*Proceedings of the*
NATIONAL ACADEMY OF SCIENCES

*Volume 49 • Number 5 • May 15, 1963*

# *HUMAN SEX CHROMOSOME ABNORMALITIES IN RELATION TO DNA REPLICATION AND HETEROCHROMATINIZATION*

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*Communicated by David Shemin, March 11, 1963*

Striking differences are demonstrable in the morphological and functional state of the two X-chromosomes in somatic cells of the female of a variety of mammalian species. Recent studies[1-10] have led to the concept that in somatic cells one of the two X-chromosomes, which may be of either maternal or paternal origin, undergoes a change of state which first becomes manifest during early ontogeny. The transformation is characterized by (1) delayed replication of DNA during the regular synthetic period of interphase,[4, 5] (2) a potential for precocious condensation during prophase and formation of a prominent heteropycnotic body during interphase, Barr's sex chromatin,[1-3] and (3) suppression or modification of the action of genes[6-10] on these parts of the X-chromosome. The other X-chromosome of females and the single X in somatic cells of males complete replication along with the majority of the complement, are isopycnotic, and genetically active. Based on the time of appearance of sex chromatin in the human embryo and the heteropycnotic behavior of X-chromosomes in sex-chromosome abnormalities in man, Grumbach and Morishima[3] proposed that the change of state, heterochromatinization, is induced in a large part of one of the two X-chromosomes of each cell during an early embryonic stage, the "fixed differentiation hypothesis." The transformation once established is then transmitted to all descendants of that particular chromosome in succeeding somatic cell generations. Lyon,[7] on the basis of mosaicism in the expression of X-linked factors in female mice, proposed a similar hypothesis which involves the genetic inactivation of the heterochromatinized X-chromosome.

Heterochromatin as originally defined by Heitz[11] is characterized by permanent heteropycnosis (highly condensed state) and relative genetic inactivity. It has often been assumed to represent a different kind of chromatin from the regular euchromatin, a special type of DNA, perhaps nonspecific in its action. On the other hand, others have proposed that heterochromatin and euchromatin represent alternative states of the chromosome related to function over the cell cycle or during morphogenesis.[12] However, the interesting feature of the mammalian X-chromosome system is that here we have a clear indication that this heterochromatin, at least, is not a special type of genetic material, but represents an induced state, a type of genetic control which we suggest may operate at many loci in autosomes as

well as sex chromosomes. For the X-chromosomes a special system appears to have evolved which can suppress one of two homologous loci in the same cell. We have previously suggested[4,9] that out-of-phase replication, heteropycnosis, and genetic suppression in an X-chromosome are all identifying features of this system which operates in the mammal. In this report we present further evidence for this view and an hypothesis for the mechanism of induction.

*Materials and Methods.*—Human leucocytes were obtained from peripheral blood of three female patients with a 47/XXX (47 chromosomes, three of which are X's) chromosome complement, one phenotypic male (48/XXXY) with seminiferous tubule dysgenesis (Klinefelter's syndrome), an eight-month-old female infant (49/XXXXX), and two phenotypic females with chromatin-positive gonadal dysgenesis associated with a structural abnormality of one X-chromosome. One of the latter has 46 chromosomes, but one X-chromosome is larger than normal and has been interpreted as an isochromosome formed from two long arms of the X[13, 14] (hereafter referred to as an X-isochromosome and by the symbol $\overline{X}$). The other patient with a similar phenotype is a mosaic with XO and XX cells.[15] Leucocytes were cultured *in vitro*[16] and labeled on the third day by adding 2 $\mu$c/ml of thymidine-H$^3$ (specific activity 5 curies/mM; New England Nuclear Corporation, Boston, Mass.). After a 15-minute exposure the cells were washed and reincubated in a culture medium containing an excess of unlabeled thymidine and colchicine sufficient to block mitosis. After 3 or 4 hours the cells were exposed to hypotonic solution before fixing and spreading. The DNA was stained by the Feulgen reaction and autoradiographs were prepared as described by Taylor.[5] Buccal smears were obtained and stained for sex chromatin.[17] In addition, the sex chromatin was studied in skin explants from several of the patients. For labeling such cells, thymidine-H$^3$ (2 $\mu$c/ml) was added to the culture medium and the cells were fixed in 95% ethanol after 20–40 minutes incubation with the isotope. Autoradiographs were prepared after staining by the Feulgen reaction.

*Results.*—In the 7 subjects studied, the maximum number of sex chromatin bodies per diploid nucleus was equivalent to one less than the number of X-chromosomes in the sex chromosome complex. Only heteropycnotic bodies with the typical characteristics of sex chromatin located at the optical edge of the flattened nucleus were counted. The sex chromatin mass was larger than normal in the two subjects who have an XX cell line.[14] Together with the 7 subjects previously reported,[4] a sufficient number of individuals with various sex chromosome constitutions have been studied to allow a few generalizations (Table 1). The maximum number of sex chromatin bodies per diploid nucleus is the same as the number of late-labeling X-chromosomes. The number of late-replicating X's is regularly one in normal females, an observation confirmed by German[18] and by Gilbert *et al.*[19] This chromosome is absent in males and XO females. In normal individuals, as well as phenotypic males and females with extra X-chromosomes, the number of late-labeling X's is one less than the total number of X-chromosomes. One XO/XX/XXX mosaic to be discussed is an exception.[3, 4]

By analogy with the labeling pattern reported for the cells of the Chinese hamster,[5] and the low frequency of human leucocytes with only the X-chromosomes labeled, we assume that the X-chromosome continues replication for only a short time after most of the autosomes are finished. A small proportion of the late prophases and metaphases accumulated during the 3 or 4 hours following exposure to thymidine-H$^3$ contain heavily labeled X's. These cells represent those which were near the end of the DNA synthetic phase (*S* period) at the time of incorporation of tritium. Since the interval between the end of *S* and metaphase varies between one and several hours, the cells with late-labeled X's do not arrive at division synchronously. However, a proportion of the late prophase and metaphase figures

TABLE 1

RELATION OF X'S IN SEX-CHROMOSOME CONSTITUTION TO LATE-REPLICATING X'S AND TO SEX CHROMATIN BODIES

| No. of subjects* | 2 | 1 | 3 | 3 | 1 | 1 | 1 | 1 | 1 |
|---|---|---|---|---|---|---|---|---|---|
| Sex chromosomes | XY | XO | XX | XXX | XXXY | XXXXX | $X\bar{X}$ | $XO/X\bar{X}$ | XO/XX/XXX |
| No. of late-replicating X's | 0 | 0 | 1 | 2 | 2 | 4 | 1(IsoX) | 1(IsoX) | 0–2 |
| Max. no. of peripheral sex chromatin bodies | 0 | 0 | 1 | 2 | 2 | 4 | 1 large | 1 large | 0–2 |

* The results in XO, XY, XX, and XO/XX/XXX subjects appeared in a previous report in these PROCEEDINGS.[4]

of the three triple-X subjects contained two heavily labeled medium-sized chromosomes with the size and morphology of typical human X-chromosomes. In a few of these cells only these two chromosomes were detectably labeled. The other X-chromosome was not conspicuously different from the majority of the autosomes in its pattern of labeling.

Identical results were obtained with the leucocytes of the XXXY individual (Fig. 1*a*). As noted previously, the Y-chromosome in these cells, as well as normal males,

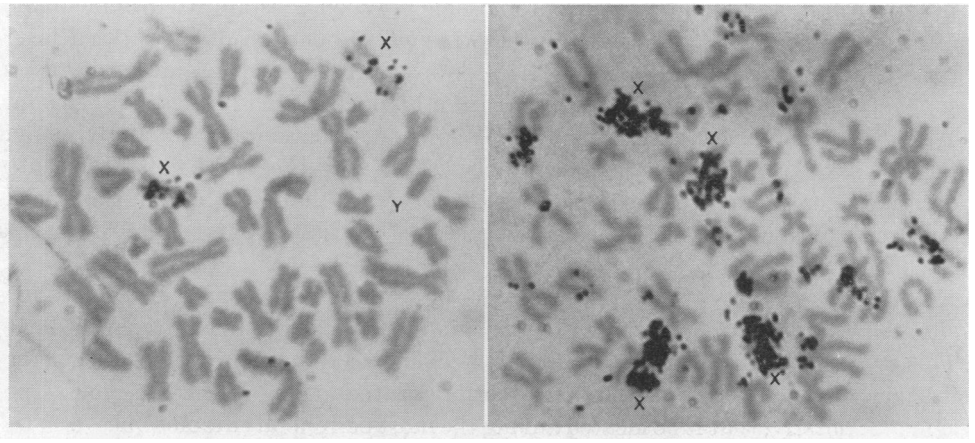


FIG. 1.—Photomicrographs of the autoradiogram of metaphase chromosomes. Left: (*a*) Two of the three X-chromosomes were heavily labeled in this cell with 48 chromosomes and an XXXY sex-chromosome complex. Note the lack of labeling of the Y-chromosome. Right: (*b*) Four X-chromosomes are heavily labeled in a cell with 49 chromosomes and an XXXXX sex-chromosome complex.

completes DNA replication before the late-labeling X's. Analysis of the XXXY complements with only the two late X's labeled revealed no detectable tritium in the Y-chromosome. This contrasts with the Y in the Chinese hamster.[5]

In all of the subjects with more than two X-chromosomes, a few cells were observed which showed differences in the intensity of labeling among the late-replicating X-chromosomes. This difference was more apparent in cells in which the autosomes were more heavily labeled; these cells were not at the very end of their DNA synthetic period.

The cells of the penta-X subject regularly had four late-labeling X-chromosomes (Fig. 1*b*). In cultured cells derived from skin biopsy specimens a maximum num-

ber of four Feulgen stained sex chromatin bodies was observed in 10.0% of interphase cells presumed to be diploid. In scoring these cells, only bodies at the optical edge of the ellipsoidal nuclei were counted. However, if all Feulgen stained chromatin bodies with the size and morphology of sex chromatin are included, the modal frequency is four (Table 2). More than 4 chromatin bodies were found in a small proportion of large, apparently tetraploid, nuclei; none of these nuclei contained more than 8 such masses. An examination of the autoradiographs of skin explants of the penta-X patient provides direct evidence that each sex chromatin body is

TABLE 2

FREQUENCY OF PERIPHERAL SEX CHROMATIN BODIES AND OF TOTAL CHROMATIN BODIES PER INTERPHASE NUCLEUS IN 500 CELLS (FEULGEN STAIN)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Peripheral sex chromatin bodies | | | | | | | | | |
| No. of sex chromatin bodies at optical edge of nucleus | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Per cent of nuclei | 8.4 | 21.4 | 33.6 | 25.8 | 10.0 | 0.2 | 0.4 | 0.2 | 0 |
| Total chromatin bodies | | | | | | | | | |
| No. of chromatin bodies (peripheral and nonperipheral) | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 |
| Per cent of nuclei | 2.8 | 2.2 | 4.8 | 16.6 | 69.6 | 1.2 | 0.6 | 0.8 | 1.4 |

produced by one late-labeling X-chromosome. When cells are fixed immediately after a short contact with thymidine-$H^3$, a small proportion of the labeled interphase nuclei have four peripheral "hot spots" (areas of high grain density). In some of these nuclei almost all of the tritium is concentrated in these four regions. When the grain density is low enough to allow identification of the underlying material, a sex chromatin body can usually be seen. Atkins *et al.*[20] have also recently described peripheral areas of high grain density over the interphase nuclei of cells cultured from a human female which they suggest originate from the late-replicating X-chromosome.

In flattened groups of metaphase chromosomes the late-labeling X-chromosome is situated at the periphery more frequently than is attributable to chance alone. This occurs in spite of the treatment with colchicine and hypotonic solution before fixation. Two criteria were used for scoring a late-labeling X-chromosome as peripheral: firstly, that it be located among the most peripheral chromosomes from the optical center of the metaphase; and secondly, that the cells scored have few or no grains over any other chromosome. In 36 cells from a normal female the late-labeled X was peripheral in 21. In one group of 92 chromosomes in a tetraploid cell the two labeled X's were side by side at the periphery. Among 10 cells from an XXX individual, 3 had both late-replicating X's peripheral, and in 5 others one of these X's was at the edge of the group. These observations are consistent with other morphological and representational evidence which indicates that sex chromatin is at the periphery of the interphase nucleus in most somatic cells.[21-23] Ferguson-Smith and Handmaker[24] and Ohno *et al.*[25] have also observed significant associations among certain autosomes in cells treated in a manner similar to these before fixation.

Leucocytes were obtained from the two patients with heteromorphic X-chromosomes ($X\bar{X}$ and the mosaic $XO/X\bar{X}$) in the hope that the labeling pattern would reveal whether or not the heteropycnotic, late-labeling X's were the result of random

induction at a multicellular stage of the embryo. The autoradiographs revealed that the cells of one female ($X\bar{X}$) had one late-labeling chromosome. However, in all cells examined the $\bar{X}$ (X-isochromosome) was the late-replicating one. Similarly, in preparations obtained from the $XO/X\bar{X}$ subject, the abnormal $\bar{X}$ was labeled in the few cells found at the appropriate stage; these preparations were technically poor because few cells were dividing.

*Discussion.*—Mammals appear to have evolved an inducible genetic control mechanism for the X-chromosome which is operative only in the *cis* configuration comparable to the operator loci described in bacteria.[26] However, in contrast to these systems which regulate small genetic loci, a large part of the X-chromosome and of autosomal loci translocated to the X can be inactivated or regulated. The control system appears to function as a dosage compensation mechanism by which both female and male somatic cells maintain one functional X per two sets of autosomes. Less is known of the dosage requirements of gonadal tissue, but the abnormalities found in phenotypic females with an XO or an $X\bar{X}$ constitution suggest that a different dosage is required for normal differentiation of the human ovary.

In a previous study,[9] the erythrocyte glucose-6-phosphate dehydrogenase activity was shown to be essentially constant over a fourfold range of X-chromosome dosage. The study of the activity of this enzyme controlled by a sex-linked gene has now been extended to include the penta-X patient (in collaboration with P. A. Marks). The results indicate that the enzyme activity is within the range of normal for males and females, and are further evidence that heterochromatinization of extra X's is an effective, but not absolute, dosage compensation mechanism in man. By contrast extra autosomes usually produce strikingly severe phenotypic changes.

The apparent preferential heterochromatinization of an abnormal X illustrated by the two patients ($X\bar{X}$ and $XO/X\bar{X}$) at first appears at variance with the concept of random heterochromatinization of parts of either the maternally or paternally derived X. However, previously reported morphological observations[3] on individuals with structurally abnormal X's suggest that the aberrant X is usually the heterochromatic one. For example, in females with one normal X and one small X, presumably the result of a deletion, the sex chromatin body was abnormally small.[14, 27] On the other hand, a preliminary report by Ohno[28] indicates that either X, the normal one or one with an X-autosome translocation, can be heterochromatic in mice. In one mouse the normal X could be identified in 90% of the prophases as the heterochromatic X by its precocious condensation. In another female 70% of the prophases showed the X bearing the piece of autosome to be heterochromatic. Similarly, the studies of Russell,[6] of Lyon,[7] and of Cattanach[8] on the variegation of autosomal genes translocated to the X-chromosome in the mouse indicate that either X, the normal one or the one bearing the translocation, may be modified in genetic expression. Further, the report by Beutler *et al.*[10] suggests that the genetic suppression may affect either the maternally or paternally derived X in individual cells.

The apparent preferential heterochromatinization of an abnormal X may be explained either on the basis of cellular selection following random induction of one of the two X-chromosomes, or as a consequence of selective induction of the isopycnotic or heteropycnotic state early in ontogeny. An analysis of the translocation in the mouse described by Cattanach[8] helps to understand how cellular selec-

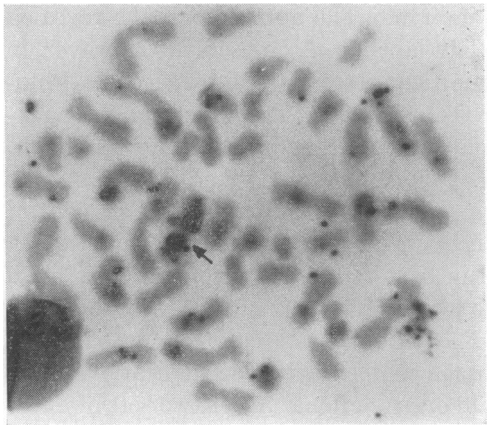

FIG. 2.—Photomicrograph of an autoradiogram of metaphase chromosomes from a patient with 46 chromosomes and $X\bar{X}$ sex-chromosome constitution. The arrow indicates heavily labeled X-isochromosome.

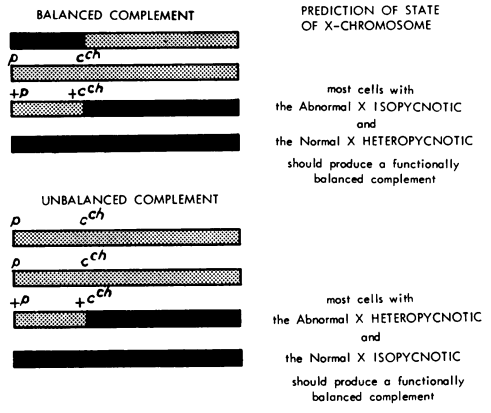


FIG. 3.—Schematic representation of balanced and unbalanced complements producing viable females from Cattanach's translocation. The stippled bars represent autosomes, linkage group I, and the solid bars represent X-chromosomes. Mice missing a piece of the autosome are not viable.

tion could operate. He described two viable types of female mice bearing a piece of an autosome, linkage group I, attached to an X-chromosome by a reciprocal translocation. One type has a balanced chromosome complement and the second is trisomic for the translocated autosomal piece but deficient for a segment of one X (Fig. 3). Ohno has found from cytological studies[28] that the autosomal segment is heteropycnotic when the X with the translocation is heterochromatinized. Cattanach's genetic data indicate that loci affecting coat color in the translocated piece produce a flecked or mosaic pattern in XX females and XXY males but not in XY males or XO females. These observations are consistent with the hypothesis of genetic inactivation of at least part of the translocated autosomal segment if the X bearing it becomes heterochromatinized. When this occurs in cells with a balanced complement (Fig. 3), these cells become, in effect, deficient for the translocated segment. Such cells might be at a disadvantage in survival, since mice deficient for this one autosomal segment are not viable. Accordingly, in females with the balanced complement most of the abnormal X's should be isopycnotic, even if the original induction of the isopycnotic or heteropycnotic state were random. Conversely, in those females which are trisomic for the translocated autosomal segment, one would expect to find more cells in which the normal X is isopycnotic and the X bearing the translocation heteropycnotic. Inactivation of the aberrant X-chromosome would leave these latter cells with a functionally balanced complement, i.e., two autosomal segments and one X-chromosome which remain genetically active.

The considerations discussed above lead us to the conclusion that the structurally abnormal "X" chromosome ($\bar{X}$) in the $X\bar{X}$ and $XO/X\bar{X}$ females we have studied may be either an isochromosome formed from the two long arms of an X-chromosome, a suggestion previously advanced,[13, 14] or an X carrying a translocation of an autosomal segment of a size to produce two equal arms, a possibility raised by

Ohno[28] from his work in the mouse. If the abnormal chromosome is an isochromosome, its inactivation should result in a functionally balanced complement. If, on the other hand, it is formed by an X-autosome translocation, we would predict that the complement is of the unbalanced type in which inactivation of the aberrant chromosome would produce a functionally balanced complement. In either case, if the normal X-chromosome carries a mutant gene, its expression should be comparable to that found in hemizygous individuals.[7]

An alternative to the cellular selection hypothesis is some mechanism which leads to selective induction of the isopycnotic or heteropycnotic state in the aberrant X. It is possible to imagine a mechanism modeled after the bacterial systems reviewed by Jacob *et al.*[29]

Let us suppose that induction involves the incorporation of an episomal factor into an X-chromosome at a specific receptive locus during early embryonic development. As soon as the episome becomes incorporated, it begins to function by producing a substance which combines with and inactivates any other such unincorporated factors in the cell. Thereby, other X-chromosomes with similar receptive sites are not induced to the potentially isopycnotic state. A factor which induces the potentially isopycnotic state is compatible with our present knowledge, since we regularly find one isopycnotic X-chromosome irrespective of the total number of X-chromosomes in the cell. After the first X-chromosome is induced, we may assume that the other X or X's in the cell remain in or acquire the potentially heteropycnotic state. Sex chromosome mosaics harboring cells with more than one isopycnotic X or none are assumed to arise by nondisjunction after the induction stage. This behavior would explain the situation previously reported[4] for the chromosomal mosaic (XO/XX/XXX), in which some cells with three X's had two late-replicating X's while others had only one.

To develop this model of regulation further, one might suppose that later the X-chromosomes begin to produce a repressor substance, at or near each receptive locus not inactivated by induction of the isopycnotic state, which effects the heteropycnotic transformation and the phenomenon of heterochromatinization. To limit the effects to the chromosome or region containing the locus, a substance with limited diffusibility and the capacity to spread along fibrous material may be necessary. Two functions might be attributed to this substance. Its primary function would be to prevent or hinder the synthesis of RNA on the DNA template; but, in addition, it must permit DNA replication. Actinomycin D has these effects on DNA;[30] however, it is a relatively small, freely diffusible molecule. The secondary function of the hypothetical repressor substance would lead to a regulatory system for DNA replication in the heterochromatinized chromosomal segments which differs from that of the isopycnotic chromosome. Such heteropycnotic regions may be representative of a large class of chromatin scattered through the complement, which is released for replication sometime after the beginning of the regular DNA synthetic period and frequently completes replication a little late.

In this model the episomal factor incorporated into the potentially isopycnotic chromosome prevents the production of the repressor substance on the X-chromosome to which it became attached, and thereby ensures its genetic function. The model provides an explanation of the preferential heterochromatinization of an aberrant X-chromosome from which the specific receptive locus is deleted or trans-

located, since the aberrant chromosome would no longer be inducible to the potentially isopycnotic state.

The association of precocious condensation, delayed DNA replication, and the apparent suppression of RNA synthesis in the segments involved may be characteristic of a regulatory mechanism that affects autosomes as well as sex chromosomes and, hence, operates in cellular differentiation. The transformation could well be reversible and induced by a variety of factors in the different parts of the complement. It should be emphasized that the above speculation concerning an episome is an attempt to explain the special case in which there is differential behavior among identical or very similar genetic loci in the same cell, i.e., the integrated episome operates only in the *cis* configuration.

*Summary.*—Tritiated thymidine and autoradiography were utilized to study the sequence of DNA replication in X-chromosomes of the human complement. Five individuals with extra X-chromosomes were found to have one X which replicates with the majority of the complement while all others replicate late. Evidence is presented which indicates that these late-replicating X-chromosomes are heteropycnotic in interphase and their genetic expression is repressed. Two patients with a structurally abnormal X were found to have this chromosome consistently heteropycnotic and late-replicating. These observations are consistent with the "fixed differentiation hypothesis" of X-chromosome behavior.

The authors gratefully acknowledge the kindness of Dr. Robert M. Blizzard, Dr. M. A. Ferguson-Smith, and Dr. Paul V. Wooley for providing the opportunity to study their patients. We wish to thank Jeanne Tung and Nancy Carmichael for their technical assistance.

* Career Scientist, Health Research Council of the City of New York. This work was supported in part by grants from the National Foundation and the National Institute of Arthritis and Metabolic Diseases;† and in part by grants from the Division of Biology and Medicine, U.S. Atomic Energy Commission, Contract AT (30-1) 1304.

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## *INCORPORATION OF PARENTAL DNA INTO GENETIC RECOMBINANTS OF E. COLI*

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*Communicated by David R. Goddard, November 5, 1962*

Two kinds of mechanisms have been considered to account for genetic recombination in bacteriophage and bacteria. One involves exchange of pre-existing genetic material between homologous chromosomes (*breakage and reunion*), and the other implies exchange of genetic information without exchange of pre-existing genetic material (*copy choice*).[1] Experiments by Meselson and Weigle[2] and Kellenberger, Zichichi, and Weigle[3] have shown that when a strain of bacteriophage λ containing labeled DNA is crossed with an unlabeled and genetically distinguishable strain, the genetic recombinants arising from such a mating contain discrete amounts of the parental DNA. We present evidence which indicates that recombinants arising from the mating of an Hfr with an $F^-$ strain of *Escherichia coli* inherit labeled DNA from the $F^-$ parent.

Bacterial conjugation involves a progressive transfer of genetic markers from the Hfr to the recipient $F^-$.[4] There is a concomitant transfer of DNA from the Hfr to the $F^-$.[5] The recipient subsequently gives rise to recombinants. The present procedure for detecting the incorporation of parental DNA into recombinants involves the mating of a T6-resistant Hfr to a T6-sensitive $F^-$ carrying specifically labeled DNA. When the mated cells are exposed to a high multiplicity of the phage T6, the sensitive cells are rapidly lysed. The T6-resistant $F^-$ recombinants can then be separated by filtration and examined for the presence of the parental label.

*Materials and Methods.—Bacteria:* The $F^-$ is a derivative of a thymine-requiring strain which originated in the laboratory of Dr. F. Ryan and was obtained through the courtesy of Miss E. Fox. Its pertinent characters for the present experiment are $Thy^-$ (requirement for thymine) and $S^r$ (resistance to streptomycin). The Hfr is resistant to phage T6 ($T6^r$) and transfers the phage resistance marker at about 5 min after mating; it is $S^r$.

*Phage:* Wild-type coliphage T6 was used. High-titer phage suspension ($5 \times 10^{12}$ particles/ml) was prepared according to the methods of Adams.[6]

*Medium:* The broth medium contained Bacto Tryptone 10 gm, yeast extract 5 gm, NaCl 5 gm, dextrose 1 gm, and distilled water one liter. The pH was adjusted to 7.4 with NaOH.

*Preparation of labeled $F^-$ and mating procedure:* An overnight culture of the $F^-$ was diluted one hundredfold into 0.5 ml of broth to which 10 microcuries of tritiated thymidine (specific activity