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# Wanderings of a DNA Enzymologist: From DNA Polymerase to Viral Latency

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## Abstract

I am a member of what has been called, perhaps too grandiosely, “The Greatest Generation.” I grew up during the Great Depression and served in the U.S. Army during World War II. Because of my military service and the benefits of the GI Bill, I was able to attend college and, later, graduate school. Early in my graduate studies, I became fascinated with enzymes and the biochemical reactions that they catalyze. This fascination has never left me during the 50 years I have been a “DNA enzymologist.” I was fortunate to have had as a mentor Arthur Kornberg, one of the great biochemists of the twentieth century, and a splendid group of postdocs and graduate students. I have studied DNA polymerases, DNA nucleases, DNA ligases, and DNA recombinases, enzymes that are critical to our understanding of DNA replication, repair, and recombination. Most recently, I have been studying herpes virus replication and inadvertently wandered into an entirely new area—viral latency.

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## INTRODUCTION

I was born in the town of Tauroggen in what was then known as Memel Territory. Memel Territory, which had been part of German East Prussia, was ceded to Lithuania by the Versailles Treaty following World War I. It was, when I was born, a German-speaking enclave in the southwestern part of Lithuania. My father, who had served in the German army during World War I (he had been wounded and was awarded the Iron Cross for valor), was deeply troubled by the anti-Semitism to which he and his family were increasingly subjected by both the ethnic Germans and the native Lithuanians. In 1927, he made the fortunate decision to emigrate to the United States. Most of the members of our extended family chose to remain and perished in the Holocaust that began a decade later. The Great Depression started within two years of our arrival in Baltimore, but because my father managed to find and keep a

job, we were spared the worst of that dreadful period.

## EARLY YEARS IN BALTIMORE

My early years were uneventful. I was a good, but not outstanding, student. During my last two years of high school, to help support the family, I worked part-time in a large meat market as a butcher's assistant. I became quite a skillful meat cutter and was promised a full-time job after high school graduation, a not inconsequential prospect because the United States was still mired in the Depression, and the possibility of college after high school graduation was very remote. All of this changed with the attack on Pearl Harbor by the Japanese on Sunday, December 7, 1941. I can still recall the radio announcer breaking in on the broadcast of the New York Philharmonic Symphony to report that Pearl Harbor had been attacked. The following day, the entire student body of my high school assembled to hear the radio broadcast of President Franklin D. Roosevelt's speech, "December 7, 1941 a date that will live in infamy." I remember discussing this momentous event after the assembly with my best friend Bobby Schwartz. Bobby and I optimistically concluded that we would probably not be affected by the war, which still seemed very far away, and it would almost certainly be over by the time we graduated. How wrong we were! Bobby became a paratrooper in the Eighty-Second Airborne Division and was killed in Holland in September 1944, just before his twentieth birthday.

I was drafted into the army at age 18, in the summer of 1943, less than three months after graduating from high school. Possessed of few if any of the skills that might have gotten me a safe desk job, I was assigned to the infantry. As a member of the Third Infantry Division, I participated in the invasion of southern France, an invasion that few know about today, and later in the battle for Germany. These were brutal experiences, incomprehensible to anyone who has not faced enemy fire in ground combat. Miraculously,

I survived without any serious wounds. However, because we were not issued watertight boots, with the onset of what turned out to be an unusually cold and snowy winter in the Vosges mountains of eastern France, I developed a gangrenous condition known as “trench foot.” After about six weeks in an evacuation hospital and a rehabilitation center I was pronounced “fit for duty” and ordered to return to my unit, which was preparing for the Rhine River crossing and the assault on Germany. Of the approximately 200 men in my company who landed in southern France in August 1944, only about 30 of us remained at war’s end, in May of 1945.

In contrast to the Viet Nam War veterans, we were treated as heroes, with very tangible economic benefits, collectively termed “the GI Bill of Rights.” These benefits included a year of unemployment benefits (\$20 per week for 52 weeks) and free college tuition for periods up to six years, depending upon length of service. Included was a monthly stipend of \$50, a more than adequate sum at the time.

The chance to attend college at government expense was a dream come true. Fortunately, I was accepted at Johns Hopkins University in Baltimore, despite a less than sterling high school record. I decided to major in chemistry, a subject that I enjoyed in high school. My ambition was to become an industrial chemist, like an uncle whom I admired.

I was an excellent student and compiled a nearly 4.0 grade-point average. In my senior year, I was very much influenced by a biology course, given by Bill McElroy, which had a strong biochemistry component. I was fascinated by the pathways of carbohydrate, lipid, and energy metabolism that McElroy revealed to us. He was an exciting teacher, and he opened my eyes to the wonders of metabolism. I abandoned my plans to become an organic chemist following graduation from college and applied to the Doctoral Program in the Department of Biochemistry at the Johns Hopkins School of Public Health.

My thesis advisor was Roger Herriott, an eminent protein chemist who had been at-

tracted to the study of bacteriophages. Roger had been at the Rockefeller Institute, the forerunner of the Rockefeller University, and was aware of and, more importantly, appreciated the significance of the discovery by Avery, McLeod, and McCarty published in 1944 that DNA was the transforming principle of pneumococcus and indeed represented the chemical nature of the gene. I recall Herriott telling me that the T2 phage with its hexagonal head and long tail was really a hypodermic syringe containing “a bag of transforming principles,” which were injected into the bacterial host by the protein coat. This was several years before the famous Hershey-Chase experiment that showed that this was indeed the case.

Because of my interest in metabolism, Herriott suggested that I study the metabolic changes that occurred in the host *Escherichia coli* after infection with an intact T2 phage and the isolated protein coats or “ghosts” of T2 that he had recently found could be produced from the phage by osmotic shock. My doctoral dissertation involved a study of the metabolic changes that occurred in *E. coli* following infection with phage T2 and T2 ghosts. There were indeed major changes in oxygen uptake and CO<sub>2</sub> release that probably reflected changes in glycolysis and the pentose phosphate pathway. There were also changes in phosphate metabolism (1). However, I failed to detect the profound alterations in nucleotide metabolism that were needed for the synthesis of T2 phage DNA and, in particular, for the synthesis hydroxymethylcytosine, which replaced cytosine in T2, T4, and T6 bacteriophages (2).

I remained convinced, after completing my doctorate, that the most excitement in biochemistry was in intermediary and energy metabolism. This was reinforced by a talk given by Irving Lieberman, then a postdoctoral fellow with Arthur Kornberg, at the 1954 Federation Meeting in Atlantic City on the discovery of phosphoribosylpyrophosphate and its role in nucleotide biosynthesis. I still recall my awe and excitement at hearing this remarkable work.

I began my postdoctoral training in the fall of 1954 with Alvin Nason at the McCollum-Pratt Institute at Johns Hopkins. Al was doing interesting work on the role of metal ions, notably molybdenum, in electron transport in *Neurospora* and suggested that I work on a particulate NAD-linked cytochrome *c* reductase from rat muscle with the aim of identifying a metal cofactor. Although I approached the project with considerable enthusiasm, I soon decided that it was not for me. Lieberman's 10-minute Federation talk was still very much on my mind, and I went to the library and read every paper Kornberg had published on coenzyme and nucleotide biosynthesis that I could find. I then wrote Arthur who had moved from the National Institutes of Health (NIH) to Washington University in St. Louis, asking if I could join his lab as a postdoctoral fellow. To my great good fortune, he accepted me. We have remained colleagues and close friends ever since.

## DNA POLYMERASE IN ST. LOUIS

I arrived in St. Louis in the summer of 1955, a summer notable for having broken a long-standing record for the number of consecutive days in which the temperature reached or exceeded 100° F. Arthur was chairman of the Department of Microbiology, whose faculty, in addition to himself, consisted of Paul Berg, Melvin Cohn, Robert De Mars, David Hogness, Irving Lieberman, and Philip Varney (the sole holdover from the previous department). Later, Dale Kaiser replaced De Mars, who had left to discharge his military obligation with service at the NIH. Shortly thereafter, Jerard Hurwitz, who had been at the NIH, joined the faculty. The department was housed in rather dilapidated quarters in the top floor of the old Washington University Clinic Building. The elevator was reputed to be the oldest still in operation in the city of St. Louis, and riding it could often be an adventure. There was a journal club that met daily at lunch. All the members of the department participated, and we

were joined by Martin Kamen, who was in the Department of Radiology, and Stanley Cohen, who was working on nerve growth factor in Rita Levi-Montalcini's lab on the main Washington University campus. The discussions were spirited, critical, often combative, and, occasionally, somewhat intimidating. But they were great fun and helped to keep us up to date in the current literature. Arthur's group, in addition to myself, consisted of Uriel Littauer, a postdoc from Israel; Jose Fernandez, a postdoc from Brazil; Arthur's late wife, Sylvy Kornberg; and Ernie Simms, a technician.

The discovery that the DNA of the T-even phages contained hydroxymethylcytosine in place of cytosine seemed terribly important, and I was eager to learn how hydroxymethylcytosine was made. Arthur felt that this was a good project and managed to acquire a small amount of [<sup>14</sup>C] β-carbon-labeled serine, a presumed donor of the hydroxymethyl group, for my experiments. Within a short time, I found that extracts of T2-infected *E. coli* did incorporate the <sup>14</sup>C-label into dCMP, and I began fractionation to purify the enzyme responsible for this activity. At about the same time, Arthur had observed that small amounts of <sup>14</sup>C-labeled thymidine, which he had obtained from Morris Friedkin in the Pharmacology Department, were incorporated into an acid-insoluble product by *E. coli* extracts. The counts were low (fewer than 100), but they were made acid soluble upon treatment with pancreatic DNase. I recall that when Arthur told me of this tantalizing finding, which suggested that he had achieved DNA synthesis in vitro. I was tremendously excited and asked if I could put my project on hold and join him. He agreed. Later, we learned that Joel Flaks and Seymour Cohen had discovered the T4 phage dCMP hydroxymethylase (3) and had opened up the whole field of virus-induced enzymes. But I have never regretted my decision.

Several months after this, Maurice Bessman, a new postdoc, arrived, and together with Ernie Simms and Arthur, the four of

us began to fractionate the activity responsible for the incorporation of the labeled thymidine into an acid-insoluble, DNase I-sensitive product. Later, we were joined by Julius Adler, another postdoc, and Steve Zimmerman, Arthur's first graduate student.

Earlier that year, Arthur and Ernie Simms had begun work on the purification of an activity in *E. coli* that converted thymidine in the presence of ATP to what they referred to as thymidine-X, later identified as dTMP. The activity was thymidine kinase. Additional products, presumed to be dTDP and dTTP, were also observed. The ability to make  $^{32}\text{P}$ -labeled dTMP was a significant step forward because we were not limited by the low radioactivity of the  $^{14}\text{C}$ thymidine that was available, and the  $^{32}\text{P}$ dTMP incorporated into the acid-insoluble product was now in the hundreds and occasionally in the thousands of counts/min. We bet that dTTP was the true substrate for our enzyme rather than dTMP or dTDP, although the latter was a distinct possibility because of the finding a year earlier by Grunberg-Manago and Ochoa that the nucleoside diphosphates rather than the triphosphates were the substrates for their ribonucleotide-polymerizing enzyme from *Azotobacter* (4), which later turned out to be polynucleotide phosphorylase, an enzyme involved in messenger RNA degradation. Once we had  $^{32}\text{P}$ dTMP, we prepared  $\alpha$ - $^{32}\text{P}$ dTTP by incubating our  $^{32}\text{P}$ -labeled dTMP with a partially purified nucleoside-diphosphate kinase and ATP and then isolating the dTTP. Our assay mixture now consisted of a crude sonic extract of *E. coli*,  $\alpha$ - $^{32}\text{P}$ dTTP, ATP,  $\text{Mg}^{2+}$ , and buffer. As in the original experiment, acid-insoluble  $^{32}\text{P}$  was measured. With this assay, we began to fractionate the crude extract for dTTP incorporation into DNA.

To begin the fractionation we added streptomycin sulfate to the extract to produce a precipitate that contained the cellular nucleic acids and a nucleic acid-free supernatant. Streptomycin sulfate was used frequently at the time to remove nucleic acids, often a

hindrance to protein purification in bacterial extracts. Assay of the nucleic acid-free supernatant (S-fraction) and the nucleic acid-containing precipitate (P-fraction) showed them to be devoid of dTTP incorporation activity. However, when the two fractions were combined, activity was restored. We also observed that prior incubation of the extract or the P-fraction for a few minutes at  $37^\circ\text{C}$  increased activity substantially. Clearly more than one enzyme was required for the incorporation of dTTP into an acid-insoluble product. The complexity of the system became even more apparent when we began to fractionate S and P. The P-fraction could be subfractionated into two fractions, one heat-labile and the other heat-stable, both of which (in combination with the S-fraction) were necessary for activity. The S-fraction could be separated into both a heat-labile fraction and a heat-stable fraction that could pass through a dialysis membrane, i.e., was dialyzable. The latter could be further fractionated by Dowex-1 chromatography into three discrete fractions. (Dowex-1, an anion exchange resin used at the time, separated low-molecular-weight acidic compounds.) Thus, incorporation of dTTP into an acid-insoluble product presumably DNA, required (a) two heat-labile fractions; (b) a heat-stable fraction; (c) three heat-stable, dialyzable, chromatographically distinct fractions; and (d) ATP. In the absence of any one of these components, the activity was significantly diminished. Clearly, a lot was going on. The heat-labile component in the P-fraction turned out to be the enzyme that catalyzed phosphodiester bond synthesis, and this enzyme we named DNA polymerase. The heat-stable, nondialyzable component in the P-fraction was DNA. The heat-labile, nondialyzable component of the S-fraction was a mixture of deoxynucleotide kinases, which together with nucleoside-diphosphate kinase produced the heat-stable, dialyzable mixture of dCTP, dATP, and dGTP.

We reconciled these complex requirements for the incorporation of  $^{32}\text{P}$ -labeled dTTP into an acid-insoluble product as

follows. The DNA in the extract and the P-fraction were degraded by endogenous nucleases to the deoxynucleoside monophosphates (dNMPs). (Recall that preincubation of the extract or the P-fraction significantly enhanced activity.) These were converted to the corresponding deoxynucleoside triphosphates (dNTPs) by the kinases in the S-fraction and ATP to generate dCTP, dATP, and dGTP. The heat-labile component of the P-fraction was the DNA polymerase. We speculated that the DNA in the P-fraction served two functions in addition to being the source of the deoxynucleoside monophosphates. First, it protected the miniscule amount of the labeled DNA that was synthesized from degradation by the nucleases in the extract. Second, Arthur had been strongly influenced by the work on glycogen phosphorylase in the Cori laboratory. In the case of glycogen phosphorylase, glycogen served as a "primer" for the addition of glucosyl units from glucose 1-phosphate to extend the glycogen chain. Similarly, he felt that dTMP from dTTP was being added to pre-existing DNA chains.

Once the outlines of the reaction became clear, we set about the task of reconstituting the reaction with purified components. We partially purified each of the deoxynucleotide kinases in the S-fraction, and with these kinases and nucleoside-diphosphate kinase, we synthesized and characterized the four dNTPs (dTTP, dCTP, dGTP, and dATP). This alone was a substantial advance, because with the exception of dTTP, none of the other dNTPs had previously been described.

To prepare the four  $^{32}\text{P}$ -labeled dNTPs, we started with  $^{32}\text{P}$ -labeled DNA, isolated from  $^{32}\text{P}$ -labeled *E. coli*, from which we generated the four  $^{32}\text{P}$ -labeled dNMPs (dAMP, dCMP, dTMP, and dGMP) by treatment with pancreatic DNase and snake venom phosphodiesterase. The dNMPs were individually purified and converted enzymatically to the corresponding  $^{32}\text{P}$ -labeled dNTPs. Because the procedure usually consumed two to three weeks, the 14-day half-life of  $^{32}\text{P}$  necessitated

the use of large quantities of  $^{32}\text{P}$  (50–100 mCi) in the 100-ml low-phosphate culture medium that we used for the growth of *E. coli*.

Purification of the DNA polymerase was a difficult and demanding task. The enzyme was present in relatively small amounts even in rapidly growing *E. coli* (about 300 molecules/cell). Fortunately, a fermenter, which had been installed in the department, for the large-scale growth of *E. coli* supplied hundreds of grams of log phase *E. coli*. Later 100-pound batches of *E. coli* cell paste were obtained from the Grain Processing Corporation in Muscatine, Iowa. DEAE-cellulose and phosphocellulose, invented by Herbert Sober at the NIH, liberated us from the sole reliance on the ammonium sulfate, alumina C $\gamma$ , and acetone, which were the major protein fractionation tools at the time. With the aid of chromatography performed with these ion exchangers, we were able to obtain a several 1000-fold purified but not yet homogeneous preparation of the DNA polymerase. A vexing problem at the time was our inability to remove deoxyribonuclease activity from the enzyme. It was found later that a 3' to 5' exonuclease, which serves a vital proof-reading function, is a component of *E. coli* DNA polymerase and, indeed, virtually all DNA polymerases, excising incorrectly incorporated nucleotides as replication proceeds.

With the progress in fractionation, the reaction requirements were now considerably simplified. Conversion of  $\alpha$ -[ $^{32}\text{P}$ ]dTTP into an acid-insoluble product, i.e., DNA, required only the partially purified DNA polymerase,  $\text{Mg}^{2+}$ , DNA, dCTP, dGTP, and dATP (5, 6). We further found that all four dNTPs were absolutely required. With omission of any one of the other three dNTPs, incorporation of  $\alpha$ -[ $^{32}\text{P}$ ]dTTP fell to background levels (7).

The requirement for all four dNTPs was puzzling. If the DNA that we added was simply serving as a primer, why would all four dNTPs be needed? Was it possible that the DNA polymerase was performing the template-directed replication proposed by Watson and Crick for their double-stranded

structure of DNA (8). To test this seemingly wild idea, we used DNAs with A+T/G+C ratios ranging from 0.5 to 1.9 as “primers.” The result was stunning. The ratio in the product corresponded closely to that of the added DNA throughout the synthesis and was independent of the relative concentrations of the individual dNTPs. Clearly, the added DNA was serving as a template to direct the polymerase as it synthesized new DNA chains, or as we cautiously put it in our initial publication, “These results suggest that enzymatic synthesis of DNA by the ‘polymerase’ of *E. coli* represents the replication of a DNA template” (9).

Having referred to the DNA added to their action as the primer, by analogy to glycogen phosphorylase, it was now clear that it also was serving as a template. However, we now know that all DNA polymerases require a primer to initiate a DNA chain. Various priming mechanisms (short RNA chains, proteins) have evolved to make up for this shortcoming in an otherwise magnificent enzyme (10, 11).

By 1958, we had established that a DNA primer, a template, and all four dNTPs were required by our DNA polymerase for the synthesis of DNA. However, we received a rude shock one day when we observed, to our surprise, that with our most highly purified enzyme DNA synthesis could proceed in the apparent absence of DNA. This episode is worth recounting in more detail. As I mentioned earlier, an important aim in purifying the DNA polymerase was to remove the contaminating DNase activity, which destroyed the product that we synthesized. Howard Schachman, the distinguished physical chemist, had come from Berkeley to spend a sabbatical year in the laboratory to analyze the DNA product of the polymerase reaction. Using the tools of the polymer chemist, the ultracentrifuge and the viscometer, Howard quickly demonstrated that the product of the reaction was indeed a large polymer. It then occurred to us that viscometry might be a very sensitive method to assay for nuclease activity in our most

purified DNA polymerase preparations. I set up a nuclease reaction that contained calf thymus DNA,  $Mg^{2+}$ , and the DNA polymerase. At Howard’s suggestion, dCTP, dTTP, and dATP were added to closely mimic the standard synthetic reaction conditions. dGTP (which was the most difficult of the triphosphates to prepare) was omitted to prevent DNA synthesis. The viscosity of the reaction mixture was then measured over an extended period. To my disappointment, the viscosity of the solution fell to that of the reaction buffer within about an hour. Obviously, the DNA was completely degraded; our best polymerase preparation was still contaminated with nuclease(s). This experiment was performed on a Saturday morning at the same time that I was proctoring a microbiology exam for second-year medical students down the hall from the physical chemistry laboratory, which Arthur had set up for Howard. (All of us in the department, including postdoctoral fellows, participated in medical school teaching at the time). After spending some time answering student questions and collecting exams, I returned to the laboratory to discard the reaction mixture and clean the viscometer. Before doing so, I absent mindedly took one last reading. To my amazement the viscosity of the solution had actually increased, and with repeated readings, the viscosity of the solution increased even further but then eventually fell back to that of the buffer. Could we be observing template-independent DNA synthesis? A number of control experiments were hastily performed that ruled out bacterial growth and contamination of the three dNTPs with dGTP. Howard Schachman and Julius Adler quickly found that the increase in viscosity required only the DNA polymerase,  $Mg^{2+}$ , dATP, and dTTP and occurred only after a lengthy lag. The product was a copolymer of alternating dAMP and dTMP, d(A-T) (deoxyadenylate-deoxythymidylate) (12). Work several years later by Arthur Kornberg, Gobind Khorana, and coworkers (13) showed that the rules had not been



violated. The synthesis of the d(A-T) copolymer was indeed template directed, but the template consisted of trace amounts of DNA present in the DNA polymerase preparation. The polymer was the result of a reiterative mode of DNA replication in which slippage of one stretch of alternating A and T residues within the contaminating DNA generated overlapping ends, and these, when filled in by the polymerase, increased the chain length; ultimately, the high-molecular-weight d(A-T) copolymer was produced in quantity (13).

The DNA polymerase that we discovered is now called DNA polymerase I. In the intervening years, four additional DNA polymerases, DNA polymerase II, DNA polymerase III holoenzyme, and DNA polymerases IV and V, have been identified in *E. coli* and purified (14). The multisubunit DNA polymerase III holoenzyme is actually the enzyme that catalyzes the synthesis of the *E. coli* chromosome (15). DNA polymerase I, by virtue of its intrinsic ribonuclease H activity, together with its DNA polymerase activity plays an essential role in processing the nascent Okazaki fragments produced during the discontinuous replication of the lagging strand at the replication fork to prepare them for ligation (16). DNA polymerases II, IV, and V serve in the repair of DNA (14).

In eukaryotes, the situation is even more complex. Fifteen distinct cellular DNA polymerases have been identified, and the list continues to grow (17). Three of these are devoted to replication of the genome (DNA polymerases  $\alpha$ ,  $\delta$ , and  $\epsilon$ ); DNA polymerase  $\gamma$  replicates the mitochondrial genome. The rest are all devoted to the repair of specific lesions in DNA. There are, in addition, virally encoded polymerases that replicate viral DNA genomes and, in the case of the retroviruses, reverse transcribe their RNA genomes into DNA. Despite the number and diversity of DNA polymerases, all of these enzymes show the same requirements that we observed nearly 50 years ago for the polymerase of *E. coli*: a template (DNA or RNA) to guide the polymerase in its base selec-

tion, a primer onto which deoxynucleotides are added, the four dNTPs, and  $Mg^{2+}$ . There are factors associated with DNA polymerases, e.g., clamps, clamp loaders, and exonucleases, which increase the efficiency and fidelity of DNA replication, but the basic mechanisms of replicating a DNA chain are all the same.

With the crystallization of many DNA polymerases, the determination of their three-dimensional structures, and the application of presteady-state kinetic analyses, much is now known about the detailed chemical mechanism of the polymerase reaction (18). This information has been invaluable in the design of effective chemotherapeutic agents, in particular antiviral drugs.

I view those days in the mid-1950s in the Department of Microbiology on the fourth floor of the old Clinic Building at Washington University to be among the most thrilling and enjoyable of my scientific career. There were new and unexpected findings being made virtually every day, and all of us in our small group shared in the joy and excitement of those discoveries.

In the summer of 1957, I received an offer of an assistant professorship at the McCollum-Pratt Institute at Johns Hopkins from Bill McElroy, the director. I had been a postdoc for two years, and under normal circumstances, it would have been time to move on. Moreover, academic positions were hard to come by. However, it was clear that I was working a gold mine, and I had no intention of leaving. I continued working as a postdoc for an additional year and then began independent research as an instructor in the department.

## FIRST INDEPENDENT RESEARCH—EXONUCLEASE I

In looking around for a project to begin independent research, I had been impressed by the importance of specific proteases in the analysis of protein structure and sequence, and I was struck by the absence of comparable enzymes that acted on DNA. Our purification of DNA

polymerase from *E. coli* extracts and constant attempts to rid the enzyme of nucleases that degraded the DNA product suggested that *E. coli* might be a good source of such enzymes. The first of these that I purified, exonuclease I, turned out to be extraordinarily useful. It was absolutely specific for single-stranded DNA and was therefore a very effective, in fact, the only reagent at the time that could distinguish single-stranded from double-stranded DNA (19). A notable example of its usefulness was in the early DNA renaturation studies of Paul Doty, Julius Marmur, and Carl Schildkraut (20). After heat or alkaline denaturation of rather heterogeneous preparations of calf thymus or salmon sperm DNA, renaturation yielded duplex DNA with single-stranded tails. These produced rather messy, often uninterpretable, banding patterns in the CsCl-density gradients that were used to measure renaturation. However, upon treatment with exonuclease I, to remove the single-stranded tails, sharp bands appeared, making it clear that true renaturation had occurred (20). Exonuclease I was also instrumental in the discovery by Fiers and Sinsheimer that the single-stranded DNA of phage  $\phi$ X174 was circular (21). Although single stranded, it was degraded by exonuclease I only after being nicked by an endonuclease. Another fortunate property of exonuclease I, which I was able to exploit, was its ability, in contrast to all other known nucleases, to degrade glycosylated T-even phage DNA completely to mononucleotides. It had been found some years before that the T-even phage DNAs contained glucose linked to the hydroxymethylcytosine that replaced cytosine. With the use of exonuclease I, my research assistant, Ann Pratt, and I were able to determine the patterns of glycosylation of the hydroxymethylcytosine residues, and we discovered to our amazement that in T4, in which all of the hydroxymethylcytosines are glycosylated, half contained glucose in the  $\alpha$  configuration, and in the other half, it was in the  $\beta$  configuration (22). Later, Kornberg and coworkers (23) discovered that T4 actually encodes distinct  $\alpha$  and  $\beta$  gluco-

syl transferases. In phage T6 DNA, the disaccharide gentiobiose was the predominant glucosyl residue. This was all very exciting, and I was convinced that these elaborate patterns of glycosylation must be terribly important. It now appears that one of their functions is to protect the phage DNA from restriction.

## THE MOVE WEST—STANFORD

In the summer of 1957, Arthur was offered the Chair of Biochemistry at the Stanford School of Medicine, and he invited the Microbiology faculty, including me, its most junior member, and Robert (Buzz) Baldwin, then at the University of Wisconsin, to join him in forming the new Biochemistry Department at Stanford. I had never been west of St. Louis, but the opportunity was too good to pass up. We arrived at Stanford in the spring of 1959 and quickly set up shop in the newly constructed Stanford University Medical Center.

## NUCLEASES

Buoyed by the success with exonuclease, I decided to begin research at Stanford by continuing the search for DNases in *E. coli* and also branched out to other microorganisms. With Stuart Linn, a graduate student, and Ian Kerr, a postdoc, we purified several of these enzymes; many were specific for single-stranded DNA and showed preferential cleavage of certain sequences, but none were truly base or sequence specific (24). We missed the jackpot—the restriction endonucleases. Stuart Linn did, however, in his subsequent postdoctoral work with Werner Arber in Geneva, discover the first restriction endonuclease, EcoB.

## BACK TO DNA POLYMERASE

In the mid-1960s, I was invited by Bob Sinsheimer to present a seminar at Caltech. During my visit, I met with Bob Edgar who told me of work in his lab with *amber* and temperature-sensitive mutants of phage T4

that were defective in DNA replication (25). As a consequence of work in the Cohen, Kornberg, and Bessman labs, it was clear that the T phages encoded a variety of enzymes required for the replication of their DNA, including a novel DNA polymerase. Edgar offered to send me a series of DNA negative T4 mutants, in the hope that we would be able to determine which was defective in the phage DNA polymerase. Adrian De Waard, a Dutch postdoc, and Aniko Vessey Paul, my first graduate student, quickly demonstrated that extracts prepared from cells infected by amber mutants in gene 43 were lacking in the T4 DNA polymerase (26). We then went on to show that temperature-sensitive gene 43 mutants produced a temperature-sensitive DNA polymerase, thus demonstrating that gene 43 was indeed the structural gene for the T4 DNA polymerase. This was an important result because it demonstrated for the first time that the DNA polymerase activity that we and others were measuring *in vitro* is required for DNA synthesis *in vivo* (27). The role of T4 DNA polymerase in T4 DNA replication *in vivo* was further reinforced by experiments carried out by Zach Hall, a temporarily transplanted neurobiologist, who showed that the DNA polymerase purified from cells infected with T4 mutator mutants in gene 43 was mutagenic *in vitro*. These results became all the more important when DeLucia and Cairns several years later, in 1969, found a mutant of *E. coli* lacking DNA polymerase (i.e., DNA polymerase I) and questioned the role of DNA polymerase in DNA replication (28). More about that later.

## DNA LIGASE

While casting about for a new area of research in the late 1960s, I heard Matthew Meselson describe his work with Jean Weigle, which demonstrated quite clearly that genetic recombination occurred by the breakage and rejoining of the recombining DNA molecules and not by a replication mechanism in which portions of the two molecules were copied al-

ternately by a DNA polymerase. What were the enzymes that were able to catalyze the joining of DNA molecules? Baldomero (Toto) Olivera joined the lab as a postdoc after training in DNA physical chemistry with Norman Davidson at Caltech, and I suggested to Toto that we look for an enzyme in *E. coli* that could promote such a joining reaction. The substrate we devised was a poly (dA) chain of about 1000 nucleotides to which was annealed multiple 100-nucleotide-long poly (dT) segments each labeled with  $^{32}\text{P}$  at its 5' terminus. This, in effect, produced a duplex DNA molecule with nicks spaced at 100 nucleotide intervals. Joining was measured by conversion of the  $^{32}\text{P}$ -labeled 5 phosphomonoester to a phosphodiester with *E. coli* alkaline phosphatase, which could hydrolyze the monoester substrate but not the diester product. Our very first experiment demonstrated a joining activity in our *E. coli* extracts (29). With this rather simple assay, we began to fractionate these extracts with the aim of purifying the responsible activity.

Early on, we noted that increasing amounts of extract did not produce a corresponding increase in activity. At low levels of extract, there was virtually no joining activity. The reactions were performed in the presence of ATP, and increasing the level of ATP did not eliminate the lag. Something was limiting. On the assumption that the limiting factor was a heat-stable cofactor, we added a small amount of "kochsaft," a boiled extract of *E. coli*, a classic maneuver that dates back to the days of Warburg and Meyerhof and the resolution of the glycolytic pathway. The lag disappeared. This, of course, provided us with an assay with which to purify the cofactor. It turned out to be NAD, whose pyrophosphate bond was cleaved to produce AMP and nicotinamide mononucleotide, an extraordinary and at that time unprecedented use of a redox coenzyme (30).

As we were purifying the polynucleotide joining activity, we became aware that a similar activity had been observed in four other labs. Martin Gellert at the NIH had also found

it in *E. coli* extracts, Charles Richardson and Bernard Weiss at Harvard, Jerard Hurwitz and Malcolm Gefter at Albert Einstein, and Arthur Kornberg and Nicholas Cozzarelli, my next door neighbors at Stanford, had all found it in extracts of T4-infected cells. The phage enzyme used ATP rather than NAD. Each group had its own name for the enzyme (Hurwitz's "sealase" was the most colorful), but we all settled on DNA ligase, the name coined by Richardson and Weiss.

There was an exciting period of intense, but generally friendly, competition among the labs studying the enzyme. My group, Toto Olivera, Zach Hall, Paul Modrich, and Richard Gumpert, were able to work out the mechanism of the joining reaction. In the first intermediate, enzyme-AMP, the adenylyl group of NAD is linked by a phosphoamide bond to a lysine in the active site of the enzyme, releasing a nicotinamide mononucleotide. Then the adenylyl group is transferred to the 5' phosphate group of the DNA, where it is linked by a pyrophosphate bond. In the final step, there is an attack of the 3' hydroxyl of the DNA on the activated 5' phosphoryl group to form a phosphodiester bond with the release of AMP. The mechanism for the *E. coli* enzyme also held for the T4 DNA ligase and, in fact, for the mammalian ligases of which there are as many as four now known. The use of NAD as a cofactor seems to be confined to bacteria; the T4 phage and mammalian ligases all use ATP instead of NAD (31).

Subsequent studies of *E. coli* and T4 mutants by several labs, including Tom Broker and Yasahiro and Naoyo Anraku in my lab, showed that DNA ligase is essential *in vivo* for the joining of Okazaki fragments during DNA replication, the joining of DNA chains during nucleotide and base excision repair of DNA, and the joining of DNA segments following cleavage of the Holiday junction in homologous recombination (32–34). And, of course, DNA ligase became a key reagent in the construction of recombinant DNA molecules. I do not believe that any of us working on the

enzyme at that time foresaw the central role it would play in the genetic engineering revolution. That was left to Paul Berg, Peter Lobban, Stan Cohen, and Herb Boyer.

## BACK TO DNA POLYMERASE AGAIN

The DeLucia–Cairns mutant announced in *Nature* in 1968 was a bombshell (28). Although ostensibly lacking DNA polymerase activity, the mutant was fully viable; its only defect was an increased sensitivity to ultraviolet irradiation. Now our DNA polymerase was relegated to the lowly role of a "repair enzyme." Some different system was thought by some to be responsible for chromosomal replication, possibly using substrates other than the deoxynucleoside triphosphates (16). Ironically, with the realization that many human cancers result from defects in DNA repair, this function is currently regarded as one of the hottest fields in biology.

Although Cairns was right in believing that our DNA polymerase (Pol I) was not responsible for chromosomal replication in *E. coli*, he was wrong in evaluating its role. With *E. coli* extracts apparently deficient in Pol I, DNA polymerase II and subsequently DNA polymerase III holoenzyme, each present in relatively few copies per cell, could be detected and purified from Pol I mutant extracts. DNA polymerase III was identified as part of a machine, responsible for replication of the *E. coli* chromosome. How did Pol I fit into the picture?

I had been deeply distressed by the Cairns paper and all of the ensuing publicity. I was determined to see if the Cairns mutant was really lacking in Pol I. It was a risky project; one that I felt was best to work on myself. With the help of my technician, Janice Chien, I was able to show that the extracts of the *pol A<sup>-</sup>* strain, which bore an *amber* mutation, did contain a low level of Pol I (1%–2% of wild type), resulting from readthrough of the nonsense codon. However, the levels of the 5' → 3' exonuclease activity associated with Pol I

were normal (35). The  $5' \rightarrow 3'$  exonuclease polypeptide was, however, far smaller than the intact enzyme. Pol I contains two domains: one with polymerase activity associated with  $3' \rightarrow 5'$  proofreading exonuclease and a second domain with a  $5' \rightarrow 3'$  exonuclease. Protease treatment of Pol I yields the “large” or “Klenow” fragment with polymerase and  $3' \rightarrow 5'$  exonuclease activities as well as a small fragment with the  $5' \rightarrow 3'$  exonuclease (36). It turned out that the *amber* mutation in the Cairns mutant was positioned in such a way that translation yielded the intact small fragment, explaining our observation that the mutant extracts contained normal levels of  $5' \rightarrow 3'$  exonuclease activity, of a size corresponding to that of the small fragment (35).

Bruce Konrad and I subsequently isolated a temperature-sensitive mutant defective in the  $5' \rightarrow 3'$  exonuclease activity but normal in its polymerase activity. Under standard growth conditions, the  $5' \rightarrow 3'$  exonuclease of Pol I is essential for DNA replication (37). The Cairns mutant was viable because it retained this activity. We now know that the essential role of Pol I in DNA replication is the  $5' \rightarrow 3'$  exonuclease removal of the RNA primers that initiate Okazaki fragment synthesis, followed by filling in the resulting gap by DNA polymerase action prior to their being joined by DNA ligase (37).

Robert Bambara, a postdoc, and Dennis Ujemura, a graduate student, purified Pol I from several of *pol A* mutants with defects in the polymerase domain. Because their  $5' \rightarrow 3'$  exonuclease activity was unchanged, they were fully viable; however, they were abnormally sensitive to UV radiation, presumably as a result of their polymerase defect. In examining various features of the polymerase activity in these mutants, Bambara and Ujemura developed what I believe to be the first quantitative way to assess the processivity of deoxynucleotide polymerization by DNA polymerases (38). High processivity is of course essential for chromosome replication, and we now know that there are complex protein assemblies that interact with DNA

polymerases to tether them to the template in order to prevent their dissociation during polymerization.

As another extension of our work on DNA polymerase mutants, Duane Eichler, a postdoc, and then later Per Olaf Nyman, a sabbatical visitor from Sweden, and Bik Kwoon Tye, a postdoc, examined the formation and processing of Okazaki fragments in these mutants. Their joining was clearly retarded to an extent that depended on the severity of the defect. We could support the claims of Reiji and Tuneko Okazaki that their pulse-labeled fragments were intermediates in DNA replication (on the lagging strand) rather than artifacts of DNA strand scissions, a possibility that had not yet been excluded (39).

## EUKARYOTIC DNA REPLICATION

At about this time, I began to think about eukaryotic DNA replication. An obvious place to start was with a DNA polymerase. Two nuclear DNA polymerases had been described: DNA polymerase  $\alpha$  (Pol  $\alpha$ ) was believed to be involved in chromosomal replication and DNA polymerase  $\beta$  (Pol  $\beta$ ) in DNA repair. The field was highly populated and rather contentious. Pol  $\alpha$  seemed to come in various sizes, and there was no agreement about its molecular weight and number of subunits. Its cellular abundance is low, so that barely microgram quantities emerged from the rather elaborate purification procedures that had been devised.

In casting about for an abundant source of Pol  $\alpha$ , I decided on *Drosophila melanogaster*. Kriegstein and Hogness (40), in our department, had shown earlier that chromosomal replication in early stage embryos of *D. melanogaster* proceeded at a frenetic pace: the entire *Drosophila* genome was replicated in about 3 min. This was a consequence of the large number of replication forks operating in tandem. It struck me that such embryo extracts must be highly enriched in DNA

polymerase (presumably Pol  $\alpha$ ). Assays of the extracts showed that this was indeed the case.

Our purification was begun by two sab-batical visitors, the late John Boezi, from Michigan State, and the late Geoffrey Banks, from Mill Hill in London (41). The project then passed on to three postdocs: Laurie Kaguni, Guiseppi Villani, and Brian Sauer. They showed that Pol  $\alpha$  consisted of four sub-units; the largest, at 180 kDa, contained the DNA polymerase activity (42). Ron Conaway, a graduate student, then demonstrated that the most purified preparations of Pol  $\alpha$  contained primase activity (primase had not yet been demonstrated in eukaryotes); he and Laurie Kaguni showed that the primase was associated with the two smaller of the four subunits (43). The association of primase with Pol  $\alpha$  in *Drosophila* was quickly confirmed by several laboratories, in eukaryotes as diverse as baker's yeast and humans.

## REC A

While this work on Pol  $\alpha$  was in progress, we had actually begun a completely new line of investigation on genetic recombination in *E. coli*, an interest of mine since the ligase days. Kevin McEntee, who joined my lab as a postdoc, had constructed, as part of his doctoral research at the University of Chicago, a specialized lambda transducing phage, containing the *rec A* gene. The *rec A* gene had been identified by John Clark in the early 1960s as essential for homologous recombination in *E. coli*, but its product had never been isolated, and no one had any idea of what it did. Kevin's postdoctoral fellowship application had involved a study of some of our Pol I mutant enzymes. But when deciding on a specific project, the idea of isolating the *rec A* gene product and determining its function seemed far more attractive. George Weinstock, who arrived in the lab at about the same time, joined forces with McEntee.

The *rec A* gene product had been known to be involved in radiation-induced mutagenesis or error-prone repair as it was known. We,

therefore, devised an assay for its purification that looked for nucleotide misincorporation during replication of single-stranded  $\theta$ X174 DNA in vitro (44). After nearly a year of failure, we decided, in desperation, to try something heretical. Induction of Kevin's transducing phage yielded large amounts of the Rec A protein, easily visible at the predicted size of about 40 kDa in a stained polyacrylamide gel of the induced crude extracts. The idea was to simply purify the 40-kDa polypeptide without a functional assay. The danger, of course, was that we would end up with a pure but totally inactive protein. This was a risky strategy particularly with a protein of unknown function. Unconventional then, this has now become almost standard procedure with cloned and overexpressed gene products.

With pure Rec A protein in hand, we could test it for various activities that are known to be associated with enzymes that act on DNA, such as DNA and RNA polymerase, nuclease, and DNA-dependent ATPase. However, before we could perform any of these assays, we heard a presentation by Tomoko Ogawa, of the University of Osaka, at the 1978 Cold Spring Harbor Meeting, in which she showed that a purified preparation of the Rec A protein had ATPase activity dependent on single-stranded DNA; it was a DNA-dependent ATPase. At about the same time, Jeffrey Roberts, Christine Roberts, and Nancy Craig, who had been studying the regulatory role of the *rec A* gene and identified the Rec A protein as a protease, also discovered its DNA-dependent ATPase activity (45). On returning to Stanford, Kevin quickly confirmed their findings with our Rec A protein preparation. He then went on to demonstrate that the DNA-dependent ATPase associated with the Rec A protein purified from a cold-sensitive *rec A* mutant was also cold sensitive. This result was important because it ruled out contamination of Rec A protein preparations with one or more of the many DNA-dependent ATPases that are present in *E. coli* (46). Coincident with these studies, George Weinstock was investigating the fate

of the single-stranded DNA (heat-denatured P22 DNA) during the ATP hydrolysis and found that the complementary P22 single strands were being reannealed. McEntee and Weinstock then quickly showed that not only could the Rec A protein promote the ATP-dependent renaturation of complementary single strands, but it could also promote ATP-driven strand exchange between a single strand and a homologous DNA duplex (47). In essence, the Rec A protein could form a Holiday junction, the key intermediate in homologous recombination, thereby explaining the essentialness of the *recA* gene for homologous recombination in *E. coli*. Findings similar to ours were made at about the same time by Charles Radding and by Steve West and Paul Howard-Flanders at Yale. Again, there was an intense, but largely friendly, competition between the various groups.

The Rec A group, which initially consisted of McEntee and Weinstock, quickly grew once we published our findings. Michael Cox, Peter Riddles, Randy Bryant, Zvi Livneh, Douglas Julin, and Daniel Soltis joined the lab in the next five years and contributed to our attempts to understand the mechanism by which the Rec A protein promotes strand exchange, and several, notably Cox and Bryant, have continued to work productively on the Rec A protein after leaving my lab.

The nine-year period that we worked on the Rec A protein was exciting, productive, and ultimately very gratifying. Although, important features of Rec A-promoted strand exchange still remain elusive. For example, a key step in the process, the mechanism by which the Rec A protein searches for and finds the homologous regions between recombinating DNA molecules, is still a mystery. Nevertheless, we did go a long way toward solving a fundamental problem in biology. There may also be broader consequences of this work. The human Rec A analogue, the Rad51 protein, seems to play an important role in embryogenesis: embryos of Rad51 “knockout” mice survive for only a few days, and Rad51 has been shown to interact with BRCA1 and

BRCA2, the human breast cancer susceptibility genes (48).

During this period, we continued to work on eukaryotic DNA replication. Although our expectation that *Drosophila* embryos would be an abundant source of replication enzymes proved correct, we were unsuccessful, despite the rapid rate of DNA replication in those embryos, in finding extracts that could promote origin-dependent DNA replication. Part of the problem was the uncertainty about a *Drosophila* origin; no origin had been unequivocally identified. At the same time, several labs, notably those of Tom Kelly, Jerry Hurwitz, and Bruce Stillman, had demonstrated SV40 origin-dependent DNA replication in mammalian cell extracts and were well on their way to identifying and resolving the components that were involved. Was there another viral chromosome with a defined origin that we might explore?

## HERPES VIRUS DNA REPLICATION

Edward Mocarski, from the microbiology department at Stanford, had told us about his work on the herpes simplex type 1 virus (HSV-1) and its life cycle. It contained not one, but three identifiable origins of replication; but it was rather large and unwieldy (152 kb). Nevertheless, introduction of one of these origins into a 5-kb plasmid permitted its replication in HSV-1-infected cells. Replication generated long concatomers, indicating that it proceeded predominantly by a rolling circle mechanism.

In recent years, we have tried to reconstitute HSV-1 DNA replication with purified enzymes. Unlike SV40, HSV-1 encodes most of the enzymes it needs to replicate its genome, including a DNA polymerase, a single-strand DNA-binding protein, a polymerase processivity factor, a primosome (helicase-primase), and an origin-binding protein (49). The last three were discovered in my lab. Again, I was fortunate to have been able to recruit a splendid group of postdocs

and students: Per Elias and Mike O'Donnell started the project and were joined later by James Crute, Robert Bruckner, Mark Dodson, Tatsuya Tsurumi, Rami Skaliter, Sam S-K Lee, Ke-Jung Huang, and Don He, as post-docs, and Tom Hernandez, Rebecca Dutch, and Boris Zemelman, as graduate students. We are enormously aided in our efforts by Ed Mocarski, a seemingly inexhaustible source of information and ideas about herpes viruses. Although we have succeeded in reconstituting rolling circle replication, the initial, origin-dependent phase of replication has eluded us and remains a challenge for the future.

## CURRENT WORK ON HSV-1 LATENCY

Very recently as an outgrowth of our work on HSV-1 DNA replication, we have begun an investigation of the molecular basis of viral latency. An important feature of the HSV-1 life cycle is its ability to establish latency in neurons. Following primary infection, HSV-1 gains access to sensory neurons and travels via axons to establish infection in the sensory neurons that innervate the site of infection. Once within the neurons, the virus can either enter a productive (lytic) cycle, resulting in the release of progeny viruses or establish latency (50).

During latency, the viral DNA is present in the neuronal nuclei and is maintained in a nonlinear (circular?) nonintegrated nucleosome-bound state; transcription is restricted to several latency-associated transcripts, whose function is unclear. During neuronal latency, HSV-1 has no apparent impact on the infected individual. However, the latent virus can be reactivated throughout the life of the individual by a variety of stimuli and produce recurrent disease. For example, recurrent HSV-1 infection can cause corneal scarring, leading to the loss of vision. In fact, HSV-1 is one of the most common infectious causes of corneal blindness in the developed countries. Although the molecular mechanisms underlying the establishment of

latency and the reactivation from latency have been extensively investigated, they are poorly understood.

In a search for binding partners for the HSV-1 replication initiator, the UL9 protein, by using a yeast two-hybrid screen, we observed that NFB42, a neuron-specific protein, which forms part of the E3 ubiquitin-ligase complex that catalyzes ubiquitination and proteasome-mediated degradation of phosphorylated proteins, formed a tight complex with the UL9 protein. The fact that NFB42 is neuron specific immediately suggested a link between the UL9 protein and HSV-1 neuronal latency. In pursuing this lead, Chi-Yong Eom and I were able to demonstrate that the phosphorylated UL9 protein is polyubiquitinated in neurons and undergoes proteosomal degradation. These findings, which are still at an early stage, suggest that in neurons the UL9 protein is phosphorylated by cellular kinase(s), is recognized by the neuron-specific NFB42, and then is degraded via the ubiquitin-proteasome pathway. They further suggest that this degradation leads to the inhibition of initiation of HSV-1 replication and possibly to the establishment of neuronal latency (51). Time and further work will tell if this conjecture is correct. In any event, in our attempts to understand the HSV-1 replication initiator, we unpredictably wandered into uncharted and, for us, exciting new territory.

## ENVOI

Looking back at my more than 50 years in biochemical research, I am grateful for having lived through an extraordinary period of biological discovery. When I began graduate studies, there was still a lively debate over whether citrate was an integral component of the Krebs citric acid cycle or only an offshoot. The structure of DNA was a matter of conjecture, and it was not completely accepted that the gene is composed of DNA. Nothing was known about how DNA was replicated. RNA was a rather ill-defined substance, and there



were only suggestions that it was somehow required for protein synthesis. Although the pathways of carbohydrate, lipid, and amino acid synthesis were being rapidly elucidated, virtually nothing was known about how they were regulated. Today, we know their regulation in great detail. The structure of DNA and the various RNAs are known, and the human genome has been sequenced. The cloning of genes, their expression, and their repression are exercises in college biology courses. Enormous strides have been made in understanding the workings of the nervous and immune systems. These discoveries and many others have had a profound effect on our understanding and treatment of human disease, and

there are certainly many more discoveries to come.

Having grown up in the Great Depression and served in World War II, I consider myself very fortunate to have survived that dreadful period and to have been able to participate, if only in a very minor way, in the extraordinary scientific advances of the past 50 years. I consider myself particularly fortunate in having had an inspirational teacher, Arthur Kornberg, and to have been associated with a talented and hard-working group of graduate students and postdocs—not all of whom I have been able to mention. None of the successful “wanderings” that I have described would have been possible without them.

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## ERRATA

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