Density matters: The semiconservative replication of DNA

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The semiconservative mode of DNA replication was originally documented through the classic density labeling experiments of Matthew Meselson and Franklin W. Stahl, as communicated to PNAS by Max Delbrück in May 1958. The ultimate value of their novel approach has extended far beyond the initial implications from that elegant study, through more than four decades of research on DNA replication, recombination, and repair. I provide here a short historical commentary and then an account of some developments in the field of DNA replication, which closely followed the Meselson–Stahl experiment. These developments include the application of density labeling to discover the repair replication of damaged DNA, a "nonconservative" mode of synthesis in which faulty sections of DNA are replaced.

DNA replication is arguably the most fundamental process required for the proliferation of all living cells. During cell division, each daughter cell must receive essentially the same genetic information that was encoded in the DNA of the parent cell. This conclusion means that DNA replication must generate a perfect copy of the genomic DNA complement. Convincing experimental evidence for a "semiconservative" mode of DNA replication was first provided by the elegant experiments of Matt Meselson and Frank Stahl (1), in which differential labeling with nitrogen-15 (^{15}N) and nitrogen-14 (^{14}N) was used to resolve parental and daughter DNA molecules by equilibrium sedimentation in a CsCl density gradient. By "semiconservative," it is meant that the parental DNA subunits are conserved but that they become equally distributed into daughter molecules as replication proceeds. It was originally thought, and is now known to be true, that these "subunits" are the complementary single strands of the double-helical DNA duplex.

A comprehensive historical description of the collaboration between Meselson and Stahl, the milieu in which they worked, and their remarkable path to success was prepared by the late Frederic Lawrence Holmes and titled *Meselson, Stahl, and the Replication of DNA: A History of "the Most Beautiful Experiment in Biology"* (2). This account highlights the personalized side of the story and provides a wonderful example of how seminal research is actually done. The crisp rendition of experiments and their clear-cut interpretations in the published journal article cannot begin to reveal the tortuous path of the research, from the germination of ideas, through the disappointments and surprises as the experimental results appear, to the ultimate success of the project.

Speculation about how DNA might replicate directly followed the proposal by James Watson and Francis Crick for its double-helical structure, in which the pairing of bases through hydrogen bonds and stereochemistry ensured that the two strands would be complementary (3). A thymine in one strand is always paired with an adenine in the other, and correspondingly, cytosine is always paired with guanine. That part of the model incorporated Erwin Chargaff's "rules" (4), based on the relative frequencies of these bases in DNA. Reflecting on their duplex DNA model, Watson and Crick stated, "It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for the genetic material" (3). Thus, the strands might separate and then serve as templates for the synthesis of the respective complementary strands—a semiconservative mode of replication, in which each daughter DNA molecule would consist of one "old" strand and one "new" one.

Whereas the suggested mechanism seemed plausible, it was not immediately apparent how it might be rigorously tested. Furthermore, there were some rather vexing topological problems with which to contend. The DNA strands in the Watson–Crick helix are wound about each other in a "plectonemic" manner—which means that "for any winding number greater than zero, the 'braid' consisting of the two chains cannot be combed" as Max Delbrück and Gunther Stent (5) pointed out in their early review on the subject. The Watson–Crick scheme assumed that unwinding and replication must proceed *pari passu*, with all three arms of the duplex DNA rotating at a replication fork. Another model suggested that periodic double-strand breaks would permit short sections of the duplex DNA to spin and then rejoin with the respective strand terminals of the same polarity. Although we now appreciate that an unprotected double-strand break in DNA is a very serious threat to cell viability, it has turned out that transient strand breaks are indeed the means by which the topological problem is resolved. As is often the case, when we are unable to explain how a plausible biochemical model might work, it may be because we have yet to discover an essential enzyme, in this case, topoisomerase. Topoisomerases are DNA "nicking-closing" enzymes and the type II topoisomerases, such as gyrase, in particular, are designed to pump negative supertwists into the DNA ahead of an advancing replication fork, thus relieving the unwinding stress and facilitating processive separation of the two strands (6). Otherwise, positive supertwists would accumulate ahead of the replication fork during replication as the parental strands are separated behind it. The topological problems of unwinding parental DNA strands and segregation of daughter DNA duplexes were resolved many years after the basic mechanism of DNA replication was revealed (7). Provocative, and perhaps clairvoyant, was the statement of Delbrück and Stent (5) regarding the putative semiconservative mode of DNA replication—"if it were possible to label differently the new material synthesized in each generation, then one could read off in each duplex the ages of the two chains."

In an exemplary set of experiments (of which Max Delbrück was surely aware) in late 1956, J. Herbert Taylor *et al.* (8) labeled the chromosomes of *Vicia faba* (English broad bean) with ^{3}H-thymidine, and then followed the distribution of the tritium label through successive generations of duplication in nonradioactive medium, by using autoradiography. The remarkable conclusions from this study were "that the thymidine built into the DNA of a chromosome is part of a physical entity that remains intact during succeeding replications . . . " and "that a chromosome is composed of

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Abbreviation: 5BU, 5-bromouracil.

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two such entities probably complementary to each other," and "that after replication of each to form a chromosome with four entities, the chromosome divided so that each chromatid (daughter chromosome) regularly receives an 'original' and a 'new unit.'" Taylor appreciated, of course, that the "chromosome is several orders of magnitude larger than the proposed double-helix of DNA." Nevertheless, he had demonstrated that eukaryotic chromosomes divide semiconservatively, in accordance with the predictions of the Watson–Crick model, if a chromosome contained a duplex DNA molecule.

The Germination of an Idea: Meselson Meets Stahl

Matt Meselson began his graduate study in chemistry at the California Institute of Technology (Caltech, Pasadena, CA) in 1953. He joined the laboratory of Linus Pauling and ultimately completed part II of his Ph.D. thesis (9) on *The Crystal Structure of* N, N′*dimethyl malonamide*, to determine whether the peptide groups contained in this molecule were planar, and thus in accord with Pauling's resonance theory. That chapter of his thesis is less widely known than is part I, which was titled *Equilibrium Sedimentation of Macromolecules in Density Gradients with Application to the Study of DNA.* As a student in Pauling's course on the chemical bond, Matt became interested in the comparative strength of hydrogen bonds when the natural hydrogen was replaced with the heavy isotope, deuterium. While developing his interest in how living organisms might fare if they incorporated deuterium into their molecules, Meselson happened to attend a seminar at Caltech by Jacques Monod, who raised the question of whether the then-poorly understood phenomenon of induced-enzyme synthesis really involved new protein synthesis. Meselson speculated that if bacteria could be grown up in deuterium (heavy) water, and then transferred to ordinary water at the same instant that an "inducer" was added, any new proteins should be of "normal" density and the density difference between "old" and "new" proteins might permit their separation. In fact, if one centrifuged the proteins in a solution of intermediate density, then the "old" protein might sink whereas the newly synthesized protein should float. Later that year, he turned his attention to the DNA replication problem, after a lively discussion with Max Delbrück about the Watson–Crick double helix and possible modes for its duplication. It occurred to Matt that the same approach he had envisioned for protein synthesis might also be applied to study

Fig. 1. Photograph taken by F. L. Holmes of Matt Meselson and Frank Stahl in 1996, standing at the site where they met at Woods Hole 42 years earlier (figure 14.1 in ref. 2). Courtesy of the Holmes family.

DNA replication. He decided at that point that he wanted to devote his energies to determine whether DNA, indeed, replicated in the manner predicted by Watson and Crick. Unrelated to that goal, he spent the summer of 1954 at the Marine Biological Laboratory at Woods Hole, MA, assisting Jim Watson with some titration experiments that were designed to provide possible support for a double-helical structure of RNA, analogous to the structure of DNA. Frank Stahl, then a graduate student in biology from the University of Rochester (Rochester, NY), was also at Woods Hole for the summer to take a physiology course. They met while Frank was sitting under a tree working on a problem in bacteriophage genetics. Fig. 1 is a photo of Matt and Frank 42 years later, standing at the same place. Whereas Matt was still quite naïve about bacteriophage genetics, he possessed the skills in calculus to help Frank solve the problem. As they became acquainted, Matt then raised the possibility that they might work in collaboration on the DNA replication problem—using phage DNA, to take advantage of Stahl's expertise. He also suggested using deuterium as a heavy label in a one-step growth experiment (by analogy with his earlier thoughts about density-labeling proteins), and to then centrifuge the sample in a solution of an appropriate density to separate the "light" DNA at the top of the tube from the "heavy" DNA at the bottom. However, they both soon recognized the complicating problem of doing their replication experiment with phage, because of the extensive recombination known to occur, which might be expected to reshuffle parental and daughter DNA, and thereby confuse the analysis. Fortunately, Stahl was already planning to go to Caltech for his postdoc, so they would be able to continue their discussion and work together there, perhaps to develop their strategy by using a "simple" cell system, the bacterium *Escherichia coli*.

Matt wanted to find out more about the chemical nature of the monomer precursors for DNA, and in the course of that literature search he learned about 5-bromouracil (5BU), an analog of thymine, which bacteria could incorporate during DNA synthesis in place of thymine. 5BU is equivalent to thymine

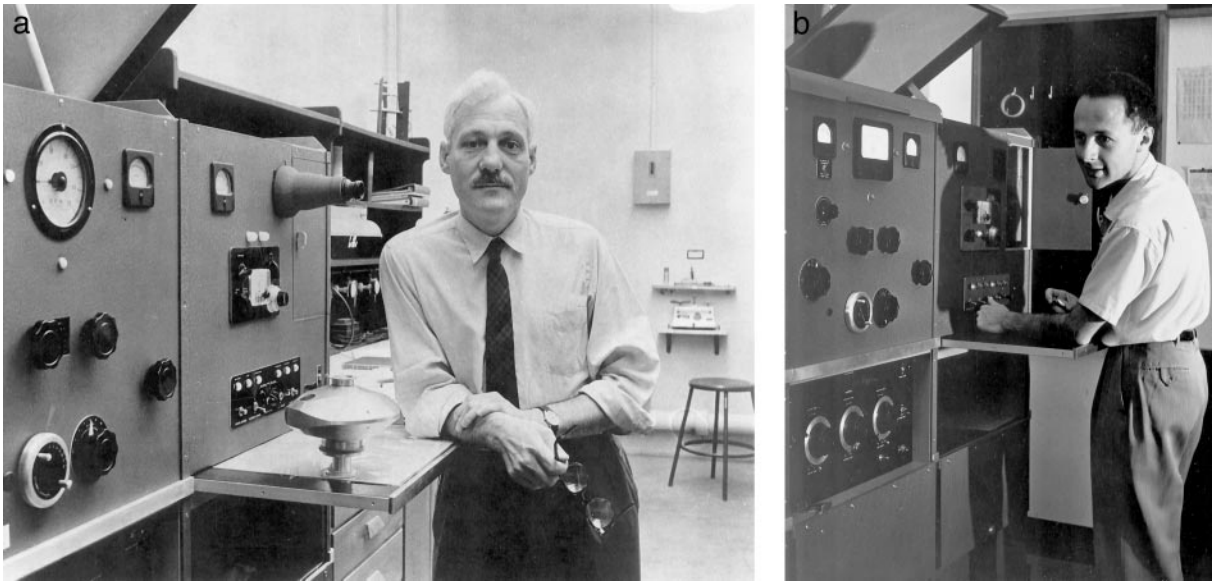

Fig. 2. Photographs of Jerome Vinograd and Matt Meselson. (*a*) Jerome Vinograd by "his" Spinco Model E analytical ultracentrifuge, serial no. 186. (Courtesy of the Caltech Archives.) (*b*) Matt Meselson at the controls for the UV optics and photography system of Model E no. 186 used for the classic experiment. (Courtesy of the Caltech Archives.)

except that bromine is substituted for the methyl group at the C5 position: the bromine conveniently has nearly the same van der Waals radius as a methyl group. Because of the different degree of ionization between 5BU and thymine, Matt considered that he might be able to separate 5BU-labeled molecules from those containing thymine by electrophoresis. However, more importantly, he appreciated the fact that 5BU would make the DNA containing it significantly heavier than normal thymine-containing DNA. He then considered using 5BU as a density label for DNA to follow its replication by the scheme considered earlier.

Matt became acquainted with Jerry Vinograd, who was the ultracentrifugation "guru" at Caltech, and he learned to operate the state-of-the-art Beckman Spinco Model E analytical ultracentrifuge (Fig. 2). With Vinograd's initial tutelage, Matt tried sedimentation of DNA in a 7-molal solution of the heavy salt, CsCl—his idea was still that an experiment could be performed with a density label and that "light" DNA should float and that the density-labeled heavy DNA would sink in a solvent of the appropriate density. However, they were both amazed at how rapidly a salt gradient formed during the high-speed centrifugation and, furthermore, that the DNA migrated to a narrow band within the gradient. The band formed at the position of the buoyant density of the DNA in that stable salt gradient.

The concept of equilibrium sedimentation in density gradients generated during the approach to equilibrium of a low molecular weight solute (e.g., CsCl) was elaborated by Meselson *et al.* (10) in a paper communicated to PNAS by Linus Pauling in May 1957. The figures in that paper and the theoretical calculations are essentially part I of Meselson's Ph.D. thesis, which, interestingly, provides no preview of the intent to apply density labeling to the study of DNA replication. The paper focuses instead on the nature of the band structure and the fact that the concentration distribution of a single macromolecular species in a constant density gradient should be Gaussian, and that the standard deviation of that band is then inversely proportional to the square root of the macromolecular weight. The model was remarkably correct, as tested with homogeneous DNA of known molecular weight from bacteriophage T4. This paper also documents the first analysis of the density distribution of DNA containing 5BU, obtained from T4-infected cultures of *E. coli* grown in media with this thymine analog. The 5BU fully substituted DNA molecules banded at a density of 1.8 g/cm^2, whereas those of normal thymine-containing T4 bacteriophage DNA were well separated from these at 1.7 g/cm^2. Although there was no mention of using this approach to study DNA replication, the application to study intact viruses and smaller molecules like proteins is discussed in this pioneering report on density gradient sedimentation.

The Classic Experiment

Matt and Frank were well on their way to design their landmark experiment on DNA replication. They might have used 5BU as the density label but they became concerned about the deleterious effects of its mutagenicity and cellular toxicity, as well as problems in obtaining uniform labeling, so they decided instead to use a synthetic growth medium in which the sole source of nitrogen was $^{15}NH_4Cl$.

The bacterium *E. coli* was grown for many generations in $^{15}NH_4Cl$ medium so that the DNA would be essentially fully labeled with the heavy isotope ^{15}N. Then, the medium was diluted with a 10-fold excess of $^{14}NH_4Cl$ as exponential growth continued. Samples were taken from the growing bacterial culture at various times to analyze the distribution of DNA densities in a CsCl gradient. There was initially a single band at the ^{15}N heavy DNA position, and then a second band began to appear at a position half way between the density of ^{15}N DNA and that of ^{14}N DNA. The parental ^{15}N band disappeared with time as this "hybrid" band formed. At precisely one generation (or division cycle), only the intermediate density hybrid band was present. It was then important, indeed essential, that the experiment was continued for a second generation, thereby to establish that when the hybrid DNA replicated in the ^{14}N medium, equal amounts of "light" and hybrid DNA were present at the completion of that second cycle. Thus, the hybrid DNA was continuously regenerated during replication and the amount of light DNA increased with each round of replication. There was the profound

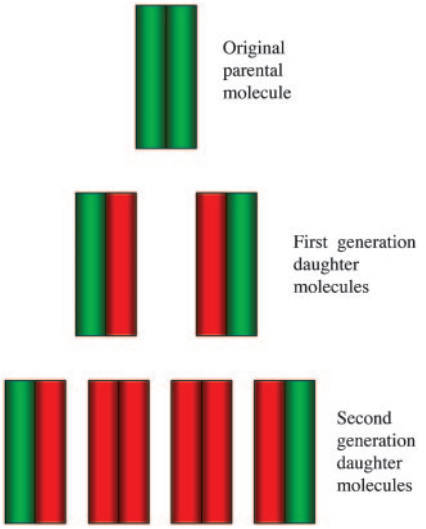

Fig. 3. Interpretation of what the density labeling data actually confirm in terms of a model for DNA replication (figure 5, from p. 677 of ref. 1). [Reproduced with permission from ref. 1 (Copyright 1958, PNAS).]

implication that the constant amount of hybrid molecules will be maintained "forever" as successive cell divisions continue.

At this point it was clear that "the nitrogen of a DNA molecule is divided equally between two subunits which remain intact through many generations," . . . "the subunits are conserved," and that each daughter molecule receives one parental subunit, according to the scheme shown in Fig. 3 [which is figure 5 in the Meselson–Stahl paper (1)]. An essential requirement of the model is that the two subunits must separate. Meselson and Stahl (1) provided convincing evidence of this through thermal denaturation studies in which the DNA samples were kept at 100°C for 30 min in the CsCl before centrifugation. The hybrid DNA clearly resolved into two bands at the respective positions of heat-denatured ^{15}N-DNA and ^{14}N-DNA under these conditions. Furthermore, the broader Gaussian bands of the denatured DNA indicated a reduction of roughly one-half in the molecular weight from that of duplex DNA, as consistent with the view that the subunits are single DNA strands. Nevertheless, the conclusions were stated with cautious restraint, leaving open the questions about the nature of the molecular structures of the subunits and the relationship of these subunits to each other in a DNA molecule (1).

I first learned of the Meselson–Stahl experiment while I was a graduate student at Yale, when I attended the second annual meeting of the Biophysical Society, in Cambridge, MA, in early 1958. In a contributed-paper session, Matt was accorded two successive 15-min slots for his talk, as I recall, as the Chair announced that this next presentation was going to be of very special significance. It was indeed an exciting and generally convincing presentation: clearly the highlight of the meeting for me and for most others.

Caveats About the Proof

In Meselson's talk and in their PNAS paper, as noted above, Meselson and Stahl (1) were very careful about what they could actually claim from their experiment. Figure 5 in their paper implies no more and no less about what can be concluded, even though the most obvious and straightforward assumption is that the conserved "subunits" must be single DNA strands. Was the Meselson–Stahl experiment definitive proof for semiconservative replication of DNA? In principle, the answer is yes, but there were additional important controls to be carried out. Although it was by no means a favored interpretation of the results, it was technically possible that the conserved "subunits" of DNA constituted an end-to-end association of parental DNA with newly synthesized daughter duplex DNA "subunits," rather than lateral association of parent and daughter DNA strands. This unlikely scenario was ruled out by Meselson's graduate student, Ron Rolfe, who showed that sonication to intentionally break the linear hybrid DNA into shorter lengths, did not alter the density of the DNA (11). Another unlikely scenario was promoted by Liebe Cavalieri *et al.* (12), who argued that the conserved "subunits" might be double-stranded DNA and that the hybrid DNA would then consist of the lateral association of two duplex DNA helices to form a four-stranded structure. Following up on several years of heated debate, definitive exclusion of the Cavalieri model was ultimately provided from the work of Robert Baldwin and Eric Shooter (13), who studied the melting of hybrid DNA, in which one subunit was labeled with 5BU. The melting profile was that expected for DNA in which the subunits were single strands rather than double helices. Meselson's graduate student at Harvard, John Menninger (14), had shown by low-angle x-ray scattering that the linear density of *E. coli* DNA corresponded to two chains rather than four.

Essential to the success of the Meselson–Stahl experiment was the fragility of the rigid linear DNA molecule, and the effect of extensive shearing of the DNA when it was handled; particularly as the sample was injected into the ultracentrifuge cell through a hypodermic syringe, now known to impose high shear. The molecular mass of the DNA fragments studied was only $\approx 7 \times 10^6$ Da. If the entire bacterial chromosome could have been isolated intact in these gradients, the interpretation of the results might have been more complicated, because there would have been a gradual shift with time of DNA from the parental to hybrid density, as sequential replication proceeded around the circular genome.

A few years after the classic replication paper was published, Meselson and Jon Weigle (15) used the combination of ^{15}N and ^{13}C to prepare heavily density-labeled λ phage DNA to determine whether recombination (with normal density λ phage) involved a "copy choice" mode or one of "breakage and reunion." In other words, was there any parental DNA in recombinant phage? The answer was that both chromosomal subunits are broken during recombination and that recombination occurs by chromosome breakage (although other mechanisms were not excluded). These studies used preparative CsCl density gradient ultracentrifugation and the enhanced resolution afforded by using two density labels. However, the procedure to prepare the ^{13}C-labeled precursors was extremely tedious, until ^{13}C-labeled glucose became commercially available some years later. Stahl and colleagues (16) then used ^{15}N^{13}C double labeling in a series of important studies to elucidate relationships between the processes of recombination and replication in λ phage. Their initial paper in this series ruled out the so-called master-strand model for replication, another unlikely alternative to the Watson–Crick scheme. It eventually became apparent that 5BU is a very convenient choice for density labeling DNA—for many reasons, including the fact that 5BU (fully replacing thymine) achieves a density shift roughly equivalent to the combined use of ^{15}N, ^{13}C, and deuterium, and at lower cost.

During my postdoc with Ole Maaløe in Copenhagen, in 1959, we found that if protein synthesis was inhibited in growing *E. coli*, then only a limited amount of DNA synthesis could occur, and we postulated that this constituted completion of those cycles of replication underway, without initiation of any new ones. The definitive proof of that hypothesis came from density labeling studies with 5BU—in which we showed that in the absence of protein synthesis only hybrid density DNA appeared during replication—thus, no second round could have been initiated to yield DNA molecules with 5BU in both strands (17). My studies with 5BU labeling

prompted additional speculation about the detailed mode of DNA replication—why did one not observe DNA molecules in which replication forks had been caught midway? These molecules would be predicted to appear in the density gradient somewhere between the parental DNA density and that of the hybrid band.

Meeting Meselson

When I arrived at Caltech in September 1960 for my second postdoc (with Robert Sinsheimer), I immediately sought out Matt Meselson—and fortunately caught him for several short discussions before he departed in early 1961 for his faculty position at Harvard. We discussed the nature of the *E. coli* chromosome and Matt speculated that it might consist of short segments of DNA held together by some sort of protein "linkers" that could help with the topological unwinding problem. John Cairns (18) used tritium autoradiography several years later to provide evidence that the bacterial chromosome consisted of one intact closed circular molecule of DNA, and that DNA replication proceeded around the circle from one (or at most two) growing points. The conclusion that the chromosome consisted of double-stranded DNA was based on the contour length of the circle, compared with the cellular DNA content. The possibility of "linkers" between DNA segments could not be excluded, however, because of the low resolution of the technique.

I thought that a possible explanation for the lack of "intermediate" density DNA between parental and hybrid bands in the Meselson–Stahl experiment could be that the replication of a DNA "segment" was essentially "all or none"—it happened so rapidly that only a negligible fraction of the DNA segments might be caught in the act. However, my student, Dan Ray, and I (19) were able to isolate partially replicated DNA fragments from growing *E. coli*, by using ^{32}P pulse labeling along with 5BU incorporation, and a very gentle cell lysis procedure before preparative CsCl equilibrium sedimentation. After mild shearing of those fragments, the labeled DNA was resolved into hybrid and parental density bands, suggesting that the replication fork DNA might be unusually sensitive to breakage. The intermediate density ^{32}P pulse-labeled DNA fragments could also be chased into the hybrid band when excess ^{31}P was added to the growing cells (19). I then reasoned that if we could stall replication forks at obstructions in the template, we might stabilize and recover those partially replicated molecules for further analysis. My student, David Pettijohn, and I (20) examined the density

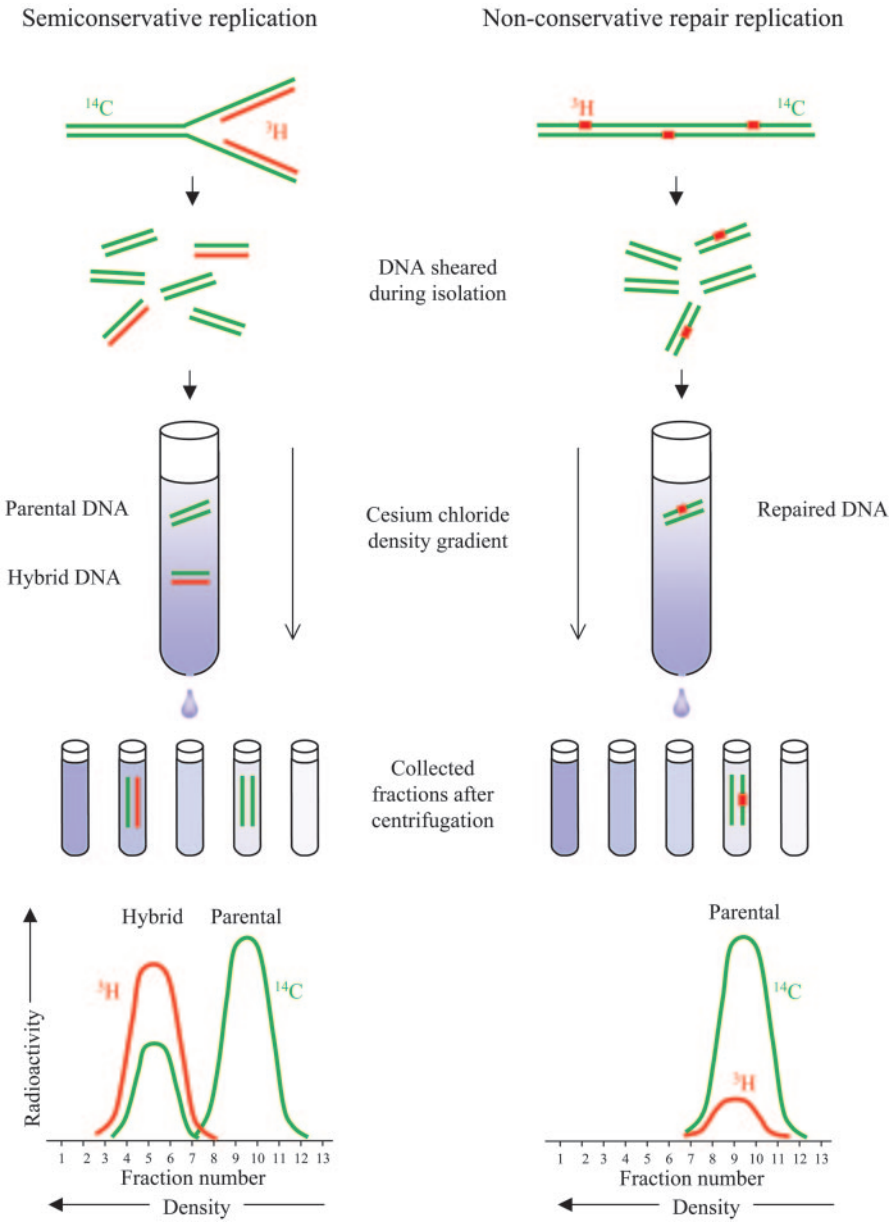

Fig. 4. Distinguishing semiconservative replication from nonconservative repair replication by using density labeling with 5BU.

distribution of DNA during labeling with radioactive 5BU in the period immediately after UV irradiation of the bacteria, to introduce cyclobutane pyrimidine dimers known to arrest DNA synthesis. We did indeed find a substantial amount of intermediate-density DNA but, curiously, there was also a significant amount of nascent DNA label at the parental density. Rebanding the parental density DNA in a second CsCl gradient verified the presence of 5BU-containing DNA with little or no evident density shift. The plausible explanation became apparent when I discussed our experiments with my former graduate mentor, Richard Setlow (21), who had just discovered that cyclobutane pyrimidine dimers are released from the chromosomal DNA in UV-resistant bacteria: he postulated an excision-repair scheme for damaged DNA. We were evidently observing the patching step in this putative process of excision repair, and the lack of a density shift was because of the fact that the patches synthesized by repair replication were too short to appreciably shift the density of the DNA fragments containing them. (Fig. 4) Thus, the approach developed by Meselson and Stahl (1) to demonstrate semiconservative DNA replication was used to first document the "nonconservative" repair replication of damaged DNA (20). Intentional shearing of the "repaired" DNA by soni-

cation did result in a measurable density shift, which, when combined with molecular weight determinations, could be used to estimate the patch size.

As with the excision repair of damage (like cyclobutane pyrimidine dimers), the heteroduplex regions generated during genetic recombination were thought to provoke localized excision of a tract of nucleotides from one strand followed by repair synthesis to fill the gap. The excision repair of mismatched bases was also postulated, and Wagner and Meselson (22) obtained genetic evidence that, although well separated mismatches were repaired independently, sometimes those separated by <2,000 nt could be repaired by a single event, if these were on the same DNA strand.

The approach pioneered by Meselson and Stahl (1) continues to be widely used for research in the fields of DNA replication, recombination, and repair. It is the method of choice when one wishes to physically separate the newly synthesized DNA from DNA existing before an appropriate density label is introduced into a culture of growing cells or a replication system *in vitro*. It has become a classic approach for the biochemical detection of DNA strand exchange in recombination, although it does not approach the sensitivity of genetic analysis. Also, it is still used for the quantification of nucleotide excision repair in a variety of prokaryotic and eukaryotic cell systems. In a 1959 letter to Frank Stahl, Matt wrote that "CsCl has an inexhaustible number of golden eggs to lay." That statement indeed has proved to be true.

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