water,

8 gm Bacto-Nutrient broth

5 gm NaCl

(v) Fraser-Jerrel medium (160)

Per liter of water,

4.5 gm KH_2PO_4

10.5 gm Na_2HPO_4

3.0	gm	NH_4Cl
0.3	gm	$MgSO_4 \cdot 7H_2O$
15.0	gm	casamino acids
0.3	ml	$CaCl_2$, 1 M
30	ml	glycerol

The $CaCl_2$ solution was sterilized separately and added to the remainder of the medium after autoclaving.

(vi) Diluting fluid

This was used for routine dilutions for plating and was 10^{-2} M K phosphate, buffered at pH 7.8, 10^{-3} M $MgSO_4$, 10^{-4} M $CaCl_2$.

D. Solid media

(i) Nutrient agar plates

This agar consisted of nutrient broth solidified with 1.5% agar and was used for all routine assays of cell concentration.

(ii) Indicator plates

Sugar-fermenting ability was assayed on indicator dye plates. EMB agar (159) was used initially; MacConkey agar was later adopted since a small minority of positive colonies could be more easily detected on the latter.

Both agars were packaged products from Difco Laboratories and were prepared according to the instructions of the manufacturer, except that they were always supplemented with 10 μg/ml thymine and 10 μg/ml thiamine. When required, sterile streptomycin solutions were added after autoclaving to make 100 μg/ml.

On EMB agar, Lac+ colonies are purple and Lac- colonies pink; on MacConkey agar the colors are bright red and yellow.

E. pH measurement

pH was measured only with a pH meter, standardized before each measurement with a fresh sample of diluted Beckman Standard Solution, pH 7.0.

F. Growth of bacteria

Overnight cultures were inoculated from an agar slant with a platinum wire loop or a sterile 0.1 ml pipette, and grown at 37° in 5 or 10 ml of liquid medium with shaking.

Exponential cultures were prepared by growth in flasks in a 37° room or a 37° water bath with rotary shaking for aeration. Growth was followed by optical density measurements using either a Klett-Summerson colorimeter (with red filter) or a Zeiss spectrophotometer (at wavelengths of 450,540, and 650 mμ for glucose-minimal medium, casamino acids medium, or nutrient broth, respectively). These instruments were calibrated by plating on nutrient agar. A culture was considered to be in exponential growth when its doubling time had remained constant through at least three doublings of optical density. Exponential cultures were used for all experiments involving mating or growth.

To prepare very large quantities of cells (10^{12} or more) a New Brunswick Bench-top Fermentor was used with nutrient broth as the growth medium. Such batches were used for preparing large quantities of non-radioactive DNA.

G. Preparation of radioactive bacteria for labeled DNA standards

E. coli DNA was labeled by growth of thymine-requiring

bacteria in minimal-casamino acids medium containing either H^3- or C^{14}- thymine.

Proteus mirabilis PM-I DNA was labeled with P^{32} by growth in a low phosphate, minimal medium prepared with phosphorus-free casamino acids and sufficient KH_2PO_4 to make 5 µg/ml total P. P-free casamino acids was prepared as follows: To 100 ml of 10% casamino acids was added 3 gm of NH_4Cl and 5 gm of $MgCl_2 \cdot 6H_2O$. The pH was adjusted to 8.5 with concentrated NH_4OH (2-4 ml), the solution was allowed to stand for one hour or more, and filtered. The filtrate was adjusted to pH 7.4 with HCl and stored for use.

Proteus mirabilis PM-I DNA was labeled with C^{14} by growth in minimal-casamino acids medium containing 1 µg/ml C^{14}-thymine and 500 µg/ml deoxyguanosine. The addition of deoxyguanosine provides a source of deoxyribose which enables prototrophic cells to incorporate exogenous thymine (H. Kammen-- pers. commun.).

H. Concentration of cells or change of medium by centrifugation

To concentrate cells or change the suspending medium by centrifugation the cell suspension was centrifuged either in a conical glass tube in a clinical centrifuge at maximum speed (ca. 2000 rpm) for 5-10 minutes or in round bottom Pyrex tubes in an angle head rotor in a refrigerated Servall centrifuge at 6500 rpm for 5 minutes. Small volumes (1 ml or less) were centrifuged in 1 ml pointed Pyrex tubes in the Servall.

I. Curing with acridine orange

Purified acridine orange was kindly provided by R. B. Uretz. Solutions at 1 mg/ml were made in 10^{-3} M versene (to retard fungal growth and to remove trace amounts of zinc) and were stored in the dark.

Male strains were disinfected of the F'Lac particle by acridine curing using essentially the method of Hirota (109). Cells were inoculated at 10^5/ml into pH 7.8 tryptone containing 20 μg/ml acridine orange. After overnight growth in the dark the culture was plated on MacConkey agar. Lac- colonies were picked, purified by repeated streaking, and tested for recipient ability.

J. Mating

Mating was performed by mixing exponentially growing male and female cells at a concentration of 1 - 5 x 10^8 cells/ml in an Erlenmeyer flask of sufficient volume that the mating mixture formed a thin layer (1 - 2 mm thick) on the bottom of the flask. The flask was usually prewarmed. In some experiments this was not possible; in these cases the flask was iced, chilled bacteria were added, and then the flask was quickly warmed to 37°. During the mating period the flask was incubated in a 37° room and swirled very gently on a Thomas platform rotator set at minimum speed. This provided aeration with minimum turbulence. This method was found to increase the rapidity and final level of F'Lac transfer by about a factor of 2 to 4 with respect to mating carried out in a stationary flask, as shown in Table III. Matings were usually

Table 3

Effect of very gentle shaking on the efficiency of transfer of F'Lac

Mating time, minutes	A Broth Shaking: +	A Broth Shaking: -	B Casamino acids Shaking: +	B Casamino acids Shaking: -
0	0	0	0	0
20	14	5	20	18
30	20	7	33	17
40	37	10	61	20

A. Strains 200P (F'Lac) and DF4 (F-) were grown in nutrient broth to exponential phase and 3 x 10^8 cells/ml. In each of two 250 ml Erlenmeyer flasks were mixed 2.5 ml of each culture. The flasks were incubated in a 37° room, one stationary (-) and the other with very gentle shaking (+) on a Thomas platform rotator. Samples were taken at the times indicated, Vortexed 30 seconds, and plated on indicator plates. The per cent transfer was calculated as [Lac+Sm^r recombinants]/[initial males]. It should be noted that this is a fairly poor mating couple; DF4 was found to show much higher mating efficiency with males subsequently developed from DF1 (e.g., DF18).

B. Same as above except that the cells were grown and mated in glucose-casamino acids medium instead of in nutrient broth.

continued for 30 - 60 minutes and stopped by violent agitation for 30 seconds with a Vortex mixer.

K. Irradiation of bacteria with ultraviolet (UV) light

Bacteria to be irradiated with UV light were suspended in cold 1X121 + P and placed in a thin (1 - 2 mm) layer in a glass Petri dish-- e.g., 5 ml in a 8 cm diameter dish. Agitation during irradiation was provided by a Thomas vibrating platform shaker. Irradiation was done with a GE 15 watt germicidal lamp. The dose rate was 70 ergs/mm^2/sec, at the level of the sample, as measured by a photoelectric cell calibrated against an Eppley thermopile. Irradiated cells were protected from light at all times to avoid photoreactivation.

L. Growth and purification of phage

Unlabeled phage were grown and purified as described in Davison and Freifelder (128). All phages were banded in CsCl before use.

To prepare radioactive phage, the host was E. coli B/r Thy- and Fraser-Jerrel medium was supplemented with 1 μg/ml C^{14}- or H^3- thymine. Banding in CsCl was replaced by sedimentation into a layer of 15% sucrose. Two ml of the phage suspension was layered on top of 3 ml of 15% sucrose and centrifuged for 15 minutes at 15000 rpm in a SW39 rotor in a Spinco Model L ultracentrifuge. The sucrose layer was fractionated by drop collection (see Section S) and the sucrose was removed from the phage fraction by dialysis.

M. Lysis-from-without (34)

This procedure was used to lyse one strain selectively

from a mixture of phage-sensitive and resistant bacteria. To broth-grown cells were added phage T6 at a multiplicity of 100 viable phage particles per sensitive cell. This mixture was incubated at 37° and 0.03 ml $CHCl_3$ was added per ml of culture until the optical density of the culture due to the sensitive cells decreased 4-fold.

N. Preparation of penicillin and lysozyme spheroplasts

Penicillin spheroplasts were prepared by inoculating exponentially growing cells into nutrient broth containing 10% sucrose, 0.01 M $MgCl_2$, and 2000 units/ml of penicillin, and incubating at 37° with very gentle shaking for 2 hours or more (161). The criterion for completion of spheroplasting was rounding up of most of the cells, as seen with a light microscope.

Lysozyme spheroplasts were prepared according to the method of Fraser et al (162) from cells grown to exponential phase in nutrient broth or glucose-casamino acids medium. The cells were resuspended in pH 8.1 Tris (0.05 M), containing 10% sucrose. A solution of crystalline eggwhite lysozyme was added to a final concentration of 50 µg/ml, and the mixture was warmed to 37°. Versene was added to make 10^{-3} M. The mixture was incubated for 3 minutes at 37° without shaking, and diluted with three volumes of prewarmed nutrient broth containing 10% sucrose and 0.01 M $MgCl_2$. The criterion for efficient spheroplasting was sensitivity of a sample to osmotic shock or mild (0.1%) sodium dodecyl sulfate (SDS) treatment, in the presence of Mg, with clearing of the cell

suspension in less than 3 minutes.

Lysozyme solutions were stored frozen at -15° at a concentration of 2 mg/ml in 0.25 M Tris, pH 7.5, for no more than two weeks.

O. Preparation of cell walls by sonication

For preparation of cell walls the cells were broken by ultrasonics using a Raytheon 10 kc sonic generator. Broth-grown cells were washed by membrane filtration and resuspended in diluting fluid at a concentration of $5 \times 10^9 - 10^{10}$ cells/ml. The suspension was placed in an ice bath and sonicated by approximately 5 pulses of 2 minutes each separated by 1 minute intervals for cooling. The criterion for complete disruption was visible clearing of the cell suspension. The sonicate was also assayed on agar plates for remaining viable cells; there was usually a 10^3-10^4 - fold decrease in viability.

P. Lysis of cells from mating mixtures-- Frankel lysis

Cell lysates, in preparation for sucrose gradient sedimentation or agar-gel analysis, were prepared by a lysozyme- detergent method developed by Frankel (163) with the single alteration that 100 μg/ml instead of 50 μg/ml of lysozyme was used. The cells were washed and concentrated and resuspended in a solution containing 0.1 M NaCl and 0.05 M versene, pH 8.0, in a 1 ml pointed glass centrifuge tube. The suspension was quickly brought to 45° and incubated for 4 minutes, after which lysozyme was added and incubation was continued for another 4 minutes; then 5% SDS was added to give 0.3% and incubation was continued for 10 minutes at 45°, by which time the

suspension had cleared completely, resulting in a viscous, water-white solution. For complete lysis it was found necessary that all glassware associated with this procedure, including that for storage of cells, be cleaned with chromic acid-- a normal detergent wash was not sufficient.

Q. Isolation of DNA by phenol extraction

In all of the phenol procedures, neutralized phenol was used. Phenol was neutralized by Vortexing one volume of phenol (Mallinkrodt--"liquefied, for chromatographic use") with two volumes of 0.02 M versene, pH 7.8. The two phases were separated by centrifugation for 1 minute at 2000 rpm in a clinical centrifuge. The lower layer was removed with a pipette and used within 10 minutes. Neutralized phenol should never be stored for more than an hour.

(i) Usual method (164,165): Bacteria were concentrated to 10^{10}cells/ml and suspended in 0.1 M versene, pH 7.8. SDS was added to make 0.5% and the mixture was heated to 70° for 10 minutes. An equal volume of phenol was added and the mixture was shaken vigorously for 10-30 minutes. The phases were separated by centrifuging for 5 minutes at 5000 rpm in a Servall refrigerated centrifuge. The aqueous layer was removed and adjusted to 0.5 M NaCl. Onto this was layered two volumes of 95% ethanol. The layers were stirred gently with a glass rod and the precipitated DNA fibers were collected by spooling. The fibers were redissolved immediately in 0.01 M versene, pH 7.8, the ionic strength was again raised to 0.5 M with NaCl, and the fibers were reprecipitated with ethanol. The fibers

were immediately dissolved in 0.01 M PO_4, pH 7.8, and stored in solution at 4°.

(ii) When radioactive DNA was prepared as test DNA for use in agar-gel columns, the precipitate was immediately dissolved in 0.01 M PO_4, pH 7.8, and to reduce losses, the second precipitation was omitted.

(iii) When P^{32}-labeled DNA was prepared from Proteus mirabilis for agar-gel analysis, it was necessary to remove RNA. Since only denatured DNA was required in the agar-gel column technique, RNA removal was simplified. The cells were lysed with SDS (1%), phenol extracted as above, and the DNA was precipitated with ethanol. The precipitate was then boiled for 30 minutes in 1 M NaOH. This procedure both hydrolyzes the RNA to nucleotides and denatures the DNA. The solution was then neutralized with HCl and used without further purification.

(iv) DNA preparations for sucrose gradient centrifugal analysis: For extraction of the DNA from mating mixtures lysed by the Frankel procedure, equal volumes (0.1 ml usually) of lysate and neutralized phenol were mixed in a 1 ml pointed Servall centrifuge tube and the mixture was Vortexed for 20 seconds. The cell concentration was always high enough that the solution viscosity provided self-protection (166) against hydrodynamic shear degradation.

R. Preparation of phage DNA from C^{14}-labeled phage

DNA from phage T7 was prepared by heating the phage for 5 minutes at 70° as described by Davison and Freifelder (164).

DNA from phage T4 was prepared by a modification of the

phenol extraction procedure of Mandell and Hershey (165). 0.1 ml of a phage suspension was placed in a conical centrifuge tube and an equal volume of phenol was added. The two phases were mixed by gentle pipetting with a Pasteur pipette. The phases were separated by centrifugation. This procedure yielded unsheared DNA using the criterion of sedimentation homogeneity (166).

Both T4 and T7 DNA were stored as frozen solutions.

S. Sucrose gradient centrifugation

Sucrose gradients were formed using the apparatus described by Britten and Roberts (167). The composition of the solutions used to form the gradient was 0.5 M NaCl, 0.01 M K phosphate, pH 6.8, and either 5% or 20% (weight/total weight) sucrose. A shelf of 60% sucrose was placed in the bottom of the centrifuge tube to prevent pelleting of rapidly sedimenting material (112). All solutions were autoclaved and stored at 4°. The material to be sedimented was layered on the gradient using a 50 µl capillary pipette ("Microcaps"-- Drummond Scientific Co., Broomal, Penna.) or a 1 ml Pasteur pipette, for 5 and 30 ml gradients, respectively. Centrifugation was done in a Spinco Model L ultracentrifuge using either the SW39 or SW25 rotor, at 3-5°. Fractions were collected by puncturing the bottom of the tube and collecting drops using the apparatus designed by Szybalski (168). Usually 60-70 drops per ml were obtained and samples of 5-40 drops were taken.

T. CsCl centrifugation

CsCl gradients with a final volume and density of 2.4 ml

and ca. 1.700 gm/ml were prepared by dissolving 2.362 gm CsCl in 1.753 gm of a DNA solution in 0.02 M versene, pH 7.8. The solution was placed in a 5 ml centrifuge tube and 2.5 ml of silicone oil was layered on top. This solution was centrifuged for 48 hours at 38,000 rpm. The gradient was fractionated by drop collecting and the radioactivity of the fractions determined

U. X-irradiation of DNA

Bacterial DNA was fragmented by X-irradiation in preparation for agar-gel chromatography (see Section V) using a Norelco MG 150 kv X-ray machine (Tube E) operated at 150 kv, 12 ma without filtration. The dose rate was determined by $FeSO_4$ dosimetry (169) and was 10,600 rads/min. The standard dose was ca. 32,000 rads, which under the conditions used, introduces a sufficient number of single-strand breaks that the single-strands of the DNA had an average molecular weight of 200,000-300,000. (170).

V. Agar-gel chromatography

The technique was essentially that of Bolton and McCarthy (171). DNA of known type was prepared by phenol extraction, denatured by boiling for 5 minutes in 0.01 M PO_4, pH 7.8, and trapped in 3% agar (Ionagar #2, Colab, Inc.) by mixing hot, denatured DNA with an equal volume of melted 6% agar. The hot mixture was rapidly solidified by pouring it into a large, iced beaker. The DNA-agar was then made into small beads by pressing through a fine (#40) stainless steel wire mesh, two or more times. The beads were washed in a sintered glass column with 2X SSC (SSC = 0.15 M NaCl, 0.015 M Na citrate) at 60° to remove untrapped DNA. Sufficiently

concentrated DNA was used so that the final concentration of trapped DNA was 300-500 μg/gm of gel. The final quantity of DNA in the gel was measured by heating 0.1 gm of agar in 0.9 ml 5 M $NaClO_4$, pH 7.4, at 60° until a clear solution resulted. The optical density at 260 mμ and 280 mμ was then read against a $NaClO_4$-agar blank and the DNA concentration thereby calculated.

In preparation for column analysis the DNA which was to be tested (usually radioactive) was suspended in 2X SSC, denatured by boiling for 15 minutes, and fragmented by X-irradiation to pieces of about 200,000-300,000 molecular weight. A mixture of 0.2 ml DNA and 0.5 gm DNA-agar was placed in a small screw-cap vial and then incubated for 15-17 hours at 60°. Under these conditions the test DNA renatures with homologous DNA in the agar-gel. After this incubation the mixture was transferred to a filter tube (cylindrical glass tube, 1 cm diameter, closed at one end by a disk of coarse, sintered glass) and eluted.

The basic elution procedure was as follows. The column was first washed with 20 ten ml aliquots of 2X SSC at 60° to remove unbound DNA. The bound material was then eluted with 10 ten ml aliquots of 0.01 X SSC at 75°, at which temperature and ionic strength the DNA hybrids melt and release the labeled fragments. Elution was accomplished in either of two ways: (i) Old method. The column was maintained at the desired temperature by wrapping plastic tubing around the filter tube and running thermostatted water through it. Column

temperatures were monitored by a thermometer inside the filter tube, the top of which was stoppered with a wad of paper tissue. The eluting liquid was preheated to the desired temperature and added to the column with a warm pipette. Each eluate was collected in a test tube. This procedure, although usable, had the disadvantage that temperature control was difficult. (ii) New method. 10 ml aliquots of eluting liquid were maintained at the desired temperature in the collecting tubes in a thermoregulated water bath. The filter tube containing the agar-gel was placed in the eluting fluid and the eluent rose through the sintered glass filter thus soaking the agar-gel. After 10 minutes the column was lifted out of the liquid; liquid remaining in the agar was blown into the collecting tube; then the column was transferred to the next tube of eluent. This method permits excellent temperature control, rapid change of temperature, and simultaneous elution of several columns.

Carrier DNA was added to the eluates and the solutions were TCA-precipitated and counted. The % test DNA which was bound was calculated as [cpm eluted at 75°]/[cpm recovered]. Melting and counting of the agar-gel after the final elution showed that less than 5% of the total radioactivity remained bound to the column.

The ability of these columns to distinguish homologous from non-homologous DNA was tested, although this has already been amply demonstrated by Hoyer et al (171). Homologous DNA from E. coli, Ps. aeroginosa, and Proteus mirabilis bound

with efficiencies of 60-70%. (For absolute values this should be multiplied by 1.2-1.5 (171)). Non-homologous DNA bound to an extent of less than 2%, an amount comparable to what is observed if the agar contains no DNA.

To obtain a melting curve of the bound material, the procedure of Falkow and Citarella (129) was used. The column was first washed 20 times with 2X SSC at 50° to remove unbound DNA and then eluted with 3 five ml aliquots of 0.1 X SSC at each of a series of increasing temperatures (usually in 2-4° intervals) up to 83°, at which temperature the gel begins to break down. A melting curve so obtained for E. coli DNA bound to an E. coli agar-gel column was very much like an optical melting curve for denatured and renatured DNA melted in 0.1 X SSC.

W. Preparation and counting of radioactive samples

Radioactive samples were precipitated with an equal volume of 10% trichloracetic (TCA) containing 100 μg/ml thymine (t). When the samples consisted of free DNA, carrier DNA was first added. The precipitate was collected by suction onto a 25-27 mm diameter membrane filter having a pore size of 0.45 μ, which had been prewashed with TCA + t, in order to saturate the thymine adsorption sites. The sample was then washed five times with TCA + t. When large amounts of soluble radioactive thymine were present, the filter was then washed with five rinses each of ethanol, to minimize adsorption of heterocyclic compounds, 1 M NaOH, to break open the cells and release any unincorporated thymine trapped

inside the cells or in protein aggregates, and TCA + t, to remove the NaOH and prevent the filters from becoming sticky while drying. This rinsing procedure was found to remove variable, large amounts of radioactivity associated with TCA-precipitated whole cells in highly radioactive medium. Evidence that the method does not remove precipitated radioactive DNA is that repeating the procedure did not significantly reduce the radioactivity and that if precipitated, labeled phage DNA was collected on a filter, application of this technique did not reduce the precipitated activity. This is probably because DNA is insoluble in ethanol and although DNA is alkali-soluble, denatured DNA will not redissolve in alkali at high ionic strength. Furthermore, DNA, and especially denatured DNA, binds strongly to membrane filters (172,173,174).

When Proteus DNA labeled with P^{32} was to be counted, because of the high concentration of P^{32}-containing free ribonucleotides present in the DNA solution (see Section Q) it was necessary to presoak the membrane filter with 1 M KH_2PO_4 prior to collecting the precipitate in order to prevent adsorption of these compounds.

After washing, the filters were dried on metal trays, either under an infra-red lamp or in a 60° oven. For liquid scintillation counting the filters were placed directly into glass scintillation vials containing 10 ml of a scintillation fluid having the following composition:

2,5-diphenyloxazole (PPO)	6.0 gm
1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP)	0.3 gm
toluene	1 l

All samples were counted for a minimum of 10 minutes or 10,000 cpm in a Nuclear Chicago Model 720 liquid scintillation counter.

Sometimes samples labeled with C^{14} only were counted with a gas-flow counter. For this purpose the membrane filters containing the precipitated sample were glued, while still wet, to aluminum planchets, dried, and counted in a Systron gas-flow counter with a 0.001 inch Mylar window.

X. Induction and assay of beta-galactosidase

Beta-galactosidase was induced and assayed by the method of Pardee, Jacob, and Monod (123). Cells were grown in 1X121 + P + casamino acids with 1% glycerol as the carbon source. The inducer, thiomethylgalactoside, was added to yield a concentration of 5×10^{-4} M and the induction mixture was incubated for 30 minutes at 37° with shaking. One drop of toluene per ml of culture was added and the mixture was shaken vigorously with a Vortex mixer and allowed to stand for at least 30 seconds. 0.2 ml of a fresh solution of the substrate, o-nitrophenylgalactoside, M/75 in 0.25 M PO_4, pH 7.0, was added per ml of solution and the mixture was incubated at room temperature for a measured period of time until color was visible-- usually about 10-20 minutes. The reaction was stopped by adding 0.5 ml of 1 M Na_2CO_3 per ml. Samples were centrifuged for 5 minutes at 5000 rpm to remove

coarse debris. The optical density at 420 mμ was read with a Zeiss spectrophotometer and corrected for turbidity by reading at 550 mμ and subtracting 1.65 x OD_{550} from the OD_{420}.

Y. Measurement of total protein synthesis by C^{14}-phenylalanine incorporation

The rate of protein synthesis in growing cells was measured by adding 2.5 μl of C^{14}-phenylalanine (5 mc/μM/5 ml) to 1 ml of culture, incubating at 37° with shaking for 10 minutes and precipitating with an equal volume of 10% TCA containing 100 μg/ml phenylalanine. The TCA-precipitable material was collected on a membrane filter, washed with TCA containing phenylalanine, and counted in a gas-flow counter.

Chapter 3-- Preliminary attempts at isolation of F'Lac

In early stages of this work several methods for isolation of F'Lac DNA were tried and abandoned. Each of these methods proved to be inadequate because of unsuspected properties of the cells. These methods are listed in Table 4 along with the principal difficulty.

In method 1, which relied upon male cells remaining intact during the various treatments, the problem of non-specific lysis of the male cells arose. Both growing and stationary cell populations were found to release DNA into the medium at the rate of 0.5-2% of the total DNA per hour at 37°. Handling of the cells such as pelleting tended to increase this leakage and no combination of ionic strength or osmolality could be found which decreased the effect. Since the chromosomal DNA is about 100 times as large as the F'Lac DNA, lysis of a small percentage of the cells can provide a background greater than four times the amount expected from the F'Lac DNA. This phenomenon was also encountered by Silver (119) and was the principal limitation in his experiments.

Method 4 was designed to make use of the observation of Frankel and Smith (pers. commun.) that if cells are treated with SDS and handled gently, lysis does not occur, although the permeability of the bacterium becomes extreme. Such cells retain all of the bacterial DNA but release soluble protein and, if infected with phage T4, phage DNA also. It was hoped that the F'Lac DNA would also be released in this way. The basic experiment was to treat H^3- thymine-labeled males and C^{14}- thymine-

labeled females with detergent, SDS, then mix, and centrifuge the mixture through a sucrose gradient. In several experiments a component with an $S_{20,w}$ of 43 ± 2 was derived from the male but not from the female. However, in some gradients male material was found in this region but small amounts of female DNA were also present. Furthermore, in many gradients no DNA was released by the male. If the cells were exposed to small shear forces so that some of the chromosomal DNA was released, it usually appeared in approximately the same region as the component described above. Whereas it is possible that the component indeed contained F'Lac DNA, this method was not pursued because of its non-reproducibility. It is hoped to explore this further in the future.

The difficulties encountered in the first five techniques plus the fact that it is necessary to ascertain that any labeled material was indeed F'Lac DNA made it clear that a method for specific labeling had to be developed. Methods 6 and 7, which were designed to accomplish this, failed for the reasons given in the Table. However, a technique has been worked out which is effective; the development of this procedure comprises a major portion of the present work and will be described in detail in Chapter 4.

Table 4 -- Methods attempted to isolate F'Lac DNA but abandoned

Method	Basic procedure	Remarks
Group I-- Preferential extraction		
1. Release triggered by female cell walls (175,176,177)	1. Female cell walls prepared by differential centrifugation of sonicated cells. Cell walls mixed with labeled males for 30 minutes. Cells spun out. Supernatant assayed for radioactivity.	1. Males show non-specific lysis. 80-90% of label in supernatant was male chromosomal DNA
Group II-- Transfer to females followed by specific lysis of females		
2. Mating labeled males with female protoplasts (178)	2. Female protoplasts prepared either by lysozyme or penicillin and stabilized with sucrose. Labeled males added. 30 minutes later, mixture treated with SDS in presence of Mg. Normal cells are insensitive to SDS-Mg, but protoplasts are lysed. Lysates examined by sucrose gradient centrifugation.	2. Lysis of males was excessive. No difference in sedimentation pattern of extracts prepared with and without protoplasts.
3. Mating labeled males with females and separation of males and females by selective precipitation at different pH.	3. Labeled males mated with labeled females and blended. Mixture to various pH in range 3-4. Males and females should precipitate at different pH (26,27,28).	3. Only slight enrichment. In mixtures, both mating types co-precipitate because of clumping.

Group III-- Differential centrifugation of cell lysate		
4. Sucrose gradient centrifugation of SDS-treated cells	4. Labeled cells treated with 0.1% SDS, 37°. Cells do not lyse but become very permeable. Mixture centrifuged through sucrose gradient.	4. Partial success. A component was found in extracts of males but not females. Procedure not reproducible. See text.
5. Sucrose gradient centrifugation of SDS lysate of lysozyme protoplasts	5. Labeled females converted to lysozyme protoplasts and then lysed with 0.1% SDS. Lysate handled with care to avoid shearing and centrifuged through sucrose gradient.	5. No difference seen between male and female extracts, due to very broad boundary of chromosomal DNA.
Group IV-- Specific labeling		
6. Inhibition of chromosome replication without inhibiting F'Lac replication. Use of amino acid starvation.	6. Males grown in minimal medium and starved for required amino acids (a.a.), in presence of acridine orange (AO). Chromosomal DNA is replicated to completion but AO inhibits F'Lac replication (109,133). Cells transferred to medium lacking a.a. and AO and containing label. F'Lac should replicate.	6. AO does not inhibit F'Lac replication in minimal medium. Therefore, F'Lac has completed its replication cycle before label is added.
7. Same as 6 but using chloramphenicol (Cm) instead of amino acid starvation.	7. Males grown in tryptone and treated with Cm in presence of AO. Transferred to medium containing Cm, lacking AO, and containing label.	7. Cm does not prevent reinitiation. AO does not stop F'Lac replication completely.

Chapter 4-- A technique for specific labeling of the DNA of F'Lac

In this section a procedure will be described for specific radioactive labeling of F'Lac DNA. In essence this procedure involves creating a situation in which neither male nor female cells can incorporate exogenous thymine into chromosomal DNA, mating these cells, and then permitting the transferred F'Lac to replicate in the female in the presence of radioactive thymine. In the following the development and properties of the requisite bacterial strains will be given, followed by details of the labeling technique, and data indicating that an appreciable fraction of the incorporated label is F'Lac DNA.

A. Prevention of chromosomal DNA synthesis (thymine incorporation) in the female

DNA synthesis can be inhibited in a wide variety of organisms by UV irradiation. With increasing doses of UV the extent to which DNA is synthesized after irradiation is decreased (179,180,181). Swenson and Setlow (181) have shown that this is a result of production of thymine dimers between adjacent thymine residues within a single polynucleotide chain of the DNA. In particular, the block is apparently the inability to replicate across such a dimer. Hence, in principle it should be possible to decrease the amount of DNA synthesis to any desired level simply by increasing the dose sufficiently since the production of thymine dimers is proportional to dose (182). It should be remembered that since the F'Lac particle probably contains an amount of DNA on the order

of 1% of that of the chromosome, it would be desirable to inhibit chromosomal DNA synthesis at least 1000-fold.

Table 5 shows the incorporation of H^3-thymine into the DNA of the thymine-requiring female, DF4, after various doses of UV. In agreement with the results of Swenson and Setlow (181), thymine incorporation is greatly inhibited. However, the inhibition is not complete. It is clear that in the 30 minutes required for mating, the total amount of DNA synthesis cannot be reduced below the 0.1% level with doses comparable to those used. Higher doses could of course be used but this would result in impairment of the protein synthesis machinery to an extent which would be undesirable for later experiments. The residual incorporation observed is probably due to the phenomenon of "repair". It is known that in normal cells after UV irradiation DNA synthesis is resumed after a delay during which excision of thymine dimers takes place; this is believed to be followed by repair replication (filling in the gap), and then normal replication (183,184,185).

It has been suggested (186,187) that the repair mechanism is inhibited by the presence of 5-bromouracil (5-BU) in the DNA. Therefore the incorporation experiment given above was repeated using cells which had been grown for three generations in a medium which gives a high degree of substitution of 5-BU for thymine. The incorporation as a function of time for UV-irradiated , 5-BU-containing cells is shown in Figure 3a. As expected, the inhibition was greater for a given dose; however,

Table 5

Incorporation of H^3-thymine by UV-irradiated F- cells

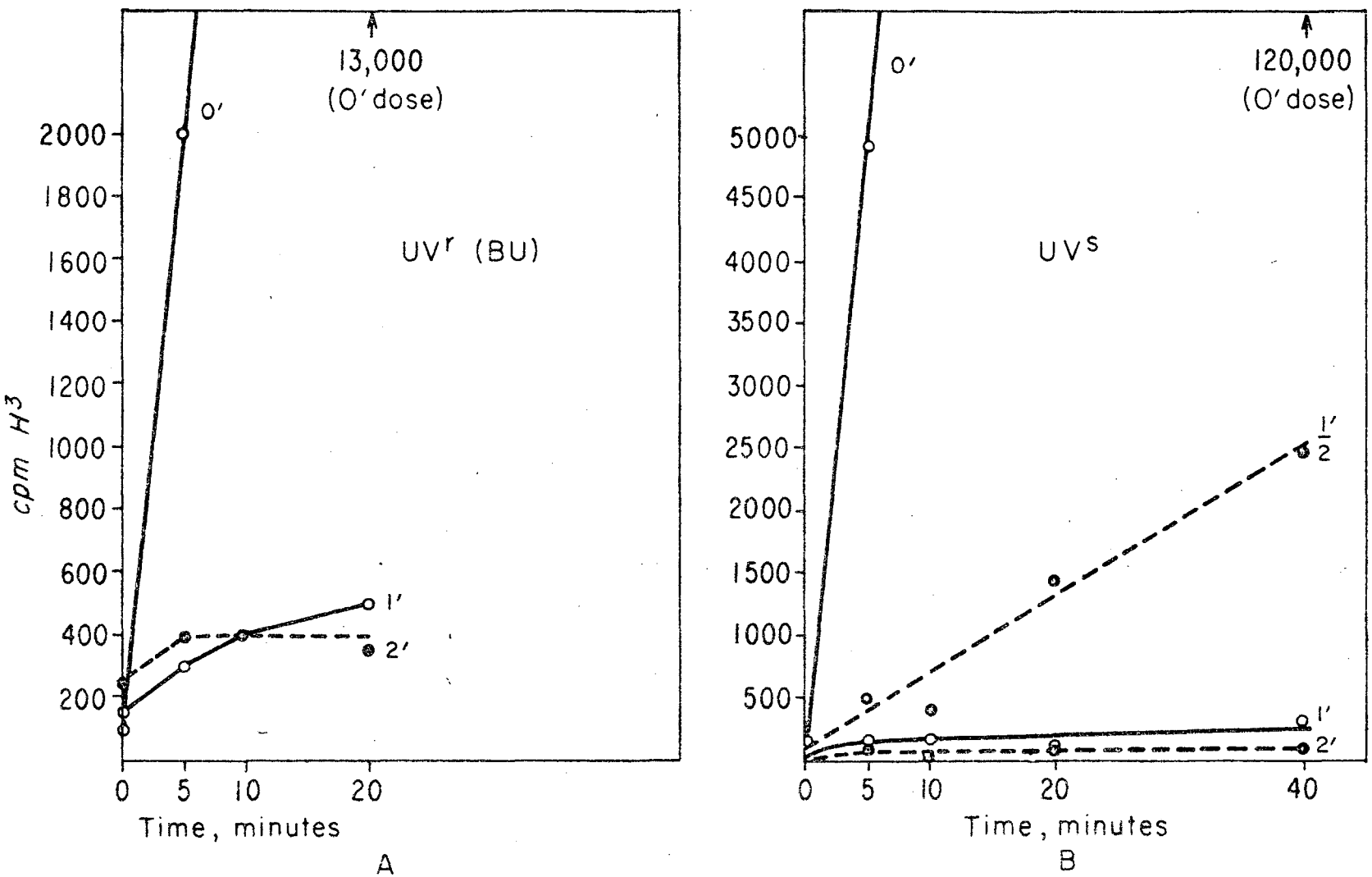
UV Dose, seconds	Incorporation in 30 min, cpm as % unirradiated control
20	33
40	12
120	6

Strain DF4 (F-Thy-) was grown in glucose casamino acids medium containing 2 μg/ml thymine to exponential growth and 4 x 10^8 cells/ml. The cells were chilled, resuspended in 1X121 + P, and irradiated with a 15 watt GE germicidal ultraviolet lamp for various times. An aliquot was reserved as the unirradiated control. Each sample was diluted ten-fold into glucose-casamino acids medium containing 1 μg/ml C^{14}-thymine and incubated for 30 minutes at 37° with shaking, and precipitated with an equal volume of 10% TCA. The precipitates were collected on membrane filters, washed as described in Chapter 2, and counted.

Figure 3-- Effect of ultraviolet irradiation on the incorporation of H^3- thymine by F- cells

A--- Strain DF4 (F-Thy-UV^r) was grown in glucose-casamino acids medium containing 2 µg/ml thymine to exponential growth and diluted into glucose-casamino acids medium containing 2 µg/ml 5-bromodeoxyuridine (BUdr) and 1 µg/ml thymine. This culture was protected from light and grown for three generations. The cells were then chilled, resuspended in 1X121 + P at 4 x 10^8/ml and irradiated with a GE germicidal ultraviolet lamp for 0, 1, and 2 minutes. Nutrients (glucose, casamino acids, and thymine) and H^3-thymine (2 µc/ml) were added to each culture and a 0-time sample was precipitated with 10% TCA. The cultures were warmed quickly to 37° and incubated at 37° with shaking. Aliquots were taken and incorporated radioactivity determined. The numbers next to each curve refer to the UV dose in minutes.

B--- Strain DF69 (F-Thy-UV_A^s) was grown in glucose-casamino acids medium containing 2 µg/ml thymine until in logarithmic growth. The cells were then chilled, resuspended in 1X121 + P, and irradiated with a GE 15 watt germicidal ultraviolet lamp for 0, ½, 1, and 2 minutes. Glucose, casamino acids, thymine, and H^3-thymine (7 µc/ml) were added to each culture, and a 0-time sample was precipitated with 10% TCA. The cultures were warmed quickly and incubated at 37° with shaking. Samples were taken at 5, 10, 20, and 40 minutes and assayed as in A. Note difference in scale of ordinate-- to correct for the difference in specific activity and cell number in the two experiments (A and B), the results were plotted so that the unirradiated controls have the same slope in each experiment.


13,000
(O′ dose)
O′
UV^r (BU)
1′
2′
cpm H3
2000
1800
1600
1400
1200
1000
800
600
400
200
0
5
10
20
Time, minutes
A
120,000
(O′ dose)
O′
UV^s
1′/2
1′
2′
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
5
10
20
40
Time, minutes
B

sufficient synthesis occurred that the criterion of a 1000-fold reduction could not easily be met (due perhaps to incomplete substitution or possibly to the fact that UV lesions in 5-BU DNA do not completely block DNA replication).

Boyce and Howard-Flanders (184) have isolated a strain (UV^s) of E. coli which is incapable of excising thymine dimers. Swenson and Setlow (181,182) have shown in a similar strain (B_{s-1}) that the block to DNA synthesis produced by UV-irradiation of these strains is permanent. Accordingly, a thymineless derivative of UV^s was made (DF 69, see list of strains, Chapter 2) and the incorporation of H^3-thymine as a function of time for various doses of UV was studied. The results are shown in Figure 3b. It is seen that two minutes of irradiation is sufficient to reduce the incorporation in 40 minutes to the desired level. Hence, this female and this dose were used in subsequent experiments.

B. Prevention of thymine incorporation by the male

Cells which do not require an exogenous supply of thymine for growth incorporate little thymine even if the thymine is present at high concentration in the growth medium (188). Hence a thymine-prototrophic male cell should be suitable for the present system, in which it is only required that the male remain unlabeled during the period of exposure of the mating mixture to radioactive thymine. To this end, a thymine-prototrophic strain (DF 20) was made, which if useful was to be infected with F'Lac. In order to determine what the

background incorporation in a mating mixture due to the male alone would be, the level of incorporation of H^3-thymine into DF 20 and DF 4 (the thymine-requiring parent) after 30 minutes was measured (see Table 6). Although the incorporation in the prototroph was considerably lower than the corresponding value for a thymine-requiring cell (less than 1%), this was significantly greater than that which could be tolerated. (Since the DNA of F'Lac amounts to about 1-2% of the DNA of the chromosome and 25-80 % of the male cells transfer F'Lac in 30 minutes, if the background is to be less than 10% as great as the label incorporated into F'Lac, then the level of incorporation must be less than 0.1% of the amount incorporated by a thymineless cell.) Hence, it was necessary to interpose an additional block to thymine incorporation in the male.

In order to accomplish this, use was made of the fact that cells containing the thymine analog, 5-BU, in their DNA are extremely sensitive to visible light (i.e., viability is lost) (155). However, when a mutagenized Thy+ stock was plated on minimal agar plates containing 100 µg/ml 5-BU and these plates were exposed to a 100 watt tungsten lamp for 24 hours, it was possible to isolate colonies which survived this treatment. This mutation we termed 5-BU^r. Such mutants must either have a repair system which is not severely hindered by the presence of 5-BU in the DNA or more likely cannot incorporate 5-BU into their DNA. Since 5-BU is an analog of thymine, it might be expected in the latter case that these mutants would also show a reduced level of incorporation of thymine, To test this,

H^3-thymine incorporation by one of the 5-BU^r strains was measured. This was found to be reduced to a tolerable level, as shown in Table 6.

A few experiments were done to characterize the 5-BU^r mutation. The reduced incorporation of thymine might be a result of impermeability of the cell membrane to thymine or of some defect in the enzymatic pathway required to utilize thymine. Kammen (pers. commun.) has shown that the poor utilization of thymine by thymine prototrophs is primarily a result of an inadequate supply of deoxyriboside compounds and that this incorporation can be greatly increased by the addition to the growth medium of a deoxyriboside such as deoxyguanosine (dG) or deoxyinosine. If dG could restore the ability of the 5-BU^r cells to incorporate H^3-thymine, the block in these cells would probably not be a permeability defect. As shown in Table 7, dG does enhance incorporation, so that the defect is probably in the biosynthetic pathway that converts thymine to thymidine triphosphate. If the block results in impaired enzymatic activity of any of these steps, then even in the presence of dG, the 5-BU^r cells should still incorporate less than the sensitive cells. However, as shown in Table 7, the level of incorporation in both strains is the same. Hence, it is tentatively suggested that 5-BU^r cells incorporate exogenous thymine to a lesser extent than 5-BU^s cells because they have an even smaller intracellular pool of deoxyribose-containing compounds. This will be investigated further.

Table 6

Incorporation of H^3-thymine in Thy-, Thy+, and 5-BU^r cells

Strain	Genotype	Incorporation in 30 minutes, cpm
DF4	Thy- BU^s	120,000
DF20	Thy+ BU^s	410
DF87	Thy+ BU^r	170

All samples were precipitated with 10% TCA and washed with TCA, ethanol, and NaOH as described in Chapter 2.

Table 7

Effect of deoxyguanosine on H^3-thymine incorporation in 5-BU^s and 5-BU^r cells not requiring thymine

Genotype	Deoxyguanosine, µg/ml	Incorporation in 30 minutes, cpm
5-BU^s Thy+	0	150
5-BU^s Thy+	500	7300
5-BU^r Thy+	0	95
5-BU^r Thy+	500	7780

C. Prevention of chromosomal transfer by the male

Male strains carrying an autonomous sex factor such as F or F'Lac transfer these factors with high efficiency in conjugation-- up to 80% in 30 minutes, using the mating conditions described earlier. F+ cells also transfer the chromosome, although with a much lower efficiency, 10^{-5}-10^{-7} per male cell, as a consequence of occasional attachment of the autonomous F to the chromosome, forming a (sometimes transient) Hfr. An F' particle, such as F'Lac, which contains one or more chromosomal markers, has an apparent affinity for the corresponding genetic loci on the chromosome, and is thereby capable of chromosomal transfer more frequently than is an F particle-- on the order of 10^{-2} per male cell (87). While this seems to be a small effect compared to the very frequent transfer of an F', episomes are in general much smaller than the chromosome, 1-2% in the case of F'Lac, so that an amount of chromosomal DNA might be transferred by a small fraction of the population which is comparable to that transferred as pure F'Lac DNA by the entire male population. It has been shown,however, that if the chromosome contains a deletion corresponding to the markers in the F'-merogenote, the amount of chromosomal transfer is markedly reduced (89,189). Although it is not known whether transferred chromosomal material replicates shortly after transfer and prior to recombination (that this is probably not the case for at least 2 hours has recently been shown by Oppenheim (190)), it seemed desirable to reduce this transfer as much as

possible. To this end a deletion for the entire lactose region was introduced into the male chromosome. As shown in Table 8, this reduces chromosomal transfer to about 10^{-3} per male cell, as measured by the appearance of recombinants for an early marker (proline).

D. Evidence that transfer occurs to UV-killed females with ca. normal efficiency

In order to reduce the level of H^3-thymine incorporation in the female to a suitably low level, the UV^S females were given a large dose of UV. The dose chosen produces several thousand lethal hits. Normally, F'Lac transfer is assayed by plating the mating mixture on dye indicator agar plates on which the females which have received F'Lac produce colonies with a characteristic color. However, in the present experiments none of the UV-irradiated females can give rise to colonies, so that some other assay procedure is necessary. One might expect that the UV-irradiation would not inhibit transfer since, as discussed in Chapter 1, Section 4, the female plays no active role in bacterial conjugation. In order to be sure that these heavily irradiated cells can serve as recipients, it is necessary to look directly at the zygotes since an assay cannot be done in any further generation. Since the damage produced by UV-irradiation of the female is primarily to the female DNA (181), it might be expected that gene products of DNA which was transferred by conjugation after UV-irradiation could still be synthesized in the female cytoplasm. Hence,

Table 8

Relative frequency of transfer of a chromosomal marker by two related F'Lac strains, one carrying a chromosomal Lac deletion

Strain	Relevant characters	Marker transfer, %		Ratio Pro+/Lac+
		Lac+	Pro+	
DF73	F-Lac-(y-)Pro-Sm^r			
DF18	Lac-(y-)Pro+Sm^s/F'Lac+	60	0.6	0.01
DF85	Lac_{del}Pro+Sm^s/F'Lac+	83	0.13	0.0015

Strains DF73, DF18, and DF85 were grown in nutrient broth to 2×10^8 cells/ml. Each male was mixed with the female in a ratio of one male to two female cells and incubated for 30 minutes with very gentle shaking. Samples were diluted, Vortexed for 30 seconds, and plated on MacConkey Lactose agar plates containing streptomycin to assay Lac+Sm^r recombinants, and on minimal proline plates containing streptomycin to assay Pro+Sm^r recombinants. % marker transfer is expressed as [concentration recombinants]/[concentration males, initial] x 100.

it should be possible in the recipients of F'Lac to detect the presence of the Lac gene by measuring the ability of the female to make one of the gene products, the enzyme, beta-galactosidase, by inducing the mating mixture. However, since the male cells are Lac+, it is necessary either to suppress enzyme synthesis in the males selectively or to remove the males from the culture before making the assay. It is clearly simpler if the assay can be carried out directly on the mating mixture. This can indeed be accomplished if the males are streptomycin-sensitive (Sm^s) and the females Sm^r, by adding Sm to the induction medium, since protein synthesis is inhibited by Sm in Sm^s but not Sm^r cells (123). Table 9, column 2, shows the amount of beta-galactosidase synthesized under standard conditions as a function of the dose of UV given to the females before mating. It can be seen that the enzyme is made but at a reduced level with increasing dose, so that one might conclude that the amount of transfer decreases significantly with dose. However, UV irradiation does itself have an effect on protein synthesis (cf., Table 9, column 3), partly due to decreased production of messenger RNA and partly due to a deleterious effect on the machinery for protein synthesis (181). Hence, a more meaningful assay is to measure the differential rate of enzyme synthesis, i.e., the ratio of induced enzyme activity to total protein synthesis. Total protein synthesis as a function of UV dose to the females was measured by incorporation of C^{14}-phenylalanine into TCA- precipitable material, as shown in the third column of Table 9. Unmated males produced little or no enzyme. It is seen that the differ-

Table 9

Differential rate of synthesis of beta-galactosidase by UV-irradiated Lac- recipient cells after mating with a F'Lac donor

UV Dose, minutes	OD_{420}, corr.*	Incorporation of C^{14}-βAla, cpm**	Differential activity, normalized
0	.578	217	1.0
2	.172	45	1.4
3	.153	52	1.1

*corrected for scattering (determined by reading at 550 mμ) and for residual synthesis in the male in the presence of streptomycin

**corrected for residual incorporation in the male in the presence of streptomycin

Strains 200P (Sm^s/F'Lac+) and DF4 (F-Sm^rLac-) were grown in glycerol-casamino acids medium containing thymine until in exponential growth and at a concentration of 3 x 10^8 cells/ml. 5-ml aliquots of the F- cells were irradiated with UV for the times shown, at 70 ergs/mm^2/sec, and each mixed with 3 ml of F'Lac cells in a 125 ml Erlenmeyer flask. The mixtures were incubated at 37° with very gentle shaking for 30 minutes. 2 ml of each mating mixture was assayed for beta-galactosidase induction and 1 ml for C^{14}-phenylalanine incorporation, each in the presence of 100 μg/ml streptomycin to inhibit synthesis by male cells.

ential enzyme activity does not decrease with UV dose. Hence we can conclude that F'Lac is not only successfully transferred to the UV-irradiated females, but that it is transferred with an efficiency not unlike that in a normal cross, and also that it can function in the "dead" female cell, at least to the extent of directing synthesis of biologically active protein. This latter point is important since it reaffirms the hope that F'Lac can become labeled by replication in the female after transfer.

E. Specific labeling of F'Lac during mating

Now that it has been shown that incorporation of radioactive label in both male and female cells can be prevented and that a functioning F'Lac can be transferred to the inhibited female, the method of labeling by transfer of an unlabeled F'Lac to a female in which it can replicate becomes a real possibility.

Specific labeling of the F'Lac particles was accomplished by mating, in the presence of H^3-thymine, the male strain, DF 87, and the female, DF 69. These strains have the following properties:

DF 87	Lac_{del} Thy+ 5-BU^r Sm^s/F'Lac	Male
DF 69	Lac- Thy- UV_A^s Sm^r F-	Female

The experiment was performed as follows. Both strains were grown in glucose-casamino acids medium; for the female this was supplemented with 1 μg/ml of thymine. When the female culture reached a concentration of 5-6 x 10^8/ml, it was quickly chilled, removed from the growth medium by centrifugation,

resuspended in chilled medium lacking glucose, thymine, and casamino acids, and irradiated in a thin layer for 2 minutes (dose rate = 70 ergs/mm^2/sec) with rapid shaking to ensure a uniform dose during the irradiation. The irradiated cells were placed in a mating flask containing exponentially growing males (ratio males:females = 3), H^3-thymine at 1 μg/ml final concentration, and concentrated glucose and casamino acids to bring the total medium concentration to the normal values. The total cell concentration was usually 3-5 x 10^8/ml. The mixture was quickly warmed to 37° and agitated gently for 30 minutes at 37°. All operations were carried out under conditions which avoid exposure to photoreactivating wavelengths of light. In parallel controls growth medium was substituted for either the male or the female in the radioactive mixture. At the end of the mating period, the controls and an aliquot of the cross were TCA-precipitated and later counted. The remainder of the cross was chilled, Vortexed, concentrated 50-fold by centrifugation, and resuspended in a solution of 0.1 M NaCl containing 0.01 M versene, pH 8.0. This concentrate was stored frozen to be used in sedimentation analyses. A parallel non-radioactive cross with unirradiated females was usually done to measure transfer by plating. In general 50-80% of the females received F'Lac in 30 minutes. The results of several such radioactiv matings are summarized in Table 10. It is clear that in each case the incorporation is very great compared to the male or female alone which suggests that the excess radioactivity in the cross is associated with the transferred F'Lac particle.

Table 10

H^3-thymine incorporation during mating of Thy+BU^r/F'Lac and UV-irradiated Thy-UV^SF-

Cell type	cpm*
F'Lac	100-200
F-	10
F'Lac x F- (X)	1000-2000
$\frac{X - [F'Lac + F-]}{X}$	80-90%

*corrected for adsorbed soluble radioactivity and normalized for cell number and specific activity. Results of many experiments.

3 ml. of exponentially-growing F'Lac males (strain DF87) in glucose-casamino acids medium were mixed with 1 ml of UV-irradiated UV^S females (strain DF69) in 1X121, in a 125 ml Erlenmeyer flask. 1 ml of 2X glucose-casamino acids medium and 0.3 ml of H^3-thymine (1 mc/ml, 15.7 µg/ml) were added. In a 50 ml Erlenmeyer flask were placed 0.6 ml of males, 0.2 ml of 1X121, 0.2 ml of 2X medium, and 5 µl of H^3-thymine; this was the F'Lac control. In another 50 ml Erlenmeyer flask were placed 0.6 ml of 1X medium, 0.2 ml irradiated females in 1X121, 0.2 ml of 2X medium, and 5 µl of H^3-thymine; this was the female control. To each control was added non-radioactive thymine to 1 µg/ml total thymine. All three mixtures were protected from light at all times. The three flasks were warmed quickly in a 40-42° water bath for 1 minute and incubated for 30 minutes at 37° with very gentle shaking. 1 ml of 10% TCA was then added to each of the controls and a 50 µl sample of the mating mixture was added to 0.1 ml of 10% TCA. The TCA-precipitable material was collected on membrane filters, washed as described in Chapter 2, and counted.

In order to demonstrate that the incorporation observed in Table 10 requires transfer, the effect of conditions which inhibit mating was examined. In the first case, the labeling procedure was repeated using male cells which had been UV-irradiated for one minute before mating. As shown in Table 11, incorporation was completely eliminated, as was F'Lac transfer in the plated cross with unirradiated females. In a second experiment, the mating mixture was shaken violently during the 30 minute mating period. The resulting incorporation and F'Lac transfer, as measured in a radioactive and a non-radioactive mating, respectively, were both reduced to about the same extent, as shown in Table 11.

Before it can be concluded that this incorporation is a result of F'Lac transfer, an alternative must be considered. The position of the UV_A marker is shown on the genetic map in Figure 1. It is possible that this marker could be transferred from the male as a chromosomal marker in a small number of cells and that this would lead to repair replication in the female. From Table 8 the number of DF 87 cells transferring chromosomal material in 30 minutes is about 0.13%. The time of entry of the UV_A marker (191) is from the map about 22 minutes. Hence, because of the time spread in marker transfer, by 30 minutes fewer than about 0.02% of the female cells should have received this marker and in these there has been little time for repair replication to occur (see Table 5). Hence, we can tentatively conclude that most, if not all, of the excess radioactivity is a result of replication of the transferred F'Lac in the female.

Table 11

Simultaneous inhibition of F'Lac transfer and H^3-thymine incorporation in a mating such as that of Table 10

Treatment	% Transfer*	Incorporation, cpm**, X - [F'Lac + F-]
None	75	1240
Violent agitation during mating	16	320
UV-irradiation of F'Lac cells	0	0

* [concentration Lac+Sm^r recombinants]/[initial concentration males]
**corrected for adsorbed soluble radioactivity

In the next section direct experiments which confirm this conclusion will be given.

F. Determination of the fraction of radioactivity contained in F'Lac DNA

In order to demonstrate directly that the excess incorporation observed in the labeled mating is F'Lac DNA, the ability to hybridize specifically to known F'Lac DNA was tested using an agar-gel column (171). These columns consist of denatured DNA of known type trapped in agar. The DNA to be tested is first denatured and fragmented and added to the column material, the mixture is incubated under renaturing conditions (usually 16 hours, 60°, 0.4 ionic strength), and the amount of test DNA which has hybridized with the trapped DNA is then determined by measuring the amount of label bound to the agar (i.e., resistant to washing at 60°, ionic strength 0.4). Homologous DNA binds to the DNA agar (171, and see Materials and Methods), whereas an unrelated DNA does not. That the binding involves specific hybridization is indicated by the fact that bound material can be released by denaturing conditions and that a melting profile determined by stepwise elution from the column is nearly the same as that found for the irreversible denaturation of native DNA of the same type in solution. Furthermore, if a fraction which elutes from the column at a particular temperature is reannealed, it will again elute at the same temperature (Freifelder, pers. commun.). Also, if a mixture of dissimilar labeled DNA's is fractionated by differential temperature elution, each fraction then binds preferentially to agar

containing DNA with the appropriate melting temperature (129).

In order to determine the fraction of the radioactive material derived from a labeled mating which is F'Lac DNA, the extent of hybridization to DNA isolated from a strain of Proteus mirabilis PM-I which contains an E. coli F'Lac particle was measured in the agar-gel. The background of non-specific binding to the column was determined using a column containing DNA from the same strain of Proteus but which lacked F'Lac. The results of this analysis are shown in Table 12, which summarizes data from several duplicate experiments.

For technical reasons (of which, for example, thermal degradation of the agar-gel, release of the column DNA during the renaturation, and self-renaturation of the test DNA are the most important (171)), the maximum efficiency of binding even of completely homologous material (e.g., E. coli to E. coli) is about 80%. This efficiency is rarely achieved and is not reproducible. A more usual value for "optimum" conditions is 50-60%. Hence the percentage of homologous material in a given sample is at least 1.2-1.5 times the observed percentage bound. In many experiments this correction may be even greater since the efficiency of a column depends strongly upon the ratio of the volume of the liquid to the volume of the agar (171) and it is difficult to control or to measure precisely the amount of liquid retained by the agar-gel. Hence, the data given in Table 12 state that in general at least 35-85 % of the radioactivity is in F'Lac DNA. To obtain a more precise value

Table 12

Binding of labeled presumptive-F'Lac DNA to various agar-gel columns

DNA in agar	% Test DNA bound*
Proteus F'Lac	30-70
Proteus F-	0-2

* Results of several experiments

it is necessary to determine the efficiency of each column. This can be accomplished by the addition of an internal standard, for instance, by adding Proteus DNA labeled with a different radioisotope.

This was at first done with C^{14}-Proteus DNA but because of difficulties involving quenching, a more energetic isotope was used. P^{32} was chosen for its high specific activity and ease in dual channel scintillation counting since quenching is not severe. P^{32}- Proteus F'Lac DNA and H^3-mating-labeled DNA were incubated together with Proteus F'Lac DNA agar. If the efficiency of selection of free P^{32}-Proteus DNA is the same as that of free H^3-F'Lac DNA, then the percentage of P^{32} which is bound should be a measure of this efficiency whereas the percentage of H^3 which is bound should reflectthis efficiency multiplied by the fraction of the H^3-labeled material which is F'Lac DNA; in other words,

$$\%H^3_{bound} = (\%P^{32}_{bound}, \text{i.e., column efficiency}) \times \frac{\text{F'Lac}}{\text{Total } H^3 \text{ material}}$$

or

$$f = \frac{\text{F'Lac DNA}}{H^3 \text{ DNA}} = \frac{\%H^3 \text{ bound}}{\%P^{32}\text{bound}}$$

Table 13 gives the results for both the C^{14} and P^{32} label. For the P^{32} case f was found to equal 0.66; for C^{14}, f was equal to 0.50. Thus, F'Lac constitutes 50-66% of the labeled material. However, this is probably also an underestimate, since the P^{32} or C^{14} F'Lac DNA was present in a much higher concentration than the H^3 DNA and probably renatures with higher efficiency. A more serious problem is that in the 75° eluates

Table 13

Binding of labeled *Proteus* and F'Lac DNA to a *Proteus* DNA-agar-gel column

C^{14}		
	$\%C^{14}$ *Proteus* bound	61
	$\%H^{3}$ F'Lac bound	31
	$f = H^{3}/C^{14} = 0.50$	
P^{32}		
	$\%P^{32}$ *Proteus* bound	23
	$\%H^{3}$ F'Lac bound	15
	$f = H^{3}/P^{32} = 0.66$	

In each case the DNA-agar contained *Proteus* F'Lac DNA. Both labeled F'Lac and Proteus DNA were added to the agar-gel, the mixture was incubated overnight at 60° in 2XSSC, and the % of each labeled material which was bound was determined. The C^{14} and P^{32} cases represent two distinct experiments.

(which measures the bound material) some agar is released which is TCA-precipitable and which quenches the H^3. The higher energy P^{32} or C^{14} is much less quenched so that fewer of the H^3 counts are seen without a proportional loss of P^{32} or C^{14} counts. A quenching correction can be done when only one isotope is present and if the quenching is not too severe. However, this is not possible in the present situation. This quenching has a large effect on the experimental data since the first high temperature fraction (i.e., the most quenched) contains about half of the observed bound radioactivity. Hence, F'Lac probably comprises more than 60% of the H^3-labeled material and if an estimate is made from the quenching seen in several experiments using single isotope labeling, we could say that this 60% value should probably be corrected to some value greater than 80%.

Chapter 5-- Physical properties of F'Lac DNA

Some physical properties of macromolecules can be determined using tracer quantities of material. Now that F'Lac has been successfully labeled with radioactivity, it becomes possible to carry out some of these measurements. In particular, studies of (1) the molecular weight, (2) denaturation characteristics, and (3) buoyant density of F'Lac DNA will be described using the techniques of zonal sedimentation through a sucrose gradient, agar-gel column chromatography, and CsCl density gradient centrifugation, respectively.

A. Molecular weight measurements: sedimentation studies

The molecular weight of the DNA derived by phenol extraction from F'Lac was measured by determining the sedimentation coefficient by sucrose gradient centrifugation and comparing this value to a DNA standard of known molecular weight.

Cells from a H^3-labeled mating, prepared and concentrated as described in Chapter 4, were lysed in versene with lysozyme and SDS and extracted with phenol. At all times precautions to avoid hydrodynamic shear degradation of the DNA were taken. The aqueous layer of the phenol extract (50-100 µl) was layered on a 5 ml 5-20% sucrose gradient (buffered at pH 7.8 with phosphate and containing 0.5 M NaCl) and centrifuged for 2 hours at 35,000 rpm in a SW39 rotor in a Spinco Model L ultracentrifuge. (The high ionic strength, 0.5., was chosen

to avoid spurious protein-DNA aggregates.) . C^{14}-labeled DNA obtained by heat release from phage T7 was usually added as a sedimentation velocity standard. After centrifugation the tube was fractionated by drop collection, carrier DNA was added to each fraction and the samples were TCA-precipitated, and then counted in a liquid scintillation counter. The boundary profile of a typical sedimentation run is shown in Figure 4. Figure 5 shows a boundary for a phenol preparation of the DNA from phage T7 for comparison. The position of the T7 DNA boundary is also shown in Figure 4. The average value of the sedimentation coefficient (S) determined from several such runs is 39 ± 2. From such a sedimentation run it is possible to calculate the molecular weight of a DNA molecule by comparing the relative distances moved by the DNA in question and a DNA of known molecular weight (e.g., T7) using an empirical equation derived by Hershey, Burgi, and Ingraham (192):

$$\frac{D_1}{D_2} = \left(\frac{\text{Mol. wt.}_1}{\text{Mol. wt.}_2}\right)^{0.35}$$

However, before proceding with the determination of molecular weight from sedimentation studies, it must first be ascertained that the DNA has not been degraded by hydrodynamic shear. Secondly, it would be premature to use any sedimentation data derived from impure systems (i.e., crude cell extracts) or from studies of radioactive molecules without first asking whether the S is constant with increasing age of the preparation, i.e.,whether degradation is occurring due to either

Figure 4-- Sedimentation of H^3-labeled F'Lac DNA

F'Lac DNA was specifically labeled with H^3-thymine, as described in the text. The cells were lysed with lysozyme and SDS and extracted with phenol. The extract was layered on a 5 ml sucrose density gradient containing an 0.1 ml "shelf" of 70% sucrose at the bottom and centrifuged for 2 hours at 35,000 rpm in a SW39 rotor in a Spinco Model L ultracentrifuge. Ten drop fractions were collected and assayed for TCA-precipitable radioactivity. The centrifuge tube bottom (TB) was cut off and counted also. Because of severe quenching, the TB value is underestimated. The corresponding position of T7 DNA is indicated by the arrow. This comes from Figure 5.

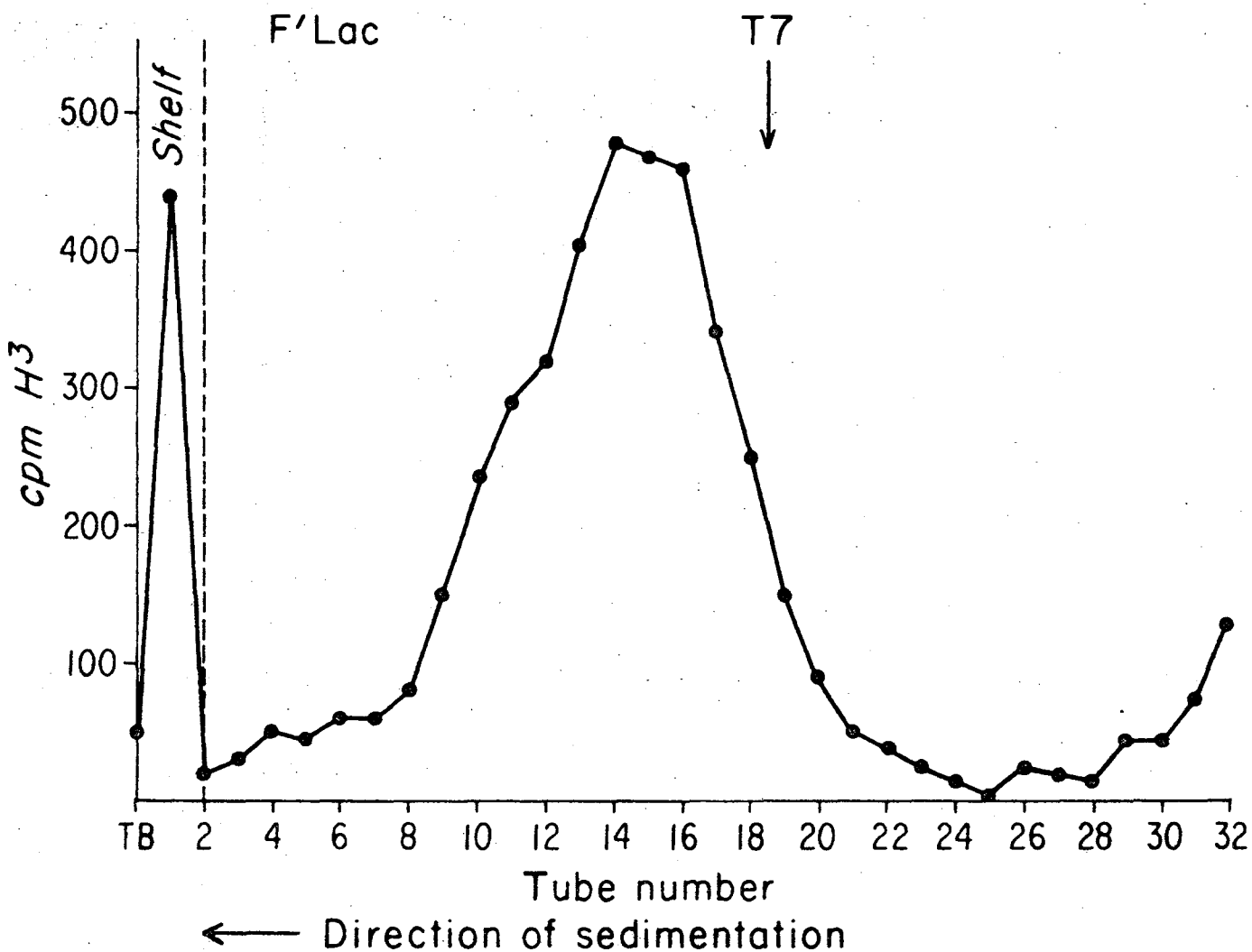
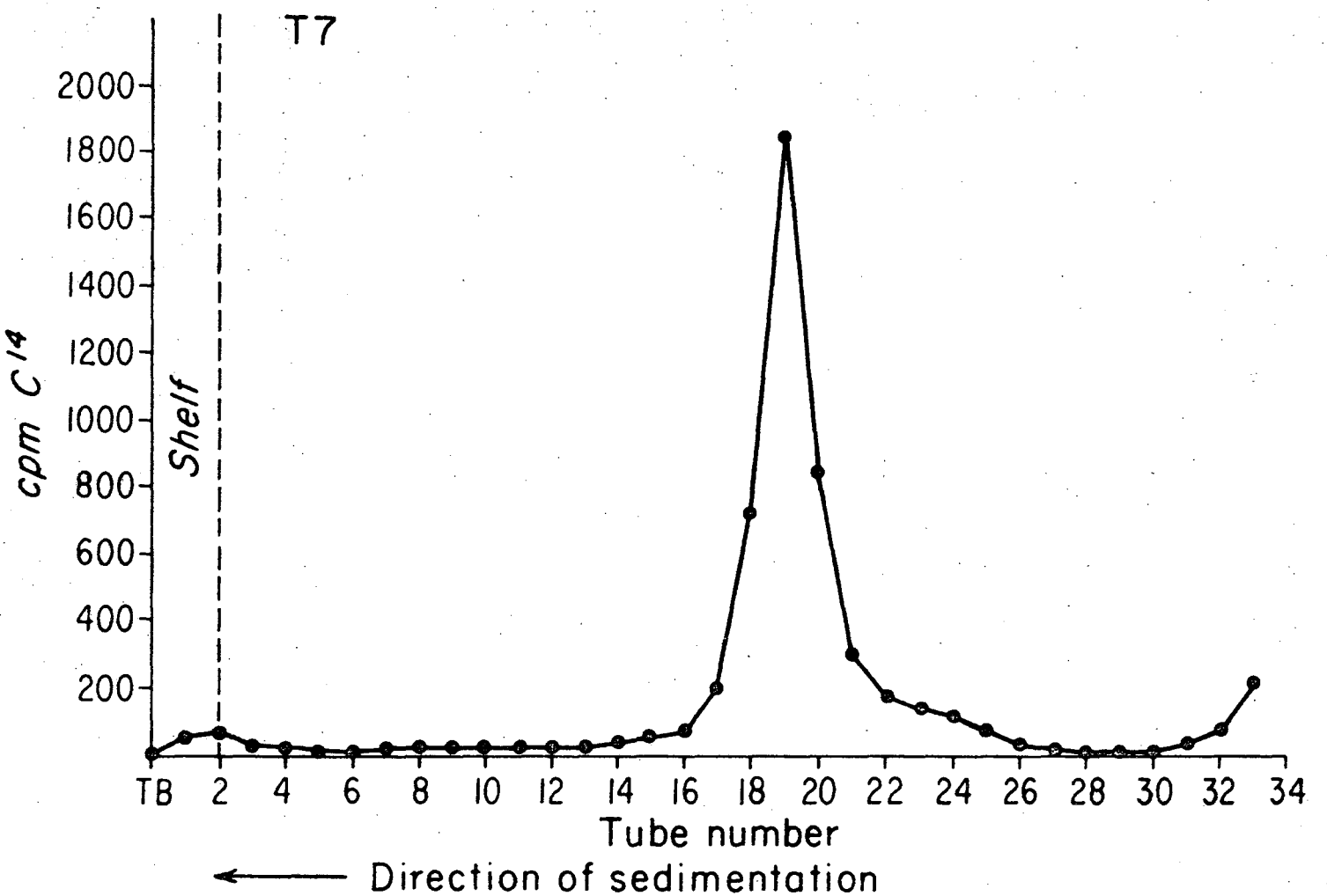

F'Lac
T7
Shelf
cpm H^3
500
400
300
200
100
TB
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Tube number
Direction of sedimentation

Figure 5--- Sedimentation of T7 phage DNA

T7 phage labeled with C^{14}-thymine was mixed with bacteria (ca. 10^9/ml) and subjected to the procedure of cell lysis with lysozyme and SDS followed by phenol extraction, as described in Chapter 5. The extract was layered on a 5 ml sucrose gradient with a 0.1 ml "shelf" of 70% sucrose on the bottom, and centrifuged in a SW39 rotor in a Spinco Model L ultracentrifuge for two hours at 35,000 rpm. Ten drop fractions were collected and assayed for TCA-precipitable radioactivity. The position of this boundary is also shown in Figure 4.


T7
Shelf
cpm C14
2000
1800
1600
1400
1200
1000
800
600
400
200
TB
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
Tube number
Direction of sedimentation

enzymatic action or radioactive decay. In addition, use of this equation requires that both DNA's have the same shape and density, two points which must be carefully considered.

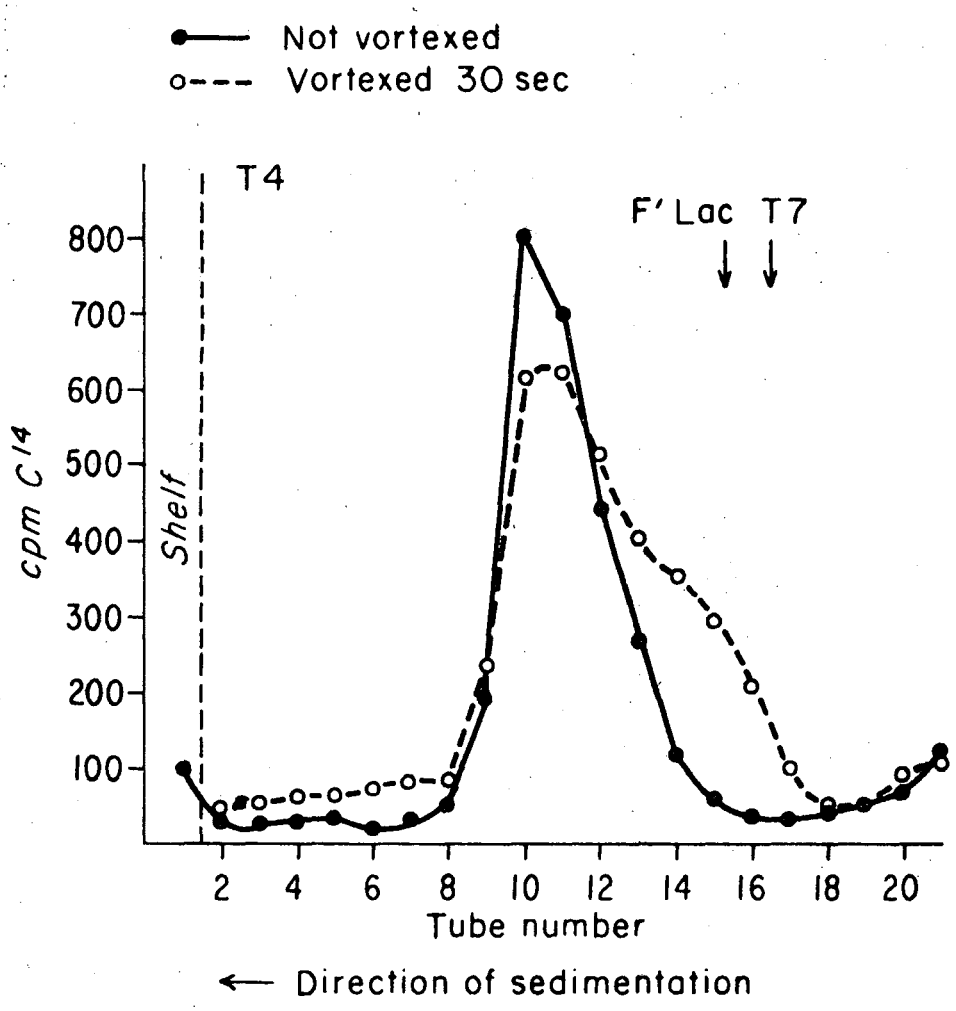
In order to determine whether the isolated DNA was a degradation product of hydrodynamic shear forces, T4 DNA (mol. wt. = 120×10^6 (193)) was subjected to the same shear conditions as in the lysis procedure (i.e., mixed with bacteria which were then lysed and phenol extracted) and sedimented under the same conditions. The resulting boundary was compared to that obtained by sedimentation of T4 DNA extracted by the standard technique, as shown in Figure 6. The position and shape of the T4 DNA boundary in the two experiments are nearly the same; a small amount of DNA has, in fact, been sheared, that in fractions 13-17, but little of the material has an S lower than that of the F'Lac DNA. It is unlikely that the F'Lac boundary represents a degradation product since the shear forces are apparently not great enough to cause significant breakage of a molecule more than twice its size. The near identity of the two boundaries also shows that the sedimentation of the T4 DNA and therefore probably of the F'Lac DNA is unaffected by the high concentration of bacterial protein and nucleic acids present in the lysate. This latter point has been confirmed in a similar experiment with T7 DNA.

The possibility of radiation damage or enzymatic degradation will be considered next.

Because of the small size of F'Lac DNA, in order to get sufficient radioactivity into the DNA to be able to locate it

Figure 6-- Effect of cell lysis procedure on the sedimentation of T4 DNA

T4 phage labeled with C^{14}- thymine was either mixed with unlabeled E. coli bacteria (2 x 10^8/ml) and subjected to the procedure used to isolate F'Lac DNA (dashed line)or the DNA was released by phenol extraction from the phage alone (solid line)using a method which avoids shear degradation of T4 DNA (195). Each extract was layered separately on a 5 ml sucrose gradient containing an 0.1 ml "shelf" of 70% sucrose and centrifuged in a SW39 rotor in a Spinco Model L ultracentrifuge for 1 hour at 35,000 rpm. Fifteen drop fractions were collected and assayed for TCA-precipitable radioactivity. The positions of T7 DNA and F'Lac DNA are indicated (from Figures 4 and 5).


Not vortexed
Vortexed 30 sec
T4
F' Lac
T7
Shelf
cpm C14
800
700
600
500
400
300
200
100
2
4
6
8
10
12
14
16
18
20
Tube number
Direction of sedimentation

accurately in the gradient, it was necessary to use a very high specific activity of thymine-- 5-8 mc/μM. This means that a DNA molecule of 55 million molecular weight (anticipating the result) experiences ca. 1.5 decays per day or, as in the present case it is likely that only one strand is labeled, 0.75 decays per day. Whereas the nature of the molecular damage to DNA by H^3 decay has not been reported, it is known that some molecular alterations occur in solutions of H^3-thymine and that chemical products other than thymine arise (detailed information supplied by manufacturer). The precise effect that this damage would have on or in a DNA molecule is not known, but it seems likely that some instability of the thymidylate residue would result, possibly leading to eventual depyrimidination and ultimately a break in that single strand of the DNA. There is evidence that a missing base in a DNA molecule results in a labile and easily hydrolysable phosphoester bond (195,196). Furthermore it is known that H^3 decay in DNA results in inactivation of bacteria and phage (197,198). It is also possible that ionizations produced by the H^3 decay could result in the production of free radicals or other radiochemical intermediates which could cause indirect damage to the DNA. Indeed, indirect effects in radiobiology are widespread (199).

Therefore, we would assume that in any but the freshest preparations, many of the F'Lac DNA molecules will have sustained some type of damage which may alter the sedimentation properties. For instance, suppose F'Lac DNA were originally

in the form of a supercoiled (twisted) circle such as is found in the case of polyoma virus (200) and certain intracellular forms of phage λ DNA (201), a single-strand break would release this coil which would result in expansion of the molecule to form a circle and reduction of S. With time, accumulation of single-strand breaks would eventually produce matched pairs, equivalent to double-strand breaks (170). If this were to occur in a circular molecule, a linear molecule having a reduced S would result; in a linear molecule, the result would be linear fragments of smaller molecular weight, the average molecular weight of the total preparation decreasing with time. To investigate the possible effects of H^3-decay, the S of the labeled material was measured after various periods of frozen storage, ranging from one day to two weeks. It was found that indeed the S decreased with time (see Table 14). The smallest S observed (after two weeks) was about 40% of the initial value, indicating about a 20-fold decrease in molecular weight, if the molecule were linear at the time the first measurement was done (202). It is evident that some sort of degradation occurs in these samples, yet from the calculations given above concerning the number of decays per day, the rate of degradation seems to be too rapid to be a consequence of direct effects of H^3 decay.

To test directly whether the degradation is a result of H^3 decay, the rate of change of S (with time of storage at -15°) was measured as a function of the specific activity of the H^3-thymine. When the specific activity was increased 3-fold

Table 14

Change of S of H^3- F'Lac DNA with time of storage

Storage time, days	Distance sedimented, fractions *
0	17 ± 1
1	15.5
4	12
8	7
10	7

*average of several experiments

(to 25 mc/μM) or decreased to half, the degradation occurred at nearly the same rate as for the original activity used. Therefore, a direct effect of H^3-decay is not likely to be the source of the degradation. However, when bacteria are treated with ionizing radiation (e.g., X-rays or gamma rays), it is known that after irradiation, there is a massive degradation of DNA with an appreciable amount of the DNA being converted to soluble nucleotides (203,204). Since this does not occur when free DNA is irradiated (170), it seems more likely that this is an enzymatic degradation. It is possible then that somehow the internal H^3-decay triggers this enzymatic system, thus resulting in breakdown of the F'Lac DNA. Since frozen preparations were thawed from time to time to take small samples, it is possible that the degradation occurred only during the higher temperature period. In fact there is some preliminary evidence that this is the case in that if two preparations are compared, one of which has been stored for four days without thawing and the other of which has been thawed several times, the one which has been thawed seems to show greater degradation. It is clear that this phenomenon deserves further investigation.

It should be pointed out that in the present work all S measurements for molecular weight determinations were made as quickly as possible after labeling. In general about three hours elapsed between initiation of labeling and completion of centrifugation.

In order to use the empirical equation of Hershey _et al_,

it is required that all physical parameters, i.e., buoyant density and shape, be the same. In general, buoyancy is a very small effect in sedimentation studies unless the density of the macromolecule is near that of the solution. Since free DNA has a density of approximately 1.7 gm/ml (205), this factor is usually not important. However, if the F'Lac DNA were associated with other substances, e.g., protein, lipid, or polysaccharide, the density could be very much lower. However, it is unlikely that there is an appreciable density effect since the ratio of the distances sedimented through a density gradient by F'Lac DNA and T7 DNA is constant for different times of sedimentation. There is no definitive evidence at present concerning the association of these substances with F'Lac DNA (see Discussion, Chapter 7). Also, in these gradients because of the high ionic strength, 0.5, nothing will be associated with the DNA by salt linkages.

It is possible that there is a difference in configuration between the DNA in the F'Lac boundary and T7 DNA since the sedimentation boundary of F'Lac DNA is somewhat broader than that of T7 DNA. This would not be expected if F'Lac were a simple, linear molecule. First, it should be remembered that the DNA is isolated from a replicating system. Even though the time required to replicate F'Lac DNA is probably short compared to the duration of the mating, so that it would be expected that only a relatively small number would be engaged in replication at the time of isolation, nonetheless such molecules

probably are present; these would contain extra DNA and therefore would tend to sediment more rapidly. It is also possible that these components or even the total collection of molecules in the boundary are replicative forms, which in analogy to other systems (206) would be more compact. Both of these effects would contribute to the breadth of the boundary. Furthermore, the isolated DNA may be a highly compact molecule (e.g., supercoil or circle) so that the breadth of the boundary would merely be the result of a very high diffusion coefficient compared to that of a linear DNA molecule.

Some properties of the degradation discussed above give some information concerning the likelihood that more compact forms of DNA are present. It should be emphasized that the question at present is not whether the "real" form of F'Lac DNA is a compact structure but what the structure is of the component observed in the sedimentation boundary.

When a supercoil sustains a single-strand break, the S decreases discontinuously by approximately a factor of two (200,201), i.e., intermediate forms are not seen. However, the decrease with time of the S of the F'Lac DNA is a continuous process so that the material being sedimented clearly cannot be a supercoil. The S of a circular molecule is only slightly greater than that of a linear molecule with the same molecular weight (200,201). In fact, circular and linear molecules of phage λ DNA cannot easily be distinguished when co-sedimented in a sucrose gradient (201) but merely result in a broader

boundary. Hence, the boundary shown in Figure 4 could consist of circles, linear molecules, partially replicated linear molecules, or a mixture. At present there are no experiments to elucidate this point.

If the boundary does not consist wholly of linear molecules, the equation of Hershey et al would not be applicable, since the shape of the molecule would be different from the linear standard (T7). However, the experimental error in determination of S is already such that this would not increase the error to greater than the limit already imposed on the mean value.

As a result of the above considerations, it is felt that the equation of Hershey et al can be applied to the present situation, with appropriate indication of the limits of accuracy. In so doing, taking 25×10^6 as the molecular weight of T7 DNA (164,207,208,209), we obtain:

$$\text{Mol. Wt. (F'Lac DNA)} = 55 \pm 8 \times 10^6$$

The probable error derives principally from the uncertainty in the S measurement.

Falkow and Citarella (129) have determined the molecular weight of the DNA of an F'Lac episome derived from the larger F'_{13}, in terms of the percentage of the size of the E. coli genome, by a quite different method. They isolated the F'Lac DNA from a strain of Proteus mirabilis harboring the episome and determined the amount required to saturate an agar-gel column containing a known amount of DNA from a Lac+ Hfr strain of E. coli. They found a value of 2.5%. Using the Cairns

value (45,131) for the molecular weight of the E. coli genome (2.2 - 2.8 x 10^9) this yields a value for the molecular weight of their F'Lac DNA of 55-70 x 10^6, in good agreement with the above. As pointed out in Chapter 1, Section 3.E., it is likely that the agar-gel method over-estimates the value.

Anticipating the future when an attempt will be made to isolate and purify the DNA of F'Lac, one experiment was done to determine the extent to which the region of the gradient containing the F'Lac DNA is contaminated with bacterial DNA (unlabeled). Male and female bacteria were uniformly labeled in their DNA by growth for several generations in C^{14}- and H^3- thymine respectively. The females were UV-irradiated, the bacteria were mixed, and the DNA extracted immediately using the procedure employed for F'Lac DNA. The bacterial DNA was found to sediment well ahead of the position of the F'Lac DNA boundary; in fact, a considerable amount of the DNA was pelleted on the tube bottom. This probably explains why no male DNA is seen in F'Lac gradients even though we know from labeling data that labeled male DNA is present. It was calculated that the amount of unlabeled bacterial DNA in the F'Lac boundary was approximately equal to the amount of F'Lac DNA. Hence this sedimentation procedure may be useful as a method for partial purification.

B. Denaturation characteristics of F'Lac DNA

When denatured DNA is renatured, the renatured DNA has a melting curve similar to that of the original native DNA (210).

This fact makes it possible to measure a melting curve by agar-gel chromatography. Although this technique lacks the precision obtainable by the usual optical methods (211), it is sufficient to determine whether there is anything out of the ordinary about the base composition of a given DNA preparation.

This melting technique, first used by Falkow and Citarella (129) to determine the melting curve of the F'Lac DNA isolated from Proteus mirabilis, involves first renaturation (in this case, the labeled DNA obtained from a mating) with F'Lac-containing DNA in an agar-gel column, as described in Chapter 2, Section V, washing at high ionic strength, and then measuring the temperature range over which the bound material is removed or "melted out" from the column. This is done by using an intermediate ionic strength and a series of temperatures, increasing a few degrees at each step, and measuring the amount of label which is eluted at each step. High ionic strength eluents cannot normally be used since temperatures high enough to melt the agar would be required to remove all of the bound DNA. 0.1 X SSC has been chosen for this reason. If the amount of eluted material is plotted as a cumulative sum against temperature (i.e., at each temperature, the total amount eluted at that temperature plus all lower temperatures is plotted), a melting curve can be obtained. The temperature at which 50% of the bound material is eluted is by definition the melting temperature, T_m.

It should be pointed out that this type of melting curve is in principle different from standard optical melting curves. As discussed in detail by Geiduschek (212), melting curves are

of two types, reversible and irreversible. A reversible melting curve is roughly a measure of the amount of hydrogen bond breakage and is obtained by reading the optical density at the temperature to which the DNA has been heated. This is the type of curve normally described. If the DNA is raised to various temperatures, T_i, but always cooled to a lower temperature (usually 25°) before reading the optical density, a melting curve can be obtained by plotting the optical density versus T_i; this is called an irreversible melting curve. This curve essentially measures the number of hydrogen bonds remaining broken after cooling. It is an irreversible curve since if all of the hydrogen bonds in a DNA molecule are not broken at a given temperature, upon cooling all of the broken bonds will reform (213). Thus, the irreversible curve tells us something about the temperature at which single strands separate. Reversible and irreversible curves are clearly different, the irreversible one having the higher melting temperature.

The melting curve determined with agar-gel columns is clearly an irreversible curve so that the T_m so obtained might be expected to be higher than the reversible curve obtained optically. However, it should be remembered that DNA must be fragmented to small pieces, 700-1000 nucleotides, before application to the column (171). It has been shown that, as might be expected, reversible and irreversible assays are not very different when the size of the DNA is small (212). The control, of course, must be to compare a T_m determined by this technique

with the T_m obtained for the same DNA by the standard optical technique, e.g., E. coli with a column of E. coli DNA-agar. This control, to be discussed in the next paragraph, has been done and is shown in Figure 7A.

The results of the determination of T_m for F'Lac DNA by the agar-gel technique are shown in Figure 7A. The source of standard F'Lac DNA (i.e., the unlabeled DNA in the gel) was Proteus F'Lac. It should be remembered that H^3-labeled F'Lac, prepared by the specific labeling procedure does not bind to Proteus DNA (see Chapter 4). Figure 7A shows two melting curves obtained with different labeled samples on different days. The reproducibility of this measurement should be noted. The T_m so determined is approximately 71°. If the agar contained DNA from E. coli strain DF3 (a F'Lac male) instead of Proteus F'Lac DNA, the melting curves were identical. For comparison, a curve obtained for totally labeled E. coli DNA and agar containing E. coli DNA is given. The two curves are not distinguishable. Since the resolution of these curves is about ±1° for the T_m, it can be concluded from the relation that $T_m = 69.3 + 0.41$ (%GC) (214) that the base composition of the F'Lac DNA is not different from that of E. coli by more than 2.5% GC, i.e., the GC-content of F'Lac is 50 ± 2.5% (see next section for amplification of this point).

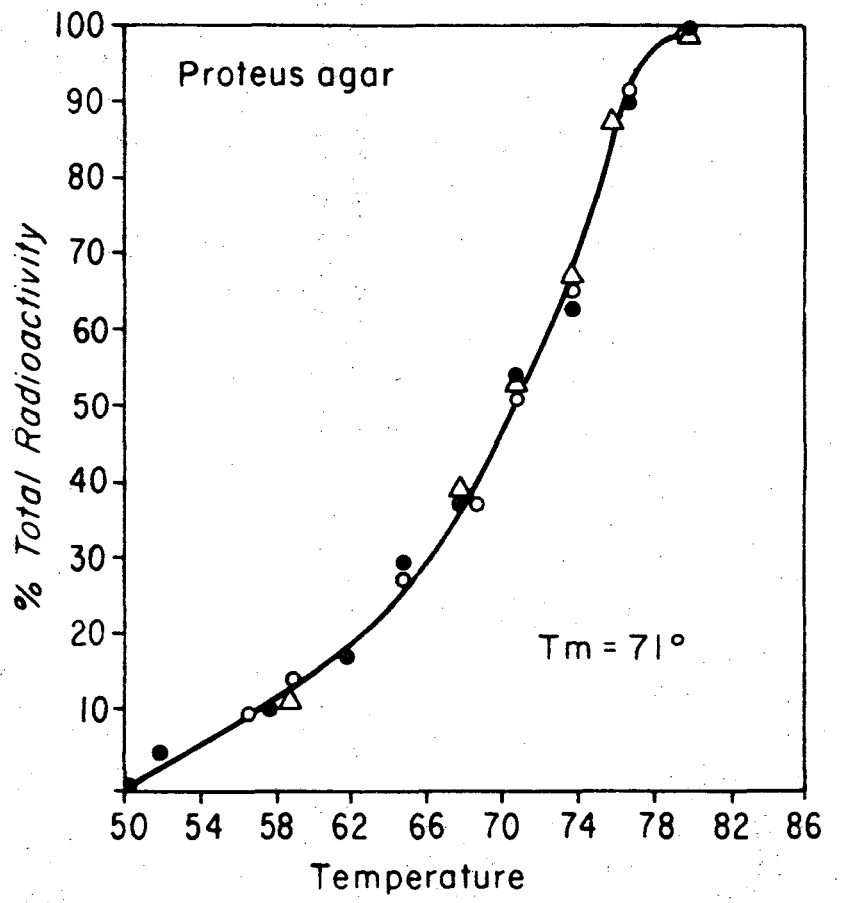
In an effort to see if some information could be obtained about the base composition of F itself or of the Lac gene, two other experiments were done. In the first, the melting curve of the F'Lac DNA homologous to strain W4032 (Hfr, Lac_{del})

Figure 7-- Melting curves of H^3-labeled F'Lac hybridized with various DNA's in agar-gel

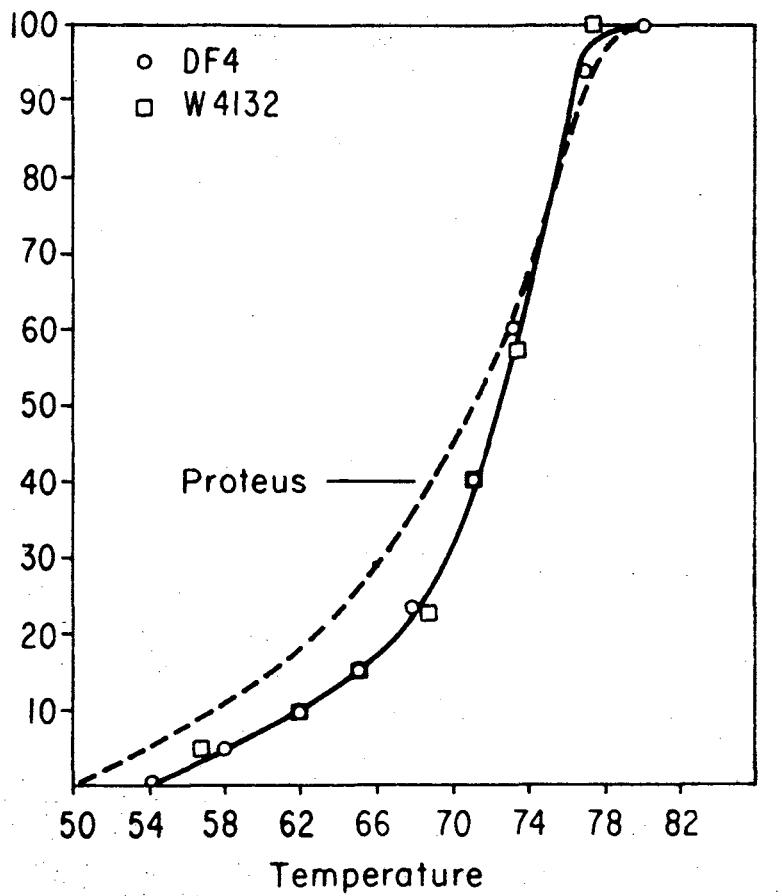
Agar-gels containing DNA from Proteus mirabilis F'Lac and from E. coli strains DF3(F'Lac), DF4(F-), and W4132(F-Lac_{del}), were prepared as described in Chapter 2. Cells from a labeled mating were lysed with lysozyme and detergent and extracted with phenol. The extract was boiled for 15 minutes and X-irradiated to denature and fragment the DNA in preparation for hybridization in agar-gels. 0.2 ml aliquots of the labeled DNA were added to 0.5 gm of each of the agar-gels, placed in small screw-cap vials, and incubated overnight at 60°. Each mixture was then placed into a sintered glass eluting tube, washed 20 times with 2XSSC at 60°, and eluted with 0.1 X SSC at a number of temperatures, increasing from 54°. The results of the elution are plotted as a cumulative sum; that is, the ordinate of each point equals the amount of label eluted at that temperature plus the total amount eluted at all lower temperatures, expressed as per cent of total bound material-- i.e., material not removed by the 60°, 2XSSC wash.

A. Three experiments are shown. The solid and open circles refer to duplicate experiments in which H^3-F'Lac DNA was melted from a Proteus F'Lac column. The triangles refer to melting of H^3-E. coli DNA from an agar-gel containing E. coli DNA also. The melting temperature (T_m) defined as the temperature at which 50% of the material has been released from the column is given for all three experiments.

B. This shows the melting curves for H^3- F'Lac DNA melted from agar-gels containing either DF4(F-) DNA or W4132 (F-Lac_{del}) DNA. The T_m is ca. 73°. The dashed curve is the curve shown in A.


Proteus agar
Tm = 71°
% Total Radioactivity
100
90
80
70
60
50
40
30
20
10
50 54 58 62 66 70 74 78 82 86
Temperature

A


DF4
W4132
Proteus
100
90
80
70
60
50
40
30
20
10
50 54 58 62 66 70 74 78 82
Temperature

B

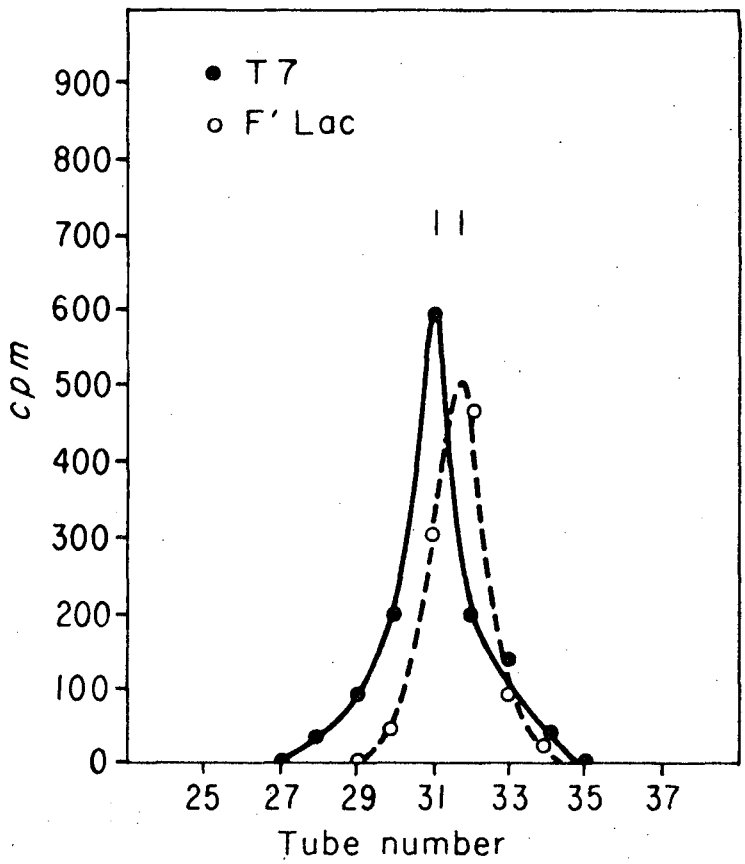
was determined. This was indistinguishable from that obtained against DF3 or Proteus F'Lac. This is presumably a result of the fact that the Lac operon probably has a molecular weight of no more than 2×10^6 and therefore comprises only a small part of F'Lac. Hence, nothing can be said about the base composition of the Lac gene. In the second experiment, the agar contained DNA from either DF4 (a female) or W4132 (female, Lac_{del}). As shown in Figure 7B, the melting curve so obtained has a higher T_m (73°) than that of the total F'Lac DNA and in particular lacks a large portion melting at low temperature. From this it can be concluded that F probably has a base composition perhaps 5-10% GC less than E. coli (i.e., 40-45 % GC). This agrees with a similar finding of Falkow and Citarella (129) that 10% of their F'Lac DNA has a mean base composition of 44% GC. Experiments to determine the base composition of F directly will be carried out in the future.

C. Determination of the buoyant density of F'Lac DNA

H^3-labeled F'Lac DNA was banded in CsCl to determine the buoyant density, using DNA from phage T7 as a density marker. The result of such an experiment is shown in Figure 8. It can be seen that the density of F'Lac DNA is slightly less than that of T7 DNA. From determinations of the density gradient and bearing in mind the limited resolution of these bands (peaks separated by about one fraction), it can be said that the density of F'Lac DNA is about 0.002-0.003 gm/ml less than that of T7 DNA, which has the same density as E. coli DNA (215).

Figure 8-- Equilibrium sedimentation in a CsCl density gradient of H^3-F'Lac DNA obtained from a labeled mating

Cells from a H^3-labeled mating were lysed with lysozyme and SDS and extracted with phenol. The extract was added to a concentrated CsCl solution and adjusted to a final density of approximately 1.700. C^{14}-labeled T7 DNA was added as a density marker. The mixture was centrifuged for 48 hours at 38,000 rpm in a SW39 rotor in a Spinco Model L ultracentrifuge. Fractions were collected, TCA-precipitated, and counted.


● T7
o F' Lac
900
800
700
600
500
400
300
200
100
0
cpm
25
27
29
31
33
35
37
Tube number

Using the equation

$$\text{buoyant density} = 1.660 + 0.00098\ (\%GC) \qquad (215)$$

the base composition of F'Lac DNA would be 2-3% GC less than that of *E. coli* DNA, or 47-48% GC. Such a difference would result in a decrease in T_m of 0.8-1.2° and would probably not be detectable using the agar-gel melting technique of the preceding section. Thus, the CsCl value for the base composition is probably more reliable than the other.

D. Summary of physical properties of F'Lac DNA

Molecular weight	$55 \pm 8 \times 10^6$
T_m (0.1 X SSC)	71°
Density	1.707-1.708 gm/ml
% GC	47-48

Chapter 6-- Experiments with F

It was expected that the specific labeling technique would be applicable to the study of any transferrable episome capable of replicating in a UV-irradiated female.

Preliminary studies have been carried out with F. An investigation of F has the weakness that an experiment equivalent to the beta-galactosidase assay, in the F'Lac case, cannot be done since no gene product of F is known which can selectively be assayed in a UV-irradiated female without interference by the males already present. Consequently the identity of the label produced by the specific labeling technique has been determined only by measuring the homology to an internally standardized agar-gel column. A determination of the molecular weight of the DNA by sedimentation velocity has also been made.

Specific labeling was carried out as described in Chapter 4 using the same female (DF69) but an F+ male strain, DF85, which has the genotype, $Sm^{s}Lac_{del}Thy+5\text{-}BU^{r}F+$ (the Lac_{del} locus is not relevant). Since the transfer of chromosomal material by F+ cells is already very low (at least 1000-fold less than by F'Lac cells), the possible contribution of replicating chromosomal DNA to the label, as discussed in the F'Lac case, has been ignored. The results of such a mating in the presence of H^{3}-thymine, as well as the male and female controls, are shown in Table 15. As in the case of F'Lac, the incorporation in a mating is significantly in excess of the combined incorporation by the males alone and by the females alone. If the mating

Table 15

H^3-thymine incorporation during mating of Thy+BU^r F+ and UV-irradiated Thy-UV^s F-

Cell type	Incorporation, cpm*
F+	140
F-	40
F+ x F-	400
F+ x F-, shaken violently during mating	150

* corrected for adsorbed soluble radioactivity and for relative cell number and specific activity

Strains DF89 (Thy+BU^rSm^s F+) and DF69 (F-Thy-UV^sSm^r) were grown in glucose-casamino acids medium containing 1 μg/ml thymine. When both cultures were in exponential growth, the female cells were irradiated with UV for 2 minutes, and 0.5 ml of females was added to 1.5 ml of males in a 50 ml Erlenmeyer flask. 1 ml of males and 1 ml of irradiated females were each placed in a 50 ml Erlenmeyer flask. 5 μl of H^3-thymine (1 mc/ml, 15.7 μg/ml) was added to each of the three flasks. The mixtures were protected from light and incubated at 37° with very gentle shaking for 30 minutes. A parallel mating mixture was made up exactly as the first flask but subjected to violent agitation during the mating period. At the end of the mating period, an equal volume of 10% TCA was added to each flask. Each TCA precipitate was collected on a membrane filter, washed as described in Chapter 2, and counted.

mixture was shaken violently during the mating to prevent formation of or to break up mating pairs, or if the male was UV-irradiated, incorporation was eliminated, as was found also for the F'Lac case. Thus, according to these criteria, this excess label is specific to the transfer of F.

DNA isolated from these cells after such a mating was fragmented, denatured, and incubated with an agar-gel containing Proteus F'Lac DNA, in the presence of C^{14}- or P^{32}-labeled Proteus F'Lac DNA as an internal standard. The results of two such measurements are presented in Table 16. It is seen that at least 30-40% of the H^3-labeled material must be F DNA. This again is probably an underestimate for the reasons given in Chapter 4, i.e., greater quenching of eluted H^3 than of C^{14} or P^{32}.

The sedimentation coefficient of F DNA was measured by sucrose gradient sedimentation of DNA extracted by phenol from a lysozyme-detergent lysate, using C^{14} T7 DNA as a sedimentation marker. The sedimentation run was done on the day of the mating to avoid the degradation discussed earlier. F DNA was found to have an $S_{20,w}$ of 34 ± 2 from which the molecular weight was calculated by the equation of Hershey et al (192):

$$\text{Molecular weight of F DNA} = 35 \pm 7 \times 10^6$$

Again the margin of error indicated derives from the uncertainty in the determination of S. The same arguments concerning the molecular configuration of F'Lac apply to F also.

Herman and Forro (115) showed by quantitative autoradiography that F contains an amount of DNA equal to 1.0-1.8% of

Table 16

Binding of labeled *Proteus* DNA and presumptive F DNA to a *Proteus* DNA agar-gel column

C^{14} Proteus label:

% C^{14} (*Proteus*) bound	63
% H^3 (F) bound	27

$f = H^3/C^{14} = 0.43$

P^{32} Proteus label:

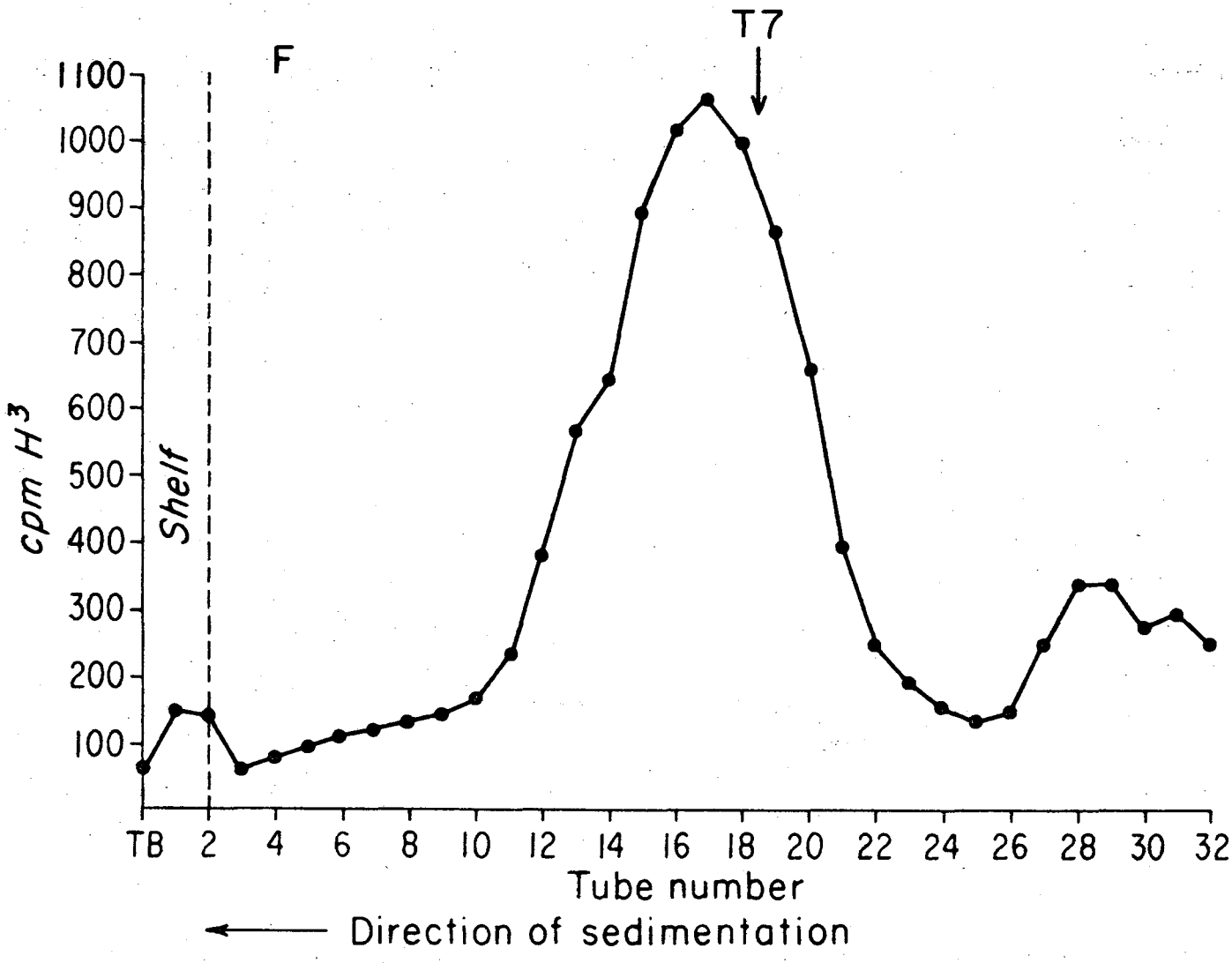
% P^{32} (*Proteus*) bound	24
% H^3 (F) bound	7.5

$f = H^3/P^{32} = 0.31$

In each case the DNA-agar contained *Proteus* F'Lac DNA. Both mating-labeled F DNA and labeled *Proteus* F'Lac DNA were added to the agar-gel, the mixture was incubated overnight at 60° in 2XSSC, and the per cent of each labeled material which was bound was determined. The C^{14} and P^{32} cases represent two distinct experiments.

Figure 9-- Sedimentation of H^3-labeled F DNA

F DNA was specifically labeled with H^3-thymine, as described in the text. The cells were lysed with lysozyme and SDS and extracted with phenol. The extract was layered on a 5 ml sucrose gradient containing an 0.1 ml "shelf" of 70% sucrose at the bottom and centrifuged for 2 hours at 35,000 rpm in a SW39 rotor in a Spinco Model L ultracentrifuge. Ten drop fractions were collected and assayed for TCA-precipitable radioactivity. The centrifuge tube bottom (TB) was cut off and counted also. The corresponding position of T7 DNA (see Figure 5) is indicated by the arrow.


T7
F
Shelf
cpm H^3
1100
1000
900
800
700
600
500
400
300
200
100
TB
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
Tube number
Direction of sedimentation

the E. coli genome. From the Cairns value for the molecular weight of the E. coli genome (45,131), this gives 22-50 x 10^6, in good agreement with the above.

Using the agar-gel technique Falkow and Citarella (129) showed that the amount of DNA in F is 70% that of F'Lac, or 38 x 10^6, in excellent agreement with the present determination.

In Chapter 5, section B, data were given which suggest that F should have a melting temperature less than that of F'Lac. Direct measurement of these values have not yet been carried out; it will first be necessary to increase the purity of the label.

Chapter 7-- General discussion and future proposals

In this work a technique for specific labeling of the F'Lac particle has been developed and evidence obtained that the majority of the labeled material is in fact F'Lac DNA. It is expected that this technique will be applicable to any DNA-containing element which can be transferred in a mating and which is capable of replication in a UV-irradiated female cell. At present F is the only other element which has been attempted. This labeling has been successful but the percentage of labeled "uninteresting" DNA is higher than in the case of F'Lac, presumably due to the smaller size of F.

This labeling procedure should prove to be a basis for numerous chemical and physicochemical experiments since it provides a solution to the principal problem encountered in the study of any cellular constituent, that of an assay. The few physicochemical experiments described herein, i.e., the measurement of molecular weight, banding density, and the melting curve, have in fact depended entirely on the ability to detect a very small quantity of DNA (usually 0.002 μg in each experiment) against a background of 100 times as much DNA and several thousand times as much general cellular material. The principal limitation of the method in its present form is that the label is not 100% pure, the contamination always being male chromosomal DNA. In the sedimentation experiments this is probably not of much importance since chromosomal DNA tends to pellet on the tube bottom and is thereby lost. However, in other

procedures, e.g., CsCl banding or agar-gel chromatography, these counts are present and not locatable. In the case of F'Lac, the contamination was sufficiently small to be negligible but this was not true when it was attempted to obtain a melting curve for F. There are several possibilities for eliminating these spurious counts. First of all, when selecting cells resistant to 5-BU, mutants with varying degrees of resistance were found. The one used in these experiments was the best of eight such mutants studied. It is possible that the isolation of mutants resistant to higher concentrations of 5-BU or possessing additional resistance to 5-bromodeoxyuridine or thiothymine might alleviate the situation. Secondly, the male cells could be eliminated from the mating mixture by lysis-from-without prior to lysis of the females.

A second limitation of the procedure is the small amount of material obtained. A detailed study of the F'Lac particle or other F' particles will undoubtedly at some time necessitate scaling up the technique so that amounts of material are obtainable which can be studied chemically and electron microscopically. For example, with sufficient amounts other procedures become feasible such as sedimentation analysis by high resolution UV absorption techniques and chemical investigation of the particle structure. Conceivably this DNA could even be used for production of messenger RNA of a single kind by *in vitro* transcription of the merogenote. However, the system cannot be scaled up simply by mating larger and larger numbers of cells;

what is required is to isolate the particle directly from male cells. This was the hope when these experiments were initiated three years ago but in the absence of an assay system, the hope could not be fulfilled. However, now that the sedimentation coefficient of the DNA is known (presumably, in the future the S of the particle will be known), it is possible to design an isolation procedure using sedimentation as the principal means of fractionation. Perhaps a start has already been made in that direction, if we recall one of the procedures that failed that was described in Chapter 3. In method 4 a thymine-containing component was found (irreproducibly) in SDS extracts of male cells (F'Lac) which had an S slightly greater (43) than that of F'Lac DNA (39), as determined in Chapter 5. Whether this component contains F'Lac DNA remains to be demonstrated; its higher S suggests that, if so, it is either more compact or contains some associated material. It should be remembered that phenol extraction was not employed in this early procedure. This must clearly be investigated further.

In investigating the properties of the F'Lac particle, thus far only the DNA has been studied-- in particular, a DNA-containing element isolated from a lysozyme-detergent lysate by phenol extraction. Since the original aim was to characterize the entire particle, it was at first attempted to avoid the use of deproteinizing agents such as phenol. However, the sedimentation patterns were not reproducible. When lysozyme and SDS lysis without the aid of phenol was used on cells which

had been grown and mated the same day or even the day before, in about ten attempts, most of the labeled material was lost as a pellet on the bottom of the centrifuge tube, except for a small fraction sedimenting with a broad boundary with an S range of about 60-100 (this may have been bacterial DNA). If the preparation was two days old or more, DNA was efficiently released from the pellet but by this time, the degradation described in Chapter 5 had set in so that little information could be gained from such experiments. After many attempts, none of which led to any understanding of the causes of this difficulty, it was decided to concentrate effort, for the present, on the DNA itself, reserving studies of the intact particle for the future. Since the present work has been completed, some newly developed lysis procedures have become available which should aid in the solution to this problem. The possibility that the difficulty is a result of association with a rapidly sedimenting membrane component, as hypothesized in Chapter 1, Section 4, must not be ignored.

These studies on the DNA nonetheless provide a basis for the investigation of the intact particle since the DNA label permits easy identification in extracts prepared in ways which would not remove associated protein or other components, if such were present. In fact, a few, rather simple observations could give valuable information about the structure of F'Lac. For example, the existence of species with different sedimentation characteristics, such as that described above and termed a "difficulty", strongly suggests either that F'Lac is not

just a piece of DNA but has a definite structure or that it is associated with some cellular component. Furthermore, if physicochemical or enzymatic treatments can be found which could convert one sedimenting species to another, some elements of the structure should be clarified. The technique of co-sedimentation with a second label permits several experiments to be done. For example, the question of protein or any other substance being associated with the DNA can also be investigated by labeling cellular protein and looking for cosedimentation of this label with the DNA radioactivity. Similarly the possibility of cell wall association can be investigated using labeled cell wall constituents such as muramic acid or diaminopimelic acid.

We must not neglect the fact that many things can be learned from sedimentation studies of the DNA alone. For example, the physical length of the DNA of a number of F' factors which carry various groups of genetic markers (Table 1) could be determined and, after correcting for the size of the F component, these lengths could be compared with the genetic length of the merogenote. Such studies could provide some badly needed information about the relation between the physical and genetic length of the bacterial chromosome. It would of course be of particular interest to determine the change in molecular weight resulting from prophage insertion: e.g., by lysogenizing with λ the F'Gal particle which contains the attachment site for λ and several other temperate phages.

A genetic map of F itself does not exist so that we do

not possess a minimum estimate for the number of functions possessed by F. Thus, in this case the heuristic value of the molecular weight determination of F becomes apparent: from the molecular weight of the DNA we can estimate that there should be 25-50 cistrons. At present only a few of these functions are known and it is clear that a large number remain to be found.

A cautionary note should be added with regard to any conclusions drawn from studies of episomes labeled by the specific labeling technique described in this work. We have of necessity studied primarily recently transferred and newly replicated F'Lac particles and it is conceivable that such particles depart in some way from the properties of F'Lac factors in an unmated, exponentially growing or stationary population of F'Lac cells. For instance, there may be a transition from circularity to linearity associated with transfer and circularity may not be re-established immediately after transfer. Also, at the moment that the cells are chilled and deprived of an energy source prior to lysis and centrifugation, the F' particles may be in various stages of replication and a partially replicated molecule would sediment differently from a single,completed one. As discussed in some detail in Chapter 5, the sedimentation peak observed was definitely broader than that of T7 DNA yet not as broad as might be expected if there were a complete and random distribution of stages of replication; thus it seems likely that many of the particles were in a similar state at the time of lysis. This would be expected if the

total time engaged in replication was short compared to the mating time. However, the possibility of some variation can certainly not be excluded and the "majority" or average state need not be one of complete replication. Kinetic studies should shed some light on these questions. If F' particles could be extracted from stationary phase cells, in which it is probable that few of them have recently replicated, their size and structure could be compared to that of the newly replicated particles.

A variation of the specific labeling technique can be devised which may avoid some of the problems of degradation which have arisen. In this procedure the F' particles would be labeled, not during mating, but after cold storage of the cells containing the transferred F'. To accomplish this, it is necessary to prevent replication of the transferred particle in the female cell, since it is likely that normally in the specific labeling technique it replicates rapidly after transfer until reaching the limiting number per cell. It is proposed to prevent replication of the transferred F' in the female cell by using a female strain requiring both thymine and adenine, and carrying out the mating in the absence of both of these substances. Starvation for thymine and adenine is known to result in complete blockage of DNA synthesis without thymineless death (D. Freifelder, pers. commun.) Otherwise the system would be the same. By subsequent addition of adenine and labeled thymine, the F' particle could be labeled

at any desired time. Also, the males could have previously been removed by lysis-from without. With this technique the kinetics of labeling could be followed independently of the kinetics of mating. By employing a density label simultaneously with the thymine, the replication time of the F' particle could be measured by the method of Meselson and Stahl (130): i.e., samples would be taken at various times after addition of the radioactive and density labels and by banding in CsCl, the time at which the thymine label reaches hybrid density and then double-heavy density could be determined. It should be noted that intact, partially replicated DNA could be isolated preparatively in this way for studies of replicating forms.

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