Chapter 9. The DNA filter elution story 220720ae3

Drugs Against Cancer: Stories of Discovery and the Quest for a Cure

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CHAPTER 9

The DNA filter elution story: a new way to measure DNA damage.

I had long wondered how the remarkable physical properties of long DNA chains might be used to develop improved methods of measuring DNA damage, in particular DNA strand breaks. I had made several attempts in that direction, but without finding anything very useful – until an answer came from a totally unexpected direction. I had imagined that DNA replication sites might be large enough to be retained on filters after passing gently dissolved cell nuclei through the filters. In trying different ways of dissolving the cell nuclei, one particular solution gave a totally unexpected result.

A research breakthrough in a long-desired direction sometimes happens unexpectedly during studies aimed in a different direction. In 1973, I was following up on a then current idea that DNA replication occurred in a part of the chromatin that was attached to the surface membrane of the cell's nucleus. I thought we could filter out the nuclear membrane-bound chromatin and test whether it mostly contained newly replicated DNA.

Working with my assistant, Regina Ewig, we deposited cell nuclei on filters that had very small pores and measured how much newly replicated DNA was retained on the filter after passing various solutions of detergents through the filter to dissolve the nuclei and wash away any DNA not attached to nuclear material large enough to be retained on the filter. We saw some selectivity for newly replicated DNA in the nuclear material retained on the filters as we had hoped and therefore tried to optimize this selectivity by using various solutions to wash away the unbound DNA. When we tested various detergent solutions to dissolve the nuclei and letting the unbound DNA drip out, we found that our hypothesis was at least partly correct: an increased amount of newly replicated DNA was retained on the filter.

However, when we tested a solution made alkaline with sodium hydroxide to dissolve the cells, the opposite happened: the newly replicated DNA quickly ran out through the filter, instead of being retained on it, whereas the bulk of the DNA was retained (Kohn and Grimek-Ewig, 1973) (Figure 9.1). I soon realized that the alkaline solution would have caused the paired DNA strands to separate and would have release the short newly replicated DNA segments, allowing them to pass freely through the filter.

It seemed that this might be a way to measure DNA damage that produced strand breaks yielding relatively short DNA single-strands that would pass relatively easily though the filter. I had been trying for a long time to think of how to measure DNA breaks in a more sensitive way than the problematic centrifugation methods then in use, and here was a new possibility to explore.

The first thing we did to check whether short DNA strands would in fact selectively pass through the filter was to expose the cells to a hefty dose of x-rays (I think it was 1000 rad in the first trial), which was known to cause DNA breaks. We trekked over to the Clinical Center with our samples in an ice bucket, to a basement facility where a radiation research group had installed a pair of x-ray tubes, one above and one below the platform for cell dishes or mice so as to subject them to a uniform dose of radiation. We were extraordinarily pleased to see that the DNA from the x-ray'd cells ran through the filter quickly as we had hoped and expected.

That was all good and well, but to make it into a useful biological measure of DNA strand breaks, we would have to increase the sensitivity of this alkaline elution phenomenon, as we called it, by a factor of at least 20. Was it possible to do that? A dictum attributed to Thomas Edison came to mind, to the effect that one could often improve a new phenomenon by a factor of 10 by systematically varying the conditions used. In accord with Edison's dictum, we systematically examined the effects of varying the conditions -- such as pH, flow rate, filter type, and compositions of both the detergent solution used to disrupt the cells and the alkaline solution used to elute the DNA through the filter.

First, we controlled the outflow from the filter by means of a peristaltic pump, thereby allowing us to control the flow rate of the alkaline solution through the filter. That turned out to be important: slowing the flow to several hours' duration increased the sensitivity of the assay several fold. Our hopes became a reality when optimizing the conditions gave us a method that was sensitive enough to measure DNA damage in mammalian cells at therapeutically relevant dosage of x-rays or other DNA-damaging agents, which no other method of the time had achieved (Kohn and Grimek-Ewig, 1973) (Kohn et al., 1976).

During several years of experience and further development, the method acquired a sound quantitative basis for measuring several types of DNA damage, including single strand breaks, double-strand breaks, inter-strand crosslinks, and DNA-protein crosslinks (Kohn, 1996).

A condition that had worried me was the number of cells that was to be loaded onto the filter. Our procedure was to lyse (dissolve) the cells on the filter with a detergent solution in order to free the DNA from other cell constituents before starting the controlled flow with the alkaline solution. I was afraid that the measurements would be sensitive to the number of cells loaded, because that would affect the viscosity of the lysed cell material. I was almost sure that the high viscosity of the DNA would slow the elution rate. It was a great surprise and a great relief, as well as somewhat of a puzzle, to find that the DNA elution rates were completely unaffected by number of cells loaded, as long as the number of cells was not so large as to clog the filter and stop the flow. Evidently, the viscosity was somehow cancelled out by some other factors, or perhaps the viscosity was irrelevant to what was happening on the filter. But, regardless of its cause, the fact that it didn't matter how many cells were loaded onto the filter was important in making filter elution a useful assay for DNA damage.

In addition to the method for measuring single-strand breaks, we worked out variations, whereby we could measure other types of DNA damage, such as double-strand breaks, inter-strand crosslinks, and DNA-protein crosslinks (Kohn, 1996). For nearly two decades, the filter elution methods were the methods used in most laboratories for measuring DNA damage and repair in cells.

Our early experiments were fraught by an annoying variability in the background DNA elution from undamaged cells, which should have shown very little DNA elution. I came to suspect that the room fluorescent lighting might cause some DNA strand breakage and raise the background measurements. What happened next may be of some interest or at least amusing. I asked Reggie Ewig, my assistant, who at that time carried out most of the assays, to arrange covers for the funnel units that had the filters with the lysed cells, in order to block the fluorescent light coming from the ceiling. Reggie was skeptical and proceeded to obey my direction by preparing dunce-like conical caps to put over the funnels, and she pasted labels on them marked "Kohn's folly." That was an example of Reggie's delightfully independent way of thinking that I treasured.

But, when she saw the results, which indeed reduced the background to almost nil, she insisted that there be only enough light to allow the work to be done. She taped the room's light switch in the off position and used only a single desk lamp turned away from the counter on which the work was done. Later, we had yellow ceiling lights installed, which solved the problem. We were now able to measure as little as one DNA strand break per average mouse chromatid (Gantt et al., 1978).

A few years later, Matt Bradley, a post-doctoral fellow in our laboratory, did quantitative experiments to determine the extent and conditions for fluorescent light-induced DNA single-strand breaks in cells (Bradley et al., 1978). He also determined the rate that high intensity fluorescent light kills cells and how it relates to production of mutations in the cells (Bradley and Sharkey, 1977).

Our new filter methods for measuring various types of DNA damage gave us a new way to study the DNA damage and its repair in cells treated with various anticancer drugs,

carcinogens, and radiation. The filter methods were more sensitive and precise than other methods of the time and allowed us for the first time to actually quantify several types of DNA damage at pharmacologically relevant dosage. The most notable result of our experiments using the filter methods was the unexpected discovery that some anti-cancer drugs blocked topoisomerases (although the term 'topoisomerase' was not yet in vogue). That story, however, will be told in the next chapter (Chapter 10), which is about drug actions on DNA topoisomerases.

In the paper by Ray Gantt et al. 1978, by the way, we collaborated with Katherine Sanford's laboratory, which she had continued to lead after Wilton R. Earle, who had founded the laboratory, died unexpectedly in 1964 at the age of 62. I am now going to take the liberty to digress with a bit of history about Katherine Sanford and Wilton R. Earle (Figure 9.3) who were the first to clone a culture from a single mammalian cell. They did so by sucking a single cell into a micropipette and letting it divide a few times before allowing the new cells to grow out of the end of the capillary (Sanford et al., 1948) (Figure 9.4). I was greatly impressed by that work, when I read their paper in 1953 while at medical school. The laboratory Earle had established at NIH and continued by Sanford was at first meticulously constrained with gloves, masks and gowns like a surgical suit in order to avoid contaminating the cell cultures, which were grown without antibiotics. Katherine had a photograph above her desk of the first mammalian cell to grow as a clone in culture. Around 1970, I used their method to clone a single mouse leukemia cell in a capillary, but without needing their elaborate apparatus, because we used antibiotics and a laminar flow hood to avoid contaminating the cultures.

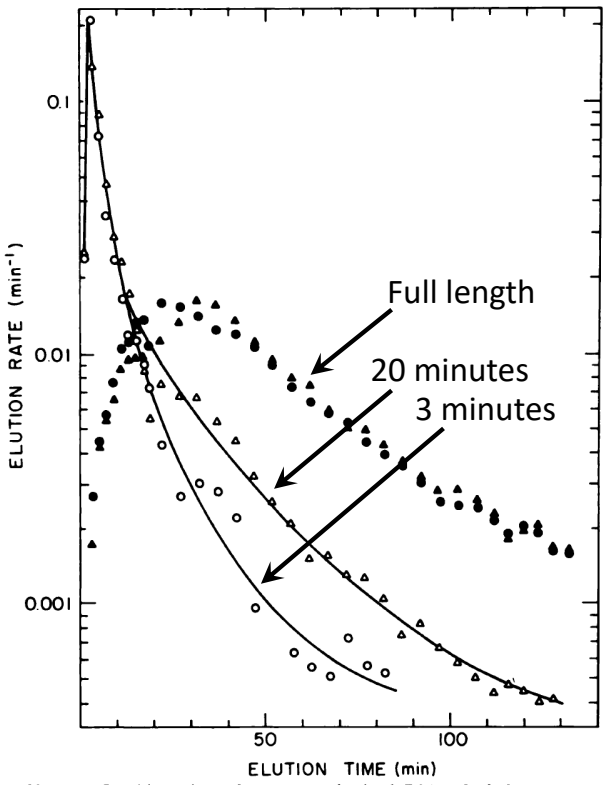

Figure 9.1. One of our first DNA alkaline elution experiments. Newly replicated DNA (after 3 or 20 minutes of replication) eluted more rapidly than the cell's full-length mature DNA. Our procedure was to deposit cells on a membrane filter and lyse them with a detergent-containing solution that would disrupt the cells and loosen proteins that were bound to the chromatin. We then pumped a solution containing 0.1 M sodium hydroxide (pH 12.0) slowly through the filter. The solid black symbols show the rate at which full-length DNA strands from eluted from the filter as a function of time. The lower two curves show the higher rates of release of newly replicated DNA from the filter (Kohn and Grimek-Ewig, 1973). The DNA that eluted during the first 10 minutes was mostly newly replicated DNA.

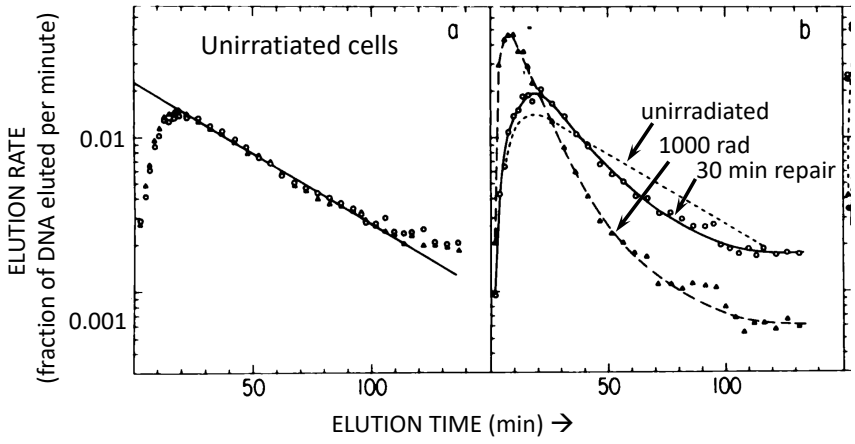

Figure 9.2. Proof that DNA single-strand segments elute from filters at rates inversely related to the length of the strands (Kohn and Grimek-Ewig, 1973). Cells were x-rayed to produce random breaks

in their DNA. The cells were lysed on a filter and the DNA was eluted with 0.1 M sodium hydroxide as described in Figure 9.1. **a)** Unirradiated cells. **b**) Cells irradiated with a 1000 rad dose of x-rays: their DNA eluted more rapidly than DNA from unirradiated cells. After 30 minutes, the cells repaired most of the damage, and the elution rate of their DNA reverted close to that of unirradiated cells.

Figure 9.3. Katherine Sanford (1915-2005) (*left*) worked with Wilton R. Earle (1902-1964) (*right*) who had come to NIH in 1937 and founded a Laboratory to study the development of mutations and cancer by cells in culture. Having joining Earle's laboratory in 1947, Sanford was appointed to assume the role of Chief of the Laboratory when Earle died unexpectedly in 1964.

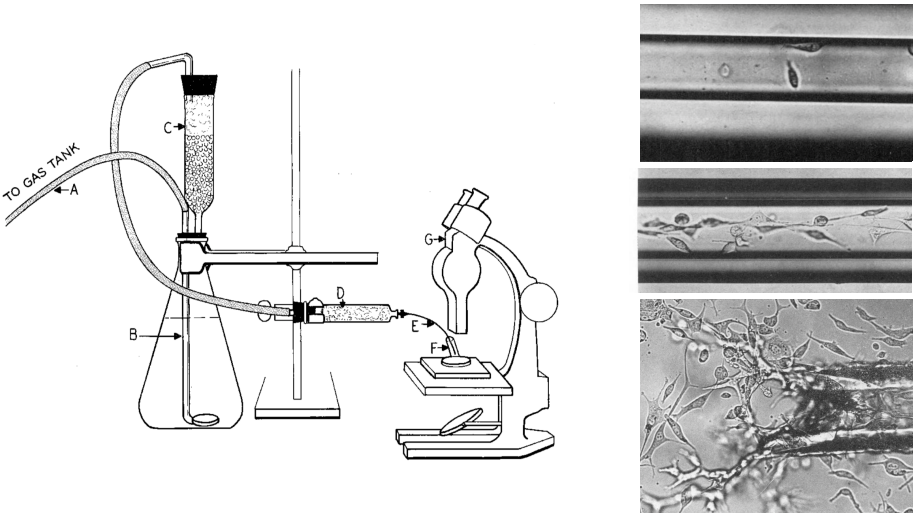

Figure 9.4. Katherine Sanford and Wilton R. Earle were first to clone colonies from single mammalian cells (Sanford et al., 1948). On the left is a diagram of the apparatus they used. A bent glass capillary (E) was inserted into a Carrell chamber (F) where cells were growing. Under a microscope, a cell was selected and sucked into the capillary. A cotton-filled syringe (D) prevented bacteria from entering into the capillary. On the right, we see pictures of the cell dividing in a glass capillary. From top to bottom, we see a single live cell in the capillary; cells having divided in the capillary; cells growing out of the end of a capillary and into a growth plate.

How the DNA filter elution phenomenon works.

The separated DNA single-strands are long enough to enter multiple pores in the filter as an alkaline solution flows slowly through the filter. The longer the DNA strand, the longer it would take to pass through the filter pores (Figure 9.5A).

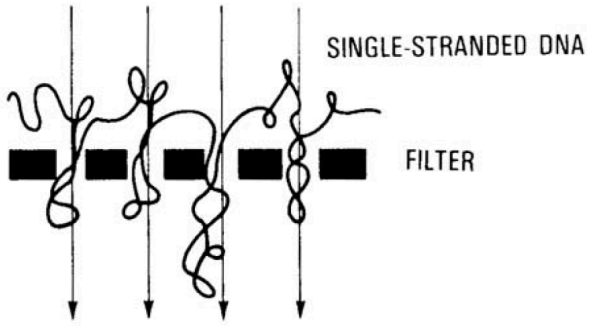

Figure 9.5A. How a filter may impair a long DNA strand from passing through. The DNA strands were long enough to enter several pores at once, longer strands would take longer to get through (Kohn, 1996). The filter elution procedure was essentially as follows. Cells were deposited on the filter and lysed by adding a detergent-containing solution. The strands of the cell's DNA were then separated by means of an alkaline solution (usually a little above pH 12.0), which caused the paired strands of DNA double helix to separate. As the alkaline solution was then slowly pumped through the filter, the shorter DNA stands came through the filter more rapidly than longer strands. The reason that the longer strands took longer to pass through may be that each strand entered a larger number of pores simultaneously, which made it more difficult for the strand to pass through. The rate at which the DNA strands eluted from the filter told us the average length of the strands, which told us the frequency of single-strand breaks in the cell's DNA.

Next, we had to find out how the DNA elution rate would tell us how many strand breaks there were. In other words, we had to calibrate the elution rate relative to strand break frequency. We did that by irradiating the cells with various doses of x-rays that produced known frequencies of DNA strand breaks and then measured the elution rate for each x-ray dose. The elution rate was beautifully proportional to x-ray dose (Figure 9.5B)! That allowed us to quantify precisely the DNA strand break frequency produced by given treatments with anticancer drugs, carcinogens, or other DNA damaging agents.

By comparing the elution rate of DNA from the x-rayed cells with the elution rate of DNA from drug-treated cells, we calculated the DNA break frequency in the drug-treated cells (Figure 9.5B). Moreover, the elution rate measurements were highly sensitive: they could detect DNA strand breaks produced by x-ray doses as low as 30 rad, which is less than 1/100 of the mean lethal dose. This was a major advance over other methods available at the time, both in sensitivity and precision.

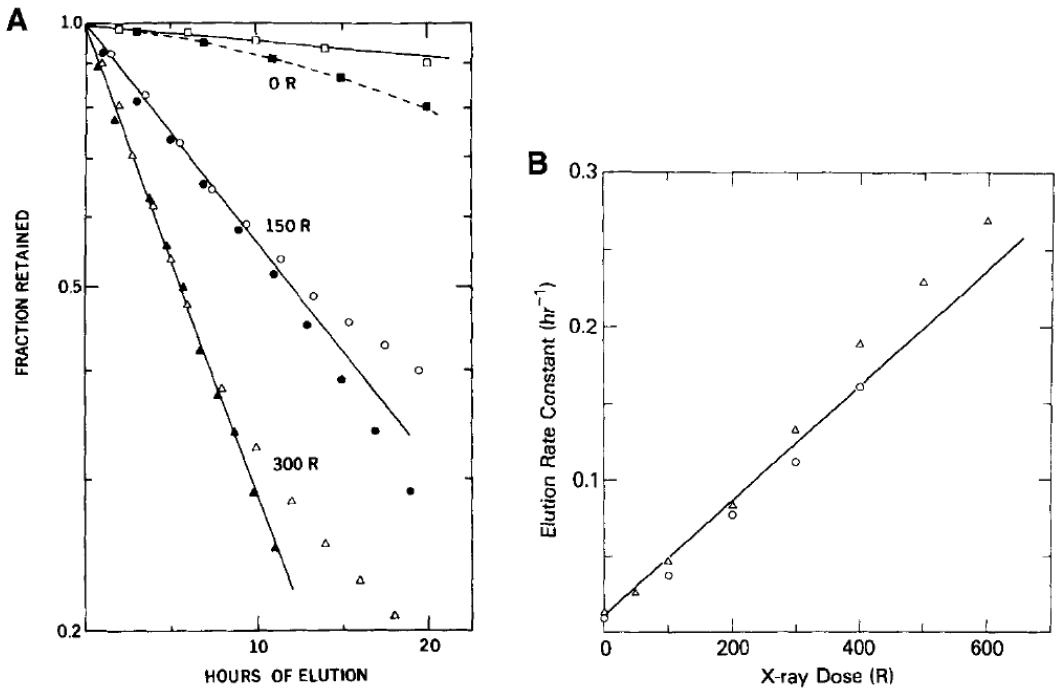

Figure 9.5B. The rate at which DNA single-strands passed through the filter depended on the average length of the strands. The DNA was cut at random places by subjecting the cells to various doses of x-rays that produced known frequencies of strand breaks. Panel A shows the rates of DNA elution from cells irradiated with 0, 150, or 300 rad of x-rays. The vertical axis indicates the fraction of the DNA remaining on the filter as a function time as an alkaline solution (pH12.1) was slowly pumped through the filter. The open and closed symbols were from experiments using alkaline eluting solutions of different pH, which showed that beyond a critical alkaline pH, raising the pH further had no effect, which was in accord with the theory of DNA strand separation developed by Paul Doty. Panel B showed that the DNA elution rate increased linearly with x-ray dose, thus linearly with the frequency of DNA strand breaks (Kohn et al., 1976). (The vertical axis in panel A is a logarithmic scale; thus, the elution rates were linear in this plot, which indicated that elution of DNA strands cut randomly by a given dose of x-ray was random in time.)

Filter methods adapted to measure several types of DNA damage.

We discovered that another property of the filters we used was that proteins tended to adhere to them. Therefore, a single-stranded piece of DNA that was linked to a protein did not pass through the filter, regardless of the length of the strand. To avoid this complication, we applied an enzyme to digest away most of the protein. Also, we used filters that had less tendency to bind proteins. Those two measures together eliminated protein binding to the filters.

However, we took advantage of the ability of certain types of filter bind proteins to create a quantitative assay for DNA-protein crosslinks. The procedure was to subject the cells to a relatively large dose of x-ray (3000 rad) to produce relatively short DNA strand segments and then carry out an alkaline elution. The strand segments that had a DNA-protein crosslink in it stuck to the filter and did not pass through, whereas the other strand segments were able to pass through. The fraction of the DNA that could not pass through the filter allowed us to calculate (with a little bit of algebra) the frequency of DNA-protein crosslinks. We validated the procedure by using the *trans*-isomer of cisplatin, which we had determined produced exclusively DNA-protein crosslinks (Chapter 3), as shown in Figure 9.6.

At that point, we were able to quantify both DNA single-strand breaks and DNA-protein crosslinks in drug-treated cells, which was to lead us to the conclusion that some drugs cause certain enzymes (later identified as topoisomerases) to produce DNA strand breaks and to become linked to the strand ends (Chapter 10).

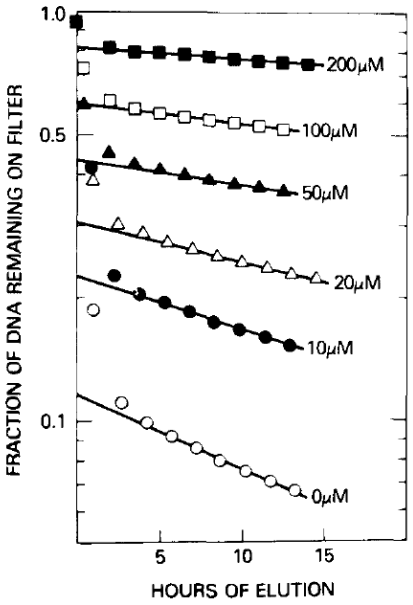

Figure 9.6. How we measured DNA-protein crosslinks. We treated cells with various concentrations of the *trans* isomer of cisplatin, which we had found produced exclusively DNA-protein crosslinks (see Chapter 3). We then subjected the cells to a relatively high dose of x-ray (3000 rad). The bottom curve (marked 0μM) showed that, for cells that received no *trans*-platinum treatment, about 90% of the DNA eluted rapidly. With increasing doses of *trans*-platinum, progressively less DNA eluted rapidly – which was the fraction of the DNA that was protein-linked. In order to get a true measure of the fraction of the DNA that was protein-linked, the curves (which were nearly linear) were extrapolated back to zero time (Kohn and Ewig, 1979).

I was pleased that my propensity for quantitative studies, engendered by my undergraduate background in physics and experience in Paul Doty's laboratory (see Introduction), yielded these quantitative measures of DNA single-strand breaks and DNA-

protein crosslinks. However, I had no idea that those quantitative measurements would lead us to a major discovery that was pertinent to cancer treatment. My background in physics and medicine seemed to come together, as if out of the blue. We discovered that certain anticancer drugs produced protein-linked DNA strand breaks that were caused by enzymes that came to be known as DNA topoisomerases. But that story will be told in the next chapter (Chapter 10).

It was an exciting time for us as additional results poured in from our DNA filter experiments. Some of our most memorable findings (aside from the topoisomerase story that is told in the next chapter):

In 1974, we measured the rate of DNA chain growth during DNA replication (Kohn et al., 1974). Then, in 1979, Len Erickson, who was a post-doctoral fellow in our laboratory, carefully investigated intermediate elution pH values and succeeded in fractionating and measuring the growth rates of newly replicated DNA chains of different lengths (Erickson et al., 1979).

In 1975, Al Fornace, who was at the time a post-doctoral fellow in our laboratory, used the filter methods to investigate the DNA repair in ultraviolet light (UV)-treated cells. He was first to detect the transient DNA strand breaks expected during nucleotide excision repair (NER) of the UV-induced thymine dimers. He showed the cells from xeroderma pigmentosum (XP) patients failed to produce those strand breaks – as expected, because XP was known to be deficient in NER (see Chapter 23). He also found that XP cells could not repair UV induced DNA-protein crosslinks. When he tested cells from the different subtypes ("complementation groups") of the disease, he noted different degrees of DNA repair deficiencies in the various subtypes (Chapter 23) (Fornace and Kohn, 1976; Fornace et al., 1976).

In 1977, we worked out how to measure DNA inter-strand crosslinks by means of the filter methods. We investigated the production and repair of those DNA lesions in cells treated with nitrogen mustard (HN2), BCNU and related drugs (Ewig and Kohn, 1977, 1978) (Figure 9.7). HN2 produced inter-strand crosslinks rapidly and then repaired them over a period of several hours (*left* panel of Figure 9.7). BCNU, on the other hand, produced crosslinks slowly, as monoadducts were slowly converted to crosslinks (*right* panel of Figure 9.7), and there was no evidence of repair, perhaps because those crosslinks, which are between paired guanine and cytosine are more difficult to repair (see Chapter 2).

We developed a quantitative model to estimate the frequencies of strand breaks, inter-stand crosslinks and DNA-protein crosslinks, even when these DNA lesions are all present at the same time. Figure 9.8, for example, shows that the calculated lesion frequencies were proportional to the BCNU concentrations and therefore were suitable for quantitative studies of drug actions on DNA (Ewig and Kohn, 1978).

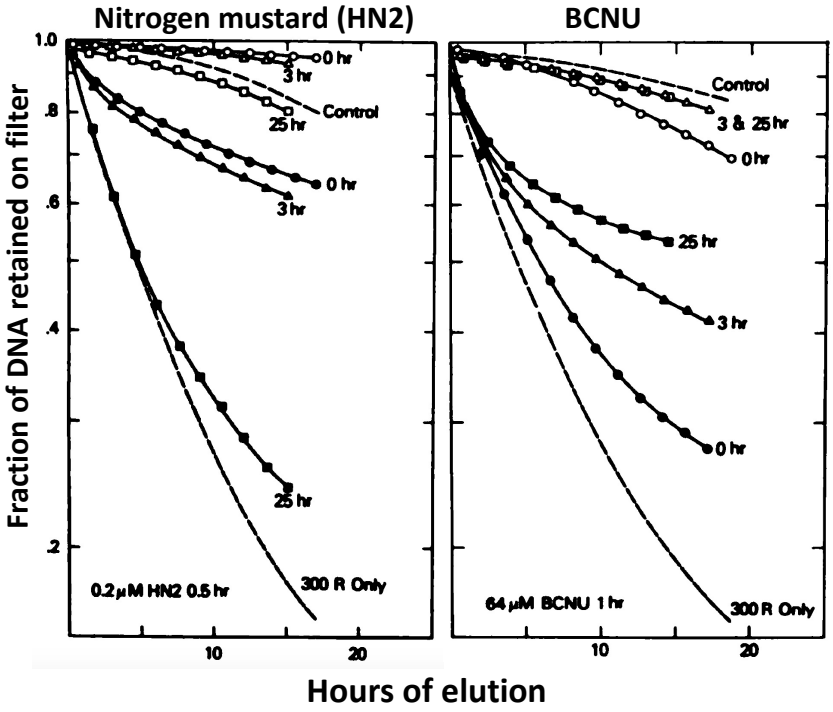

Figure 9.7. DNA inter-strand crosslinking by nitrogen mustard (HN2) (*left*) and BCNU (*right*) (Ewig and Kohn, 1977).

Left panel: after treating the cells with HN2 for 0.5 hours (solid symbols), there was a marked reduction of DNA elution in 300-rad-irradiated cells, indicating that there were many DNA inter-strand crosslinks (curve marked "0 hr"). There was little change after 3 hours, but by 25 hours the crosslinks were almost all repaired as shown by the elution curve being nearly back to 300 rad without drug.

Right panel: after treating the cells with BCNU for 1 hour (solid symbols) there was only a modest reduction of DNA elution in 300-rad-irradiated cells (curve marked "0 hr"). After further incubation, progressively more crosslinks appeared (curves marked "3 hr" and "25 hr"). Thus, BCNU was slow to produce inter-strand crosslinks, whereas HN2 produced them rapidly.

These experiments were repeated under conditions where DNA-protein crosslinks had no effect (by digesting the proteins with proteinase-K and using filters having low protein binding ability) and gave essentially the same results.

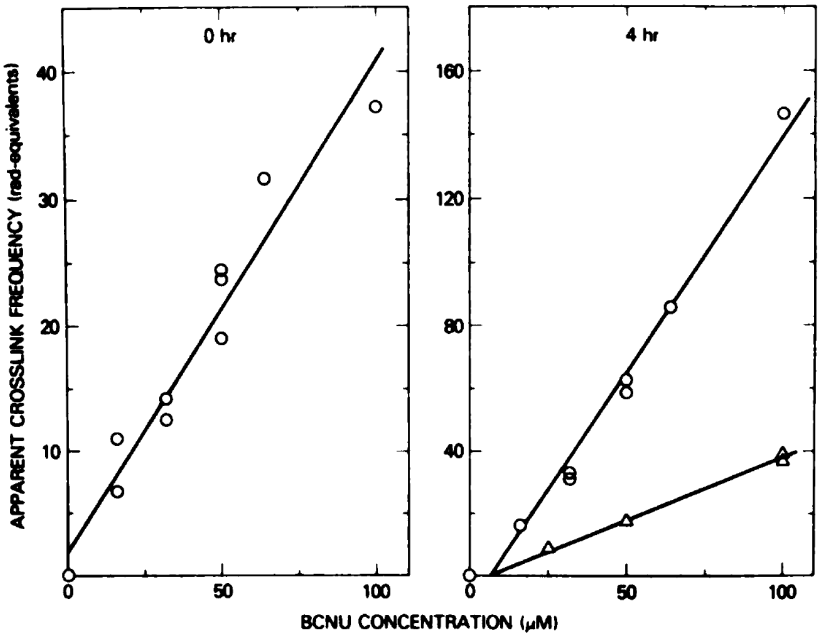

Figure 9.8. A quantitative model for inter-stand crosslink and DNA-protein crosslink production gave estimates that were proportional to drug concentrations shown on the horizontal axis of the graphs. Cells were treated with BCNU for 1 hour and were then, either assayed immediately (*left*) or incubated for 4 hours to allow time for more crosslinks to form (*right*). *Circles (upper line)*, assay combination of inter-strand and DNA-protein crosslinks (without proteinase). *Triangles (lower line)*, inter-strand crosslinks, which developed slowly. At zero time (immediately after 1-hour treatment with BCNU) almost no inter-strand crosslinks had yet formed; hence the results showed DNA-protein crosslinks only (*left)*. After 4 hours (*right*), inter-strand crosslinks appeared (*triangle*) (Ewig and Kohn, 1978).

In 1978, Matt Bradley, as already mentioned, discovered that fluorescent lights caused DNA single-strand breaks in cells illuminated in culture medium (Bradley et al., 1978). Tissues, however, normally have enzymes that destroy the free radicals that are the likely cause of the observed DNA damage -- the cells in the experiments were illuminated in solutions that lacked those enzymes.

In 1979, Bradley and I worked out how to measure DNA double-strand breaks by using solutions of neutral pH for elution from filters (Bradley and Kohn, 1979). Using that method, he together with Warren Ross later observed double-strand break production in cells treated with doxorubicin or other DNA intercaltors, and inferred that the double-strand DNA breaks were produced by a trapped topoisomerase (Ross and Bradley, 1981).

In 1985, Neil Gibson and Len Erickson found that a new drug related to the chloroethylnitrosoureas (see Chapter 2) produced inter-strand crosslinks and that the ability of cells to survive the treatment was related to their ability to repair or prevent the formation of those crosslinks (Gibson et al., 1985). Then, in 1987, we found that brain

cancer cell lines grouped according to whether or not they were able to prevent DNA crosslink formation by chloroethylnitrosoureas and that this was related to the ability of the cells to survive (Sariban et al., 1987). As explained in Chapter 2, the ability of the cells to survive these treatments was dependent on whether or not the cells produced an enzyme (MGMT) that quickly removed the chloroethyl groups from the DNA before they could go on to form inter-strand crosslinks.

The next chapter will relate what was for us the most exciting finding: the discovery that a DNA topoisomerase enzyme was the target of action of several anti-cancer drugs.

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