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Mini-review

# On the origins and development of the $^{32}$P-postlabelling assay for carcinogen–DNA adducts

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ABSTRACT

The $^{32}$P-postlabelling method for the analysis of carcinogen–DNA adducts originated 30 years ago from Baylor College of Medicine in Houston and was the work of a team comprised of Kurt and Erica Randerath, Ramesh Gupta and Vijay Reddy. With subsequent modifications and developments, it has become a highly sensitive and versatile method for the detection of DNA adducts that has been applied in a wide range of human, animal and *in vitro* studies. These include monitoring human exposure to environmental and occupational carcinogens, investigating genotoxicity of chemicals, elucidating pathways of metabolic activation of carcinogens, mechanistic studies of DNA repair, analysing the genotoxicity of complex mixtures and in ecotoxicology studies. Its use has been instrumental in providing new clues to the aetiology of some cancers and in identifying a new human carcinogen.

## 1. Introduction

### 1.1. Origins of postlabelling

I first heard of the $^{32}$P-postlabelling assay at a symposium in Keystone, Colorado USA in February 1981 on Mechanisms of Chemical Carcinogenesis, where a poster presentation on it attracted a great deal of interest. The ensuing publication in *Proceedings of the National Academy of Sciences USA* [1] was co-authored by Kurt Randerath, Vijay Reddy and Ramesh Gupta. It described the detection of covalent adducts formed in DNA by *N*-methyl-*N*-nitrosourea, propylene oxide, formaldehyde and several other agents by digestion of the DNA to deoxyribonucleoside 3′-monophosphates, followed by 5′-labelling with T4 polynucleotide kinase (PNK) and with [$\gamma$-$^{32}$P]ATP as the donor of the radiolabelled phosphate group. The labelled modified and unmodified nucleotides were then separated on thin-layer plates of polyethyleneimine(PEI)-cellulose and visualised by autoradiography. A pattern of spots on the 2-D chromatograms revealed the presence of the four radiolabelled normal nucleotides and additional spots indicated the presence of the adducted nucleotides. A limit of sensitivity of 1 adduct per $10^5$ nucleotides was reported.

These adducts were generated by incubating the direct-acting chemicals with calf thymus DNA in solution. While this demonstrated, as a proof of principle, that DNA adducts could be detected without the requirement to radiolabel the test compound, for the method to be useful for detection of DNA adducts formed in cells or tissues under physiological conditions or environmental levels of exposure, greater sensitivity would be required. The publication that followed in 1982, co-authored by Gupta, Reddy and Randerath [2], represented a major step forward from the *PNAS* paper and opened the way to a much more versatile and sensitive series of techniques for detecting DNA adducts at physiological levels. Realising the potential of this method, I contacted Kurt Randerath and asked to visit him to learn the method. He kindly agreed and with a travelling fellowship from UICC I spent a month in Houston in April 1983 learning at firsthand how to postlabel. In the ensuing 30 years the method has been a core technique in my research and it has been instrumental in pursuing many interesting and important questions in environmental carcinogenesis that have been addressed and answered by a large number of investigators.

The 1982 paper focused on DNA adducts formed by bulky, aromatic and/or hydrophobic carcinogens, rather than ones forming small chemical modifications to DNA (e.g. methyl groups), and it used high molarity urea-containing solvents to resolve these non-polar adducts on PEI-cellulose. In consequence it was now possible to remove the radiolabelled normal nucleotides from the chromatograms and detect the presence of modified nucleotides from a wide variety of bulky carcinogens at much lower levels, around 1 adduct in $10^7$ nucleotides.

These techniques had evolved from earlier work by Kurt and Erica Randerath, who had developed over several years both $^3$H- and $^{32}$P-postlabelling methods for sequence analysis of RNAs, especially tRNA which has many modified nucleotides [3]. This led onto a method for labelling normal DNA nucleotides [4], underpinned by Kurt's pioneering studies into the anion-exchange thin-layer

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chromatography (TLC) of nucleic acids [3], and thence to the detection of DNA adducts.

## 2. Enhancement of sensitivity

As already mentioned the original assay consisted of three basic steps: (1) digestion of DNA; (2) $^{32}$P-labelling of nucleotides by transfer of $^{32}$P-orthophosphate from [$\gamma$-$^{32}$P]ATP; (3) chromatographic resolution and detection of the radiolabelled adducts. Under these conditions, the vast majority of the radioactivity is incorporated into normal nucleotides, limiting the sensitivity of the technique. Methods that target a given amount of radioactive material preferentially to modified nucleotides would have the potential to increase the sensitivity substantially.

The first such approach was the serendipitous discovery that by leaving carrier (unlabelled) ATP out of the labelling mix, an enhancement of labelling of adducts was achieved [5]. This has become known as the "limiting ATP" method of sensitivity enhancement. By using limiting amounts of high specific activity (carrier-free) ATP, rather than a molar excess of much low specific activity material (because of isotope dilution), the same amount of total radioactivity in the reaction nevertheless results in enhanced sensitivity, because some modified nucleotides are labelled in preference to normal nucleotides. This appears to be the case for a number of carcinogen–DNA adducts, e.g. those formed by polycyclic aromatic hydrocarbons (PAHs), but quantification of adducts is not straightforward and the degree of enhancement may be subject to day-to-day variation due to subtle changes in labelling conditions and batch-to-batch variation of some materials (such as ATP and PNK).

A more systematic approach to sensitivity enhancement is to separate the adducted nucleotides from the normal ones prior to postlabelling. A method for this was described in 1985 by Gupta [6], with butanol as the extracting solvent, coupled with as a phase transfer agent. The method is suitable for many aromatic/hydrophobic adducts and has been widely used. Because of the low levels of adducts under most experimental conditions, carrier-free [$\gamma$-$^{32}$P]ATP can be used with it being in excess over the amount of substrate (i.e. adducts).

An alternative approach, developed around the same time by Reddy et al. [7], involves additional digestion of the DNA sample with nuclease P1 prior to labelling. This has the effect of converting normal 3′-mononucleotides to nucleosides, which are not substrates for PNK and thus not able to be labelled. Fortuitously, many 3′-mononucleotide adducts are resistant to dephosphorylation, so that when carrier-free [$\gamma$-$^{32}$P]ATP is used in the ensuing labelling reaction its concentration is in excess over substrate.

An advantage of this method over the butanol extraction method is that little additional manipulation or handling of the DNA digest or reaction mixture is required, merely the addition of another component to the reaction mixture. A disadvantage is that not all adducts are resistant to nuclease P1 digestion; empirically, it seems that many that are formed at the C8 position of guanine (e.g. adducts formed by aromatic amines) are not resistant [8,9].

Both the butanol extraction and the nuclease P1 digestion procedures can achieve a limit of detection of 1 adduct in $10^{10}$ nucleotides from the analysis of 10 $\mu$g DNA. For most experimental and analytical purposes, this is more than adequate, making the $^{32}$P-postlabelling assay highly versatile and applicable to a wide range of human, animal and *in vitro* studies.

Another method of sensitivity enhancement, less widely used, also takes advantage of the resistance of many adducts to nuclease P1 digestion. If the DNA samples are first incubated with nuclease P1 and prostatic phosphates (PAPs), instead of with micrococcal nuclease (MN) and spleen phosphodiesterase (SPD), the digest is converted to normal nucleosides (N) and adducted dinucleotides (XpN). Only the latter are then substrates for PNK, and the resulting labelled dinucleotides can be converted to labelled 5′-mononucleotides by further incubation with venom phosphodiesterase (VPD) [10].

Other methods that have been developed by other investigators for sensitivity enhancement include separation of adducts on HPLC [11] or using small cartridge columns prior to postlabelling [12,13], or using immunoaffinity columns containing antibodies to the adducts of interest [14,15].

## 3. Validation and standardisation

Ten years after the seminal 1982 paper [2], a conference on Postlabelling Methods for Detection of DNA Adducts was held at the International Agency for Research on Cancer (IARC) in Lyon, France, and the proceedings of this conference have been published [16]. As a prelude to the meeting, interlaboratory trials were conducted to assess the reproducibility of the assay and as a step towards validation. The results revealed that in the ensuing 10 years since the origin of the assay, many variations in assay conditions had been introduced, resulting in quite a wide quantitative range in the results obtained in different laboratories [17]. There then followed a series of workshops and trials aimed at producing a more standardised protocol to be recommended for use. The results of these trials and the recommended conditions to be followed were published subsequently [18].

These interlaboratory studies demonstrate the potential for standardisation, but also some limitations. Being an assay that relies on enzymatic digestion of DNA and enzyme-mediated transfer of radiolabel, it is vulnerable to different activities of enzymes derived from different manufacturers and even from different batches from the same manufacturer. Different sources of chromatographic media present another potential source of variability. While such matters limit the realisation of a truly standardised protocol, the lessons learned from interlaboratory trials do at least give investigators some guidelines and "points to consider" when devising assay conditions. Furthermore, the versatility of application of the assay means that no single set of assay conditions is likely to be suitable or optimum for the detection and characterisation of all types of DNA adduct. Nevertheless, recommendations for conducting the $^{32}$P-postlabelling assay [19] can serve a useful purpose for new users and as a quality control check for established practitioners.

## 4. Variations to the $^{32}$P-postlabelling assay

The versatility of the assay has been amply demonstrated by the many variations developed to suit the particular aims of different investigators.

Several other methods for resolution of labelled adducts have been used; for example, solvents based on ammonium hydroxide and 2-propanol have been used in place of urea-based TLC systems [20]. Some investigators have found it advantageous to resolve the labelled adducts as 5′-mononucleotides [10,21,22]. High performance liquid chromatography (HPLC) has been used in place of TLC to resolve adducts [23–25], but can also be used in addition to TLC to characterise further the nature of the material in adduct spots on TLC plates [26,27]. Reverse-phase TLC systems have been adapted to detect smaller aromatic adducts [28]. Another approach has been to use polyacrylamide gel electrophoresis (PAGE) to resolve and detect $^{32}$P-labelled adducts [29].

It should also be mentioned that another isotope of phosphorus, $^{33}$P, can also be used in place of $^{32}$P [30]. Its longer half-life (25.3 d versus 14.28 d) means a slightly lower specific activity of ATP is