July editorial

The "modified" RNA (and DNA) world

A few months ago, as COVID-19 vaccinations were getting underway in the U.S., a non-scientist friend said he was uneasy because had heard that the RNA in them had been "doped". Leaning in, I asked "How?" (I knew of course, *vide infra).* "With some chemical" he replied.

This struck me as a perfect storm of an educated, reasonably informed non-scientist being led astray by how the media often doesn't get it quite right, though we all recognize that too much detail can be narcoleptic. The art is to convey the science in just the right dose, as Lewis Thomas and Carl Sagan did for example (1). I told my friend what the "doping" was, using lay terms. He listened thoughtfully and then I came in with my final shot: nature is full of RNA that is "doped", and even DNA is as well. These chemical modifications are not done by mad scientists but the very biological systems in which these RNAs and DNAs reside, using their own enzymes.

He left somewhat convinced and hopefully is now vaccinated. This encounter gave me the thought that I, and my readers, should take a step back and think about all the "modified" RNAs and DNAs out there.

For transfer RNAs alone, there are 120 known base modifications, with their prevalence as high as 13 of the 76 nucleotides in human cytosolic $tRNA^{tyr}$(2). N^6-adenosine methylation of messenger RNA, discovered in 1974, has recently come to the fore, although not all experts agree on its functional significance (3,4). And as to my friend's angst, the accounts he had seen in the media were based on the fact that in both mRNA-based vaccines, all the uracil positions are replaced by N^1-methylpseudouridine (Fig. 1).

Discovered in transfer RNA (5), pseudouridine is thought to be a stabilizer in which its N^1 hydrogen can bond to the hemi-acetal oxygen in the ribose of a paired adenosine. Thus, the triple helix of poly(A):poly$(U)_2$ has a lower T_m than

one in which the U strands are all pseudouridine (6). The elegant structural work on pseudouridine in RNA has been complemented by other studies that have, *inter alia*, raised the possibility that the cancer chemotherapeutic action of 5-fluorouracil, the first nucleic acid base analog, pioneered by Gertrude Elion and George Hitchings, might not be limited to its substitution for thymine in DNA, but also its impairment with the biosynthesis of pseudouridine-containing RNA (7).

Why do the Moderna and Pfizer vaccines have N^1-methylpseudouridine substituted all throughout where uridines would otherwise be? This came from two groups, working independently, who found that inserting pseudouridine into RNA caused it to outwit the machinery that normally degrades exogenous RNAs in mammalian cells (8,9). As far back as 1990 my laboratory succeeded in introducing a (naturally) pseudouridine-containing RNA into mammalian cells and showed that it was not only not degraded but accurately went through the same steps of ribonucleoprotein assembly and intranuclear localization as we had previously established for its endogenous counterpart (10). But this was a very small RNA (ca. 200 nucleotides) and, in hindsight, needed no further chemical protection added by us. Subsequent to the pseudouridine-substituted mRNA findings (8,9) it was found that N^1-methylpseudouridine conferred even more resistance to degradation and also displayed enhanced translational activity (11) and thus it was chosen by both Pfizer and Moderna for their COVID-19 vaccines.

But there is more. OK- so cells modify their RNAs and now companies do too, for good therapeutic reason. What about DNA? The first base modification of DNA was discovered in 1948 (12) and later it was recognized that these are, in bacteria, part of a host-restriction mechanism against bacteriophages (13). Later, one DNA modification, methylation of the C5 in cytosine, was found to be part of an epigenetic process in which genes can be programmed in the mammalian life cycle (14). In due course, the idea of using DNA as a drug arose, specifically in the form of short single-stranded molecules that could attenuate protein synthesis by hybridizing with a targeted RNA (15). It soon became apparent that these synthetic oligodeoxynucleotides would need modification to persist long enough in cells to achieve the intended effect and the first step in this direction was to synthesize them with phosphorothioate inter-nucleoside bonds, in which a sulfur atom replaces the non-bridging oxygen. in each inter-nucleoside bond. Many

other modifications were later developed as the antisense DNA therapeutics field evolved (16), including the strategic placement of phosphorothioates at certain positions to trigger degradation of the mRNA by RNase H but with these flanked by different inter-nucleoside modifications to achieve even higher resistance of the oligonucleotide to degradation (17).

But returning to my friend's query about the mRNA vaccines and how his angst may have lessened upon learning that the modifications occur naturally, I want to close on a stunning point: phosphorothioate substituted DNA occurs in nature and plays a role in phage host-restriction and bacterial epigenetic mechanisms (18,19). And unlike all else we have discussed here so far, these are not base modifications but rather *sequence-directed* modifications of the inter-nucleoside linkage itself, and if that were not enough, also stereo-specific. The enzymes that accomplish this have been identified and this is a new frontier in prokaryotic evolution and biology. And I cannot resist commenting that given an unproven idea as to the origin of SARS2/COVID-19, one of the leading groups in this elegant work on naturally occurring DNA phosphorothioate linkages is at Wuhan University, reminding us of all the great science being done throughout China, and the support it deserves here in the U.S. and throughout the world, as I have emphasized previously (20).

For RNA, I suspect almost all of the modifications have been discovered although a few more may show up as RNAs from extremophiles continue to be studied. As for DNA, some years ago a group reported a bacterium that lives in Lake Mono, California, in which arsenate inter-nucleoside linkages were claimed to occur (21). Arsenate and phosphate share tetrahedral anatomy and electronic features so this seemed plausible at first blush, given the high concentration of arsenic in this lake. (It is a rare one in having no effluent, thus accreting very high dissolved mineral concentrations over its 750,000-year geological history.) Alas, subsequent work revealed that it was not the case, but this did get everyone thinking about the notion, not just like natural phosphorothioate linkages, but others in which at the origin of life or later chemical opportunities might have led to an atom other than phosphorus. But phosphorous "checks all the boxes" as a great essay once beautifully articulated (22). Meanwhile, we remain ready to be excited by all that awaits us- the innocent children that we are, riding on the endless frontier.

All this has come to me, with gratitude, from a brief, appreciated encounter about a vaccine.

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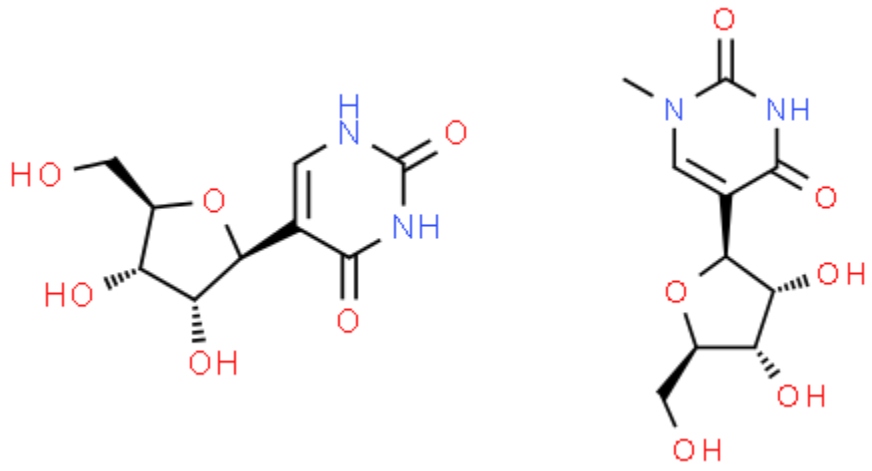

Fig. 1. Pseudouridine (left) and N^1-methylpseudouridine.