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Enzymatic shot-gun 5′-phosphorylation and 3′-sister phosphate exchange: a two-dimensional thin-layer chromatographic technique to measure DNA deoxynucleotide modification

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ABSTRACT

DNA adducts occur through environmental, therapeutic, dietary, oxygen stress, and aging processes. A modified thin-layer chromatographic (TLC) technique can assess base composition and adduct formation. This requires labeling DNA by "shot-gun" 5′-phosphorylation of representative ^{32}P-α-deoxyribonucleotide monophosphates. Subsequent 3′-monophosphate digest "sister exchanges" a radioactive $^{32}PO_4^{2-}$ to the neighboring cold nucleotide. Separation in two-dimensional polyethyleneimine-cellulose TLC is carried out in acetic acid, $(NH_4)_2SO_4$, and $(NH_4)HSO_4$. The technique was applied to control DNA, cold substition of dUMP, methylation, depurination, and pBR322. This technique quantifies low-molecular-mass adducts and DNA integrity both *in vivo* and *in vitro*.

INTRODUCTION

DNA is a highly reactive structure that undergoes numerous reactions *in vivo* and *in vitro* [1]. Repair *in vivo* may or may not be complete, given variable repairabilities by the host and different disease states. Further, repair outside the cell is impossible. The ability to assess DNA quickly and reproducibly impacts on both understanding gene function and misfunction. Also, many molecular biologic analyses are dependent on high-quality, unaltered DNA, *e.g.* polymerase chain reaction, which can also introduce artefact.

The present technique derives from a long history of thin-layer chromatographic (TLC) separation techniques carried out to separate tRNA [2,3] and mRNA polynucleotides [4]. Also, the technique derives impetus from ^{32}P-postlabeling techniques, which have been critical in the separation of high-molecular-mass carcinogen DNA adduct formation and other "indigenous" ("I" spots) adducts [5–9]. The present technique, an alternative technique to the others described [10–12], labels representative fractions of all four deoxynucleotides in DNA, *in situ*, affording better adduct detection, since the configuration of many adducts will not allow postlabeling after DNA digestion to monophosphates. The TLC separation retains normal deoxynucleotides retention values and allows rapid visual assessment of DNA quality. The technique employs commercially available enzymes. Also, the assay is car-

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ried out in two dimensions on standard polyethyleneimine (PEI)-cellulose, and not by a more typical four-dimension solvent system. The technique does not require specially prepared TLC sheets. The assay can assess maintenance of DNA integrity and quality. This technique can impact on results obtained in molecular biology and DNA from patients with many disease states.

EXPERIMENTAL

Nucleic acids

Calf thymus DNA (Type I; highly polymerized), 2′-deoxyuridine 5′-triphosphate (dUTP), 2′-deoxyinosine 5′-triphosphate (dITP), and 2′-deoxy-5′-nucleotides (dAMP, dCMP, dGMP, dIMP, dTMP, and dUMP) were purchased from Sigma (St. Louis, MO, USA). pBR322 DNA was obtained from Boehringer-Mannheim (Indianapolis, IN, USA). α-^{32}P-Radiolabeled (3000 μCi/mmol; 8–16 μCi per "nick") dATP, dCTP, dGTP, and dTTP were purchased from Amersham or New England Nuclear (Dupont, Cambridge, MA, USA). Transfer ribonucleic acid (tRNA) from Type XXI *Escherichia coli* strain W was purchased from Sigma.

Enzymes

Micrococcal nuclease (EC 3.1.31.1; activity: 100–200 μmol/mg of protein) and spleen phosphodiesterase II (EC 3.1.16.1; activity: 13.5 U/mg of protein) were purchased from Sigma. DNase I and *E. coli* DNA-polymerase I were purchased from Boehringer-Mannheim. Contamination of the spleen phosphodiesterase II with adenosine deaminase accounts for the formation of deoxyinosine 3′-monophosphate, which is blocked by the addition of deoxycorfomycin (0.1 m*M*; the kind gift of Dr. Vern Schramm, Department of Biochemistry, Albert Einstein College of Medicine).

Kits

DNA extraction ASAP columns and nick translation kits were purchased from Boehringer-Mannheim.

Chromatographic solvents and plates

Solvent A (1 *M* acetic acid, pH adjusted to 3.5 with NaOH) was purchased from J.T. Baker (Piscataway, NJ, USA). Solvent B consisted of 74 g of $(NH_4)_2SO_4$, 0.4 g of $(NH_4)HSO_4$ (Aldrich, Milwaukee, WI, USA), and 4 g of Na_2EDTA in 100 ml of distilled water (pH 4.0). TLC sheets, polyester PEI-cellulose, were purchased from Sigma.

Reagents

The following reagents were used: 30.7 μ*M* dimethyl sulfate (DMS; EM Science, Gibbstown, NJ, USA); DMS reaction mix [200 m*M* sodium cacodylate (Sigma), 130 m*M* sodium perchlorate (Sigma), and 1 m*M* EDTA at pH 7.0]; 5.0 *M* sodium chloride, 0.5 *M* sodium acetate, 4.0 *M* ammonium acetate (Fisher Scientific, Springfield, NJ, USA); 100% ethanol, 70% ethanol, Tris-Cl EDTA (TE) buffer at pH 8.0, phosphate-buffered saline (PBS), DNA digest buffer (20 m*M* succinate and 8 m*M* calcium chloride at pH of 6.0).

Equipment

Filters (Millipore Nylon filter; 0.22 μm 25 mm nylon syringe) were purchased from Fisher. Laser densitometry equipment was from LKB (Bromma, Sweden), and TLC scintillation counting was performed with a computerized Ambis (Boston, MA, USA). Kodak XAR-5 films (20.3 × 25.4 cm) came from Eastman Kodak (Rochester, NY, USA).

Substrate DNA, postlabeling and nick translation methods for TLC analysis of formed DNA adducts

In brief, the technique requires labeling DNA by "shot-gun" 5′-phosphorylation of representative deoxyribonucleotides with all four [^{32}P]-α-deoxyribonucleotide monophosphates (dNMPs) by nick translation. Subsequent 3′-monophosphate digest "sister exchanges" a radioactive $^{32}PO_4^{2-}$ to the neighboring cold nucleotide. Calf thymus DNA is incubated at 0.5 μg/μl concentration (PBS buffer). This technique of detection is sensitive from one adduct per 10^5 to 10^8 nucleotides [5,6]. ^{32}P has been incorporated into DNA constituent mononucleotides by [^{32}P]-α-dNTP nick translation [13]. (Nick-translation will remove a small percentage of adducts. Neverthe-

less, equal amounts of adducts are labeled, *in situ* in DNA, and some may be uniquely labeled as opposed to postlabeling after enzymatic digest, which may not label all adducts.) At the end of nick translation native DNA is coprecipitated with 2 μl (10 μg) of tRNA [14]. Unincorporated counts are meticulously removed by three cold ethanol washings and buffer (TE at pH 8.0) resuspension of pellet. Subsequent digest is carried out by 40 μl of spleen phosphodiesterase II (activity: 0.03375 U/μl) and 10 μl of micrococcal nuclease (activity: 0.2–0.4 μmol/μl) in 40 μl of 20 m*M* sodium succinate, 8 m*M* $CaCl_2$, pH 6.0, at 37°C for 16 h. Deoxycorfomycin can be added to prevent deamination of deoxyadenosine moieties to their respective deoxyinosine moieties. Acetone precipitation and collection of supernatant precede evaporation to dryness (vacuum dessicator). Filtration through a Millipore Nylon filter (0.22 μm, 25 mm syringe filter) is utilized only with significant protein or insoluble contaminants. Resuspension in 5 μl of distilled water and typical spotting of 20 000–100 000 dpm (up to 10^6 dpm; range: 1–10 μl) occurs next. The monophosphate separation is easily carried out by two-dimensional PEI-cellulose TLC employing solvent A in the first dimension and solvent B in the second dimension [10,15,16]. Ambis quantification of ^{32}P, with control dpm to account for quenching, is next carried out. This is then followed by autoradiography at 24 and 72 h at −70°C with dual screens. Laser densitometry follows.

Conditions for thin-layer chromatography [17]

1. Solvents and composition: first dimension = 0.1 *M* acetic acid (pH 3.5 with NaOH); second dimension = 5.6 *M* $(NH_4)_2SO_4$, 0.12 *M* Na_2EDTA, and 0.035 *M* $(NH_4)HSO_4$ to pH 4; stable for two weeks.
2. TLC sheet: PEI-cellulose (Sigma), 200 × 200 mm; method of storage, refrigerator "crisper" at 4°C; preparation, no prerun and constant room temperature and humidity; treatment, dried (dehumidified) during spotting; heterogeneity (R_F lower with thicker layer), less than 1–3% variation over each TLC sheet.
3. Developing tank: Sigma; 275 mm × 275 mm × 75 mm with lid.
4. Application: 1.0–10.0 μl volumes (typical: 20 000–100 000 cpm).
5. Drying (origin, plate, after first dimension): at 1 cm, 1 cm *X,Y* axis; cold dryer.
6. Direction of development: ascending, both dimensions.
7. Distance of origin from solvent reservoir (closer to solvent reservoir produces a higher R_F): 1.0 cm.
8. Depth of immersion: 5 mm.
9. Volume of solvent in reservoir: 15 ml.
10. Duration of development: first dimension, 4 h; second dimension, 15 h.
11. Temperature: 17°C; 50–60% humidity constant.
12. Equilibration humidity of tank: complete prior to TLC placement.
13. Character of solvent front: regular, linear.
14. Comparison of R_F *versus* R_X: consistency of chemical migration *versus* relative standard, less than 3% variability. All R_F are given as R_X with *X,Y* coordinates. *N.B.*: conversion of R_X to R_F requires all numbers divided by 19 cm (if R_F usually multiplied by 100 = hR_F).

Stoichiometric dUMP replacement of dTMP

dUTP (2.0 μl) was added beginning at 4 mmol/l to standard nick translation of calf thymus DNA (final volume 20 μl). Dilutions ranged from 4.0 mmol/l to 0.4, 0.04, and 0.004 mmol/l.

Base methylation. Prelabeled calf thymus DNA was co-incubated in 3.87 μl of DMS (30.7 μ*M*) and 40 μl of DMS reaction mix for 1 h at 37°C. Incubations to methylate nucleobases in DNA were carried out at standard, 1:10, 1:100, and 1:1000 dilutions [21]. Alteration of DNA deoxynucleotides is shown by the contrast of known cold marker overlying ^{32}P counts on autoradiograms or overlaying developed film.

Depurination. Depurination was carried out on ^{32}P prelabeled DNA. Calf thymus DNA (0.5 μg) was treated with 1 μl of 0.5 *M* sodium acetate and 1 μl of 5.0 *M* sodium chloride, in 43 μl of distilled water, for 10 min at 70°C. Ethanol precipitation and DNA digestion with subsequent TLC separation were carried out.

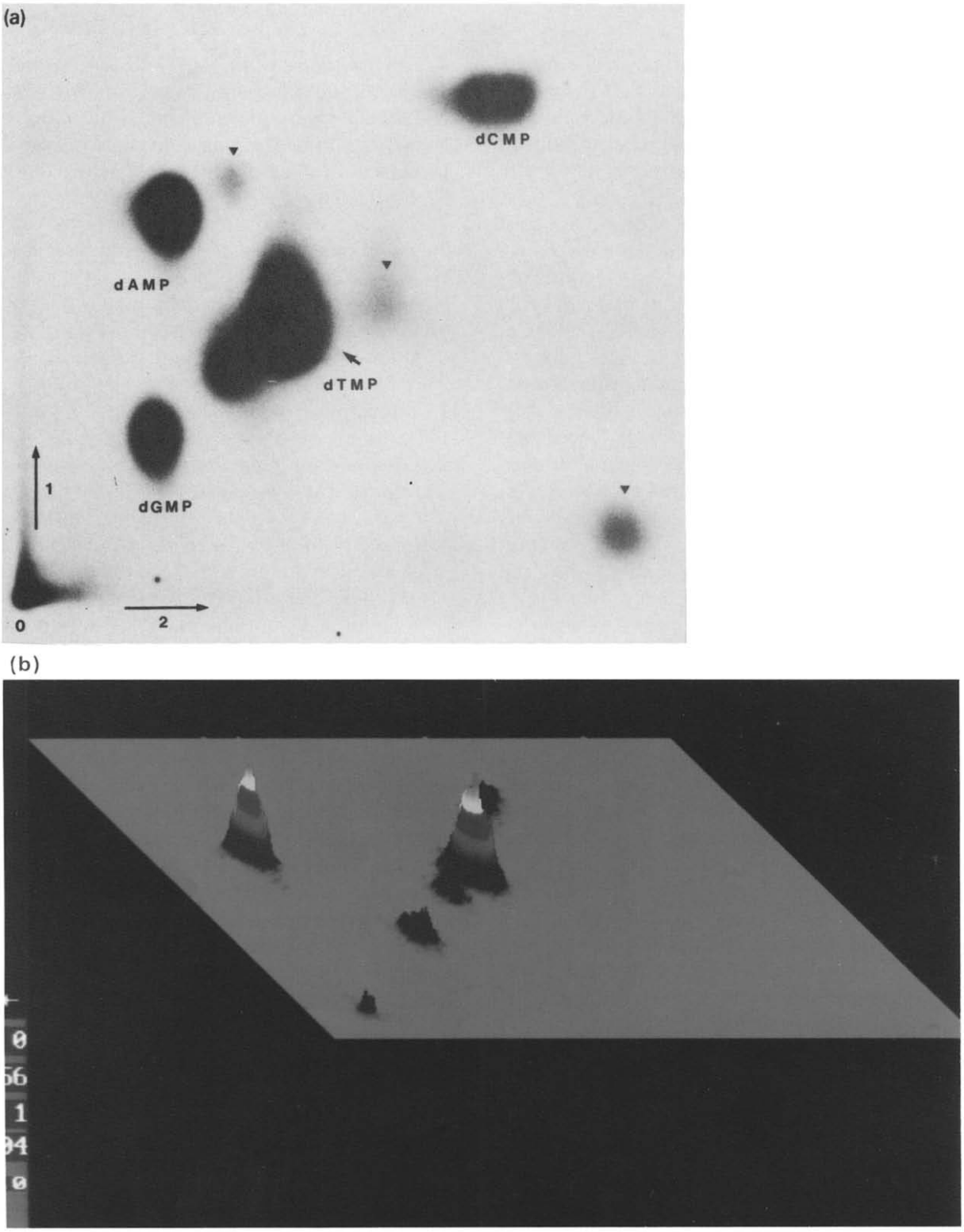


Fig. 1. Control calf thymus DNA: (a) 24-h autoradiogram and (b) Ambis three-dimensional representation. Autoradiogram demonstrates, in clockwise position, the major bases of DNA as their nucleotides: dAMP [retention factor (R_X) X, Y = 1.8, 9.5 cm); dTMP (the arrow; R_X = 8.3, 7.0 cm); dGMP (R_X = 3.7, 3.3 cm); and dCMP (R_X = 10.9, 13.5 cm). The arrow head at dAMP represents methyl-dAMP; the arrow head at R_X 13.1, 1.1 represents a constant finding that is unknown.

Ambis computer-assisted TLC scintillation counting

This was carried out directly after drying the second phase. Typical readings required 15–30 min and chromatograms were faithfully reproduced. Controls were added to the TLC plate to establish quenching, which is significant below 500 dpm (1:10). This means that at low dpm, quenching blocks 90% of counts detectable, but at high dpm, quenching blocks only 50% of counts.

LKB laser densitometry computer-assisted analysis of the TLC XAR-5 autoradiograms

This was helpful in determining adduct-to-nucleotide ratios, retention factor analyses, area of spot exposure, and spot density (on gray scale) which reflects quantity of each labeled phosphate. The ability to superimpose graphically each film with control spots ultimately eases the analyses of product.

RESULTS

Control calf thymus DNA

Findings for calf thymus DNA digest include the following (Fig. 1a, b; 24-h autoradiogram and Ambis three-dimensional representation).

1. Normal retention times of all radiolabeled monophosphates to cold UV markers. The autoradiogram demonstrates, in clockwise position, the major bases of DNA as their nucleotides (Table I): dAMP [retention factor (R_X) X,Y = 1.8, 9.5 cm), dTMP (R_X = 8.3, 7.0 cm), dGMP (R_X = 3.7, 3.3 cm), and dCMP (R_X = 10.9, 13.5 cm). Literature-reported percentages of dNMP label of calf thymus DNA are: dTMP, 31%; dAMP, 31%; dCMP, 19%; dGMP, 19%; 5-Me-dCMP, 1.2% [19]. Our percentages (Table II), based on scintillation counts, are: dTMP, 44.6%; dAMP, 22.3%; dCMP, 10.6%; dGMP, 12.7%; 5-Me-dAMP, 0.0%, dUMP, 3.8%. These differences in percentages may be due to selective over/under-labeling AT/GC rich areas, but is consistent.

2. One additional major spot (6.0%) exists at 24 h which represents deaminated dAMP to dIMP via contaminating adenosine deaminase in the spleen enzyme preparations. After 72 h, four additional minor spots are more clearly visible: 5-Me-dAMP, two spots close to dCMP representing 5-Me-dCMP and dUMP. Overlabeling of dTMP is consistent and unexplained. Further, some variation in cpm exists due to differences in quenching at lower counts (10:1 at 200 dpm) *versus* higher counts (2:1 over 10 000 dpm). Lastly, densitometry integrates area, and not gray scale density; these numbers appear in Table I.

Cold stoichiometric replacement of dUMP for dTMP

We have been able to routinely detect dUMP on our TLC system. Naturally occurring dUMP coincides with cold dUMP, and incorporated, radiolabeled dUMP (densitometry R_X, 12.6, 5.2; densitometry percentage, 25% at highest millimolar substitution). Fig. 2 demonstrates our ability to easily detect these abnormal cold nucleotides from normal. The concentration of dUTP replacement began at 4 m*M* (0.1 nmol added). dUMP's incorporation in DNA produces one unique spot in each case. The addition of 4 m*M* dUTP, after incorporation as dUMP, significantly competes with dTMP labeling and its undetermined neighbor. Further, the addition of dUMP may enhance our ability to detect methyl-dAMP, methyl-dCMP, and possibly hydroxymethyl-dCMP.

Base methylation

Calf thymus DNA was co-incubated in 3.87 μl of DMS (30.7 μM) and 40 μl of DMS reaction mix for 1 h at 37°C. Incubations to methylate nucleobases in DNA were carried out at standard, 1:10, 1:100, and 1:1000 dilutions. Autoradiograms of standard methylation protocols at 24 and 72 h demonstrate the following (Tables I and II; Fig. 3): 1. complete abolition of dGMP and much of the dIMP near dTMP; 2. significant preservation of dTMP; 3. reduction of dAMP and dCMP; 4. enhancement of methyl-dAMP, and methyl- and hydroxymethyl-dCMP; 5. a new adduct at 16, 2; 6. many polyphosphates along the "O" X axis; 7. many monophosphate fragments along the "20-cm" X axis; 8. many polyphosphates along the "O" Y axis. More gentle methylation preserves methylated dAMP and dCMP. Further confirmation of dIMP as neighbor to dTMP is the observation that both meth-

TABLE I

DENSITOMETRY RESULTS

Methylated dNMPs refer to dAMP', dCMP'1, or dCMP'2 as likely N3-methyl-dAMP, methyl-dCMP, and hydroxymethyl-dCMP, respectively. This is justified by forming N3-methyl-dAMP by chemical methylation, methyl-dCMP by chemical methylation and obtaining cold control, but incomplete evidence is available for hydroxymethyl-dCMP.

Nucleotide	Calf thymus DNA	dUTP	Methylated DNA	Depurinated DNA	pBR322
R_X values					
dAMP	2.7/11.2	2.9/11.6	1.6/9.6	2.3/9.7	1.4/8.6
dAMP'	–	–	1.9/10.3	2.8/10	1.6/9.1
dCMP	12.8/14.9	12.7/15.3	10.6/13	12.2/10.3	10.6/11.9
dCMP'1	–	–	9.5/14.8	–	–
dCMP'2	–	–	10.4/13.6	–	–
dGMP	3.6/5.5	3.6/5.7	–	–	3.7/3.0
dIMP	5.8/8.5	5.8/8.8	7.3/6.5	–	6.5/4.8
dTMP	7.5/10.0	7.4/10.3	8.6/7.3	11.4/10.4	8.3/5.8
dUMP	–	10.5/9.1	–	13.1/4.4	11.5/6.1
Area (mm^2)					
dAMP	644	631	241	420	263
dAMP'	–	–	56	279	31
dCMP	336	271	187	450	384
dCMP'1	–	–	57	–	–
dCMP'2	–	–	69	–	–
dGMP	387	417	–	–	386
dIMP	291	318	178	–	397
dTMP	967	407	1114	2015	636
dUMP	–	875	–	454	49
Percentage volume (A.U. mm^2)					
dAMP	21.35	19.61	9.3	7.8	11.2
dAMP'	–	–	1.1	6.1	0.7
dCMP	11.05	8.48	4.2	7.2	15.3
dCMP'1	–	–	0.2	–	–
dCMP'2	–	–	0.2	–	–
dGMP	12.31	16.17	–	–	21.5
dIMP	6.22	7.44	–	–	13.8
dTMP	47.38	8.66	84.3	41.8	37.3
dUMP	–	39.42	–	5.3	0.1

TABLE II

AMBIS RESULTS

Nucleotides	Calf thymus DNA	dUTP	Methylated DNA	Depurinated DNA	pBR322
dAMP	12863/22.32%	15426/26.50%	2690/12.7%	13130/8.1%	3337/14.80%
dCMP	6121/10.62%	6244/10.73%	3138/14.8%	6059/3.7%	3529/15.65%
dGMP	7338/12.73%	8298/14.25%	2254/10.6%	–	3563/15.80%
dIMP	3444/5.98%	4394/7.55%	3239/15.3%	–	3086/13.68%
dTMP	25686/44.57%	4589/9.77%	7652/36.1%	132869/81.6%	6634/29.42%
dUMP	2178/3.78%	18161/31.20%	2238/10.6%	7033/4.3%	2402/10.65%

ylation and depurination obliterate the dIMP spot. Additional assays of DNA methylation by DMS have demonstrated that standard methylation procedures are overzealous. Three orders of magnitude less DMS than suggested produces significant adduct formation. We are presently methylating at seven orders of magnitude less than published.

Depurination

A depurinating treatment of calf thymus DNA obliterated dGMP and dIMP, alters the retention and diminishes dCMP and 5′-methyl-dCMP, significantly increases (eighteen or greater) apurinic and 2′-deoxyribose phosphates moieties. Another spot about dUMP is also significant (Fig. 4a: R_X: 13.1, 4.4; Ambis: 3.4%; densitometry: 5%). Ambis β-scintillation quantifies the group of depurinated moieties produced and accounts for 17.5% of total counts (Fig. 4b; illustrated by the asterisk). DMS studies may elucidate methylation at N-7 of dGMP, N-1 and N-3 of dAMP, and N-3 of dCMP [20].

pBR322

To both control for artefact present in genomic DNA and validate the technique for shorter DNA fragments, we extended the technique to pBR322 (4.36 kilobases). Results in pBR322 support the findings in calf thymus DNA (Fig. 5): the same retention times for all dNMPs; the miniscule presence of dUMP; some formation or suggestion of other adducts near dAMP and dCMP, probably representing methylated moieties.

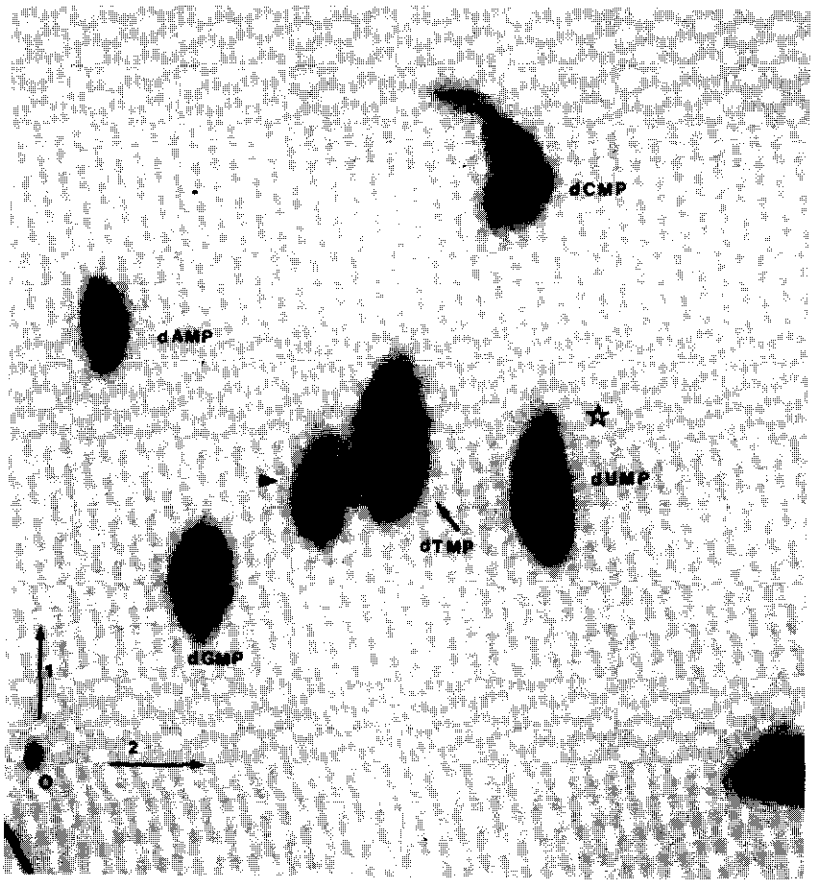
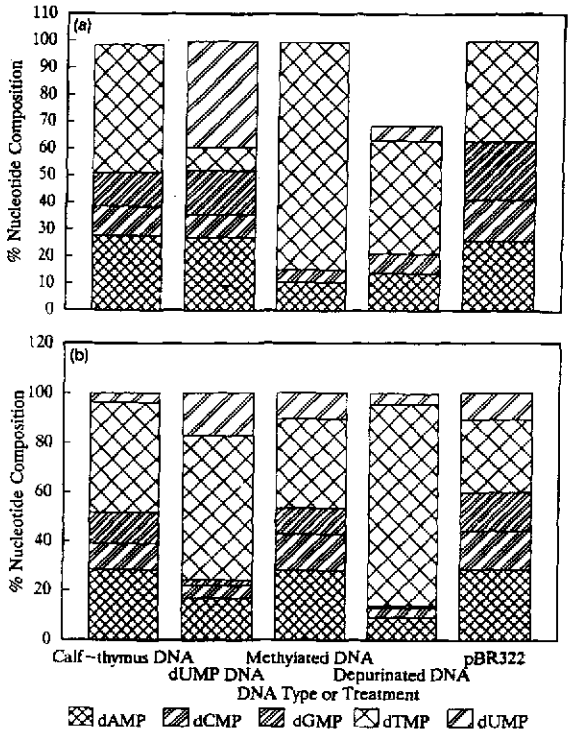


Fig. 2. Cold stoichiometric replacement of dUMP for dTMP. Autoradiogram demonstrates radiolabeled dUMP (densitometry, R_X, 12.6, 5.2; densitometry 25% at highest millimolar substitution). The arrow head represents dIMP, the open star dUMP, and the arrow dTMP.



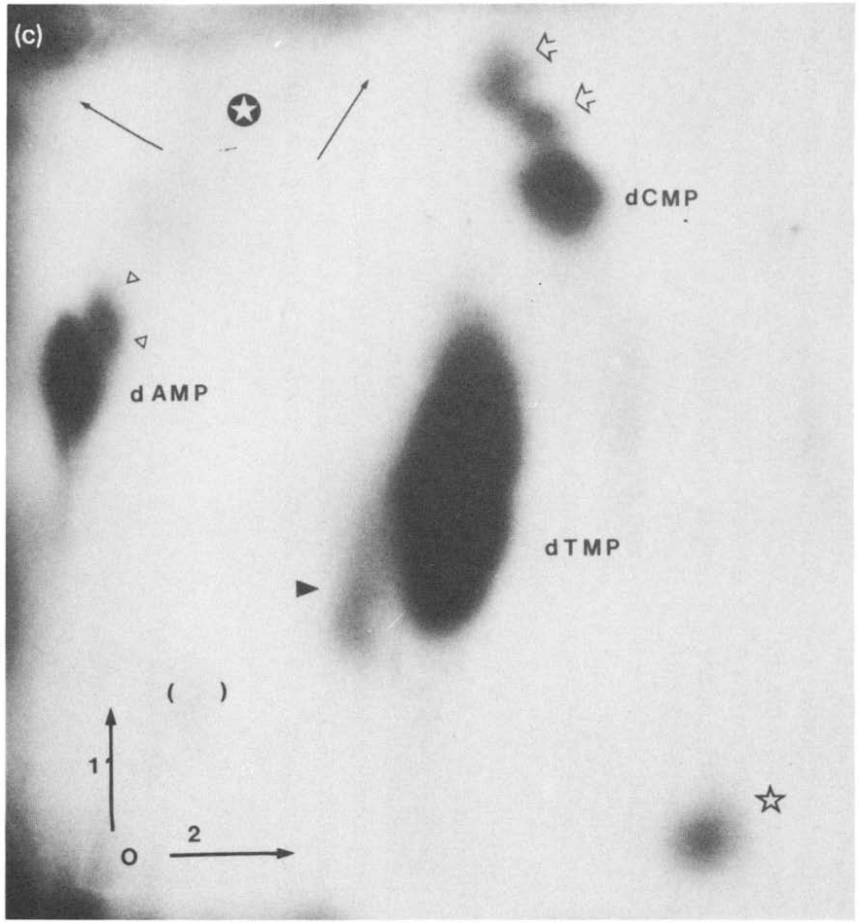


Fig. 3. Histograms of DNA dNMPs and adduction. Densitometry (a) *versus* scintillation counts (b). Ambis correlated closely with densitometry, but exceeded densitometry by 6.5% overall, with an average range of − 2.7% (dGMP) to + 36% (dCMP). Ambis is more successful for less discrete dNMPs than densitometry. Yet, densitometry better dissects away borders between migration patterns of close dNMPs, *e.g.* especially methylated dNMPs. Lastly, the diminished histogram for depurination, as demonstrated by densitometry, is a result of the exclusion of many dozen adducts formed (seen in Fig. 4) and loss of normal nucleotide composition. (c) DNA methylation demonstrating: 1. complete abolition of dGMP (demonstrated by open parentheses) and much of the unknown spot near dTMP (closed arrow, likely dIMP); 2. significant preservation of dTMP; 3. reduction of dAMP and dCMP; 4. enhancement of methyl-dAMP (open arrow heads) and methyl- and hydroxymethyl-dCMP (lower and upper open arrows, respectively); 5. an enhanced adduct at 13.1, 1.1 cm (open star); 6. many polyphosphates along the "O" *X* axis; 7. many monophosphate fragments along the "20-cm" *X* axis; 8. many polyphosphates along the "O" *Y* axis; 9. depurinated moieties are partially shown by slender arrows and the blacked rounded border star.

Sensitivity and reliability

Sensitivity. The technique can ultimately detect one adduct per 10^5–10^8 nucleotides. Scintillation counting can detect as few as 50 dpm over background, from approximately $6.0 \cdot 10^6$ dpm. Further, we have detected less than 1 nmol of a foreign dNMP incorporated into DNA by diminishing dNTP pools during enzymatic incorporation. Lastly the technique requires a small amount of DNA, which can be readily obtained from endoscopic biopsies.

Reliability. Control DNA is run with every batch of experimental TLC. The experience with this technique approaches 800 chromatograms, with alterations of R_F ranging from 1 to 3%. Further, we have compared laser densitometry to Ambis scintillation counting. A linear regression statistical analysis provides a correlation coefficient of $r = 0.93$, $n = 23$ pairs, $p < 0.001$, providing the formula of: Ambis dpm = 62.4 (mm^2 area from densitometry) − 17 410.

In this system, nothing beyond a mononucleotide migrates into the TLC field above the origin. Minute amounts of inorganic phosphate and undigested polynucleotides remain at the origin. Smaller phosphate polynucleotides remain unmoved after the first-dimension acetic acid run, but migration within the field represents normal minor base nucleotides or adducts. Autoradiography not only demonstrates four primary spots, but is also accompanied by a specific "fingerprint".

Our conclusions from these TLC studies are the following. The DNA enzymatic label and digest do not introduce artefacts, with the exception of dIMP which is easily obliterated by deoxycorfomycin. Some adducts are lost by nick translation, yet others which are not available to postlabeling techniques, will be measured. We believe we can determine base modification or damage in DNA and in kilobase size fragments or motifs. The technique is able to measure DNA

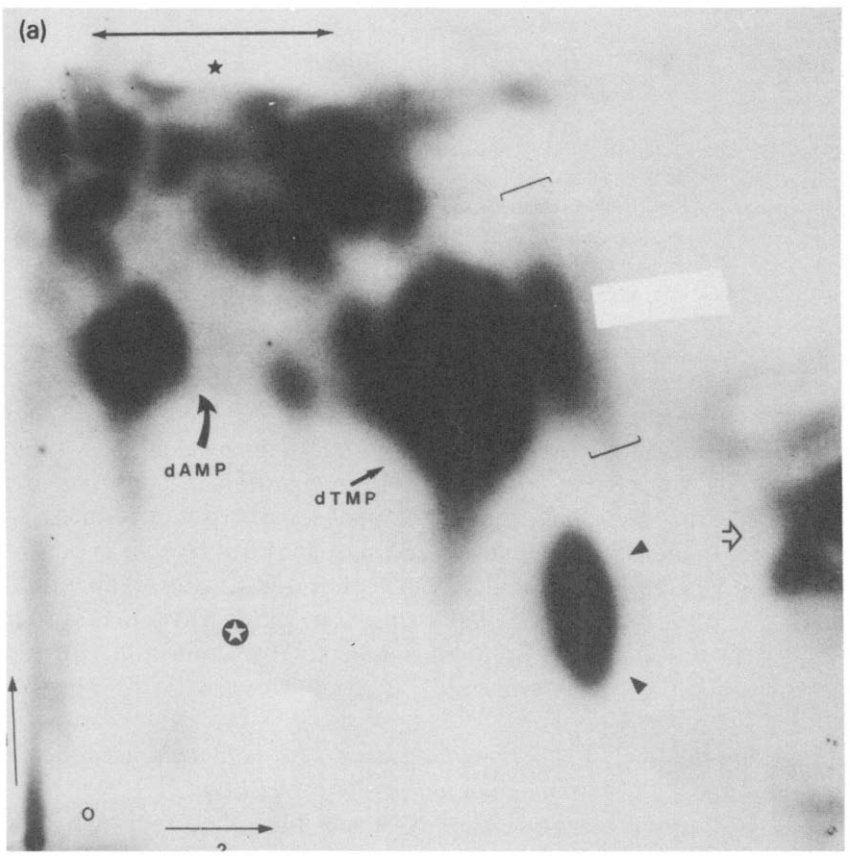


Fig. 4.

(Continued on p. 50).

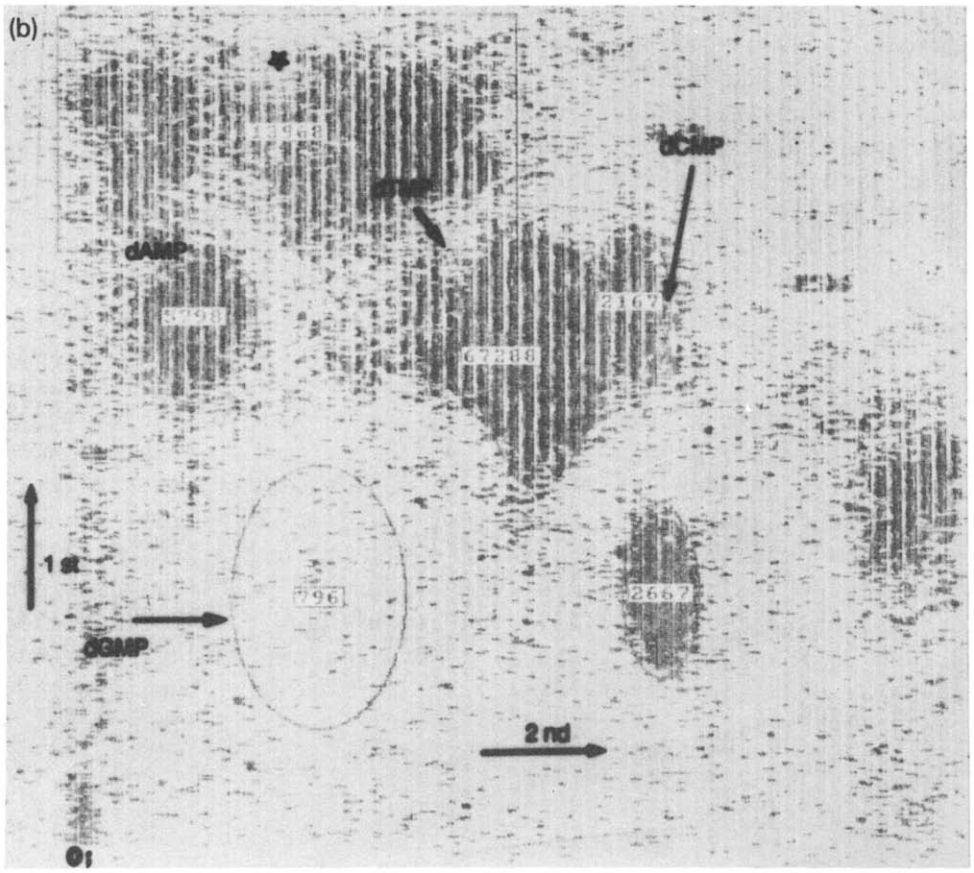


Fig. 4 (a) Depurination of calf thymus DNA. Obliterated dGMP (blacked rounded border star) and dIMP alter the retention and diminishes dCMP and 5-methyl-dCMP, significantly increase (eighteen or greater) apurinic and 2'deoxyribose phosphates moieties (double arrow and black star at front of chromatogram). dUMP is between two broad square parentheses. Another spot (black closed arrow heads) below dUMP's R_X is also significant (R_X: 13.1, 4.4; Ambis, 3.4%; densitometry, 5%). The open arrow head represents unknown adducts. The closed straight arrow is dTMP; the curved closed arrow is dAMP. (b) Ambis β-scintillation. Quantifies the group of depurinated moieties produced (closed black star) and accounts for 17.5% of total counts. Other markers reflect autoradiogram represented in a.

damage from tissue samples. The strength of this technique is based on its ability to ^{32}P label even significantly altered DNA, and DNA to which significant chemical alteration has occurred. It offers a rapid test of DNA integrity prior to more extensive DNA manipulations, *e.g.*, polymerase chain amplification or sequencing.

DISCUSSION

The benefits of this TLC technique are the following.

Improved ability to radiolabel nucleotide adducts versus standard postlabeling techniques

Postlabeling after digestion may prevent the enzymatic labeling of some classes of adducts. T4 polynucleotide kinase may not be able to postlabel all deoxynucleotide adducts due to alteration of the planar ring. Yet, within DNA the transfer of the 5'-phosphate to the 3'-position is easily carried out.

Higher resolution on TLC than high-performance liquid chromatography (HPLC)

One employs a new TLC plate with each TLC experiment. The ability to resolve nucleotides on

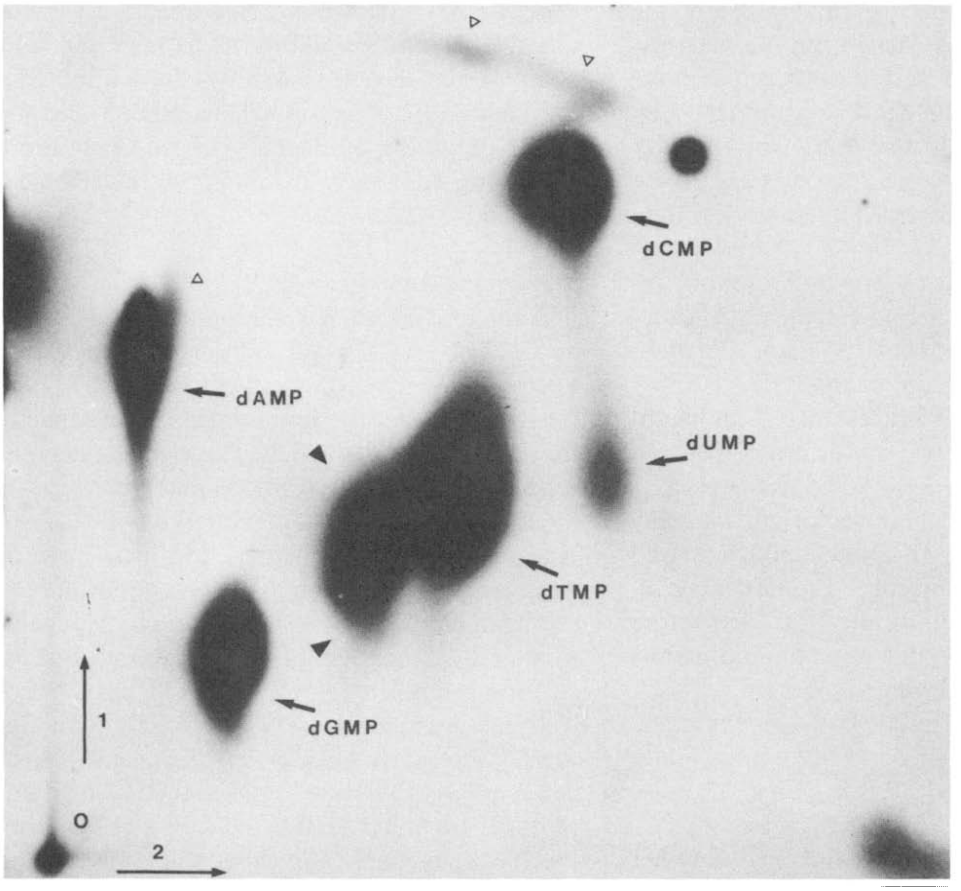


Fig. 5. pBR322. Results in pBR322 support the findings in calf thymus DNA: the same retention times for all dNMPs; the miniscule presence of dUMP. Closed black arrow head represent dIMP; open triangular arrow heads represent methylated species described in text.

HPLC diminishes after each run. Further, greater visible separation is more easily affected with TLC.

Ease of technique

The technique employs "off-the-shelf" items, without long preparations of TLC plates, or multiple-dimension TLC analyses, as most postlabeling techniques suggest.

Quantification of low-molecular-mass adducts

Standard postlabeling technology has been geared to chemotherapeutic or carcinogenic adduct formation, *e.g.* benzo[*a*]pyrene, 7,12-dimethylbenz[*a*]anthracene (DMBA), aflatoxins. Typically, with other techniques, the normal and abnormal low-molecular-mass nucleotides (less than MW 400) are washed off the TLC plate during the first and second dimensions. Heavier nucleotide adducts, which would not be visible through the denser ^{32}P-labeled normal dNMPs, are then visualized after the third and fourth dimensions. The authors' technique enhances low-molecular-mass adduct identification, nucleotides that may differ from the parent molecule by a hydroxyl or alkyl moiety.

We have attempted to validate the technique by directing attention to a number of concerns including: R_F reproducibility, role of solvent and mobile phase, sorbent (paper) phase, quality and quantification of zones, solvent strategy, and estimation of spots.

R_F reproducibility

Strict attention must be adhered to the following [17].

1. Temperature and humidity: is kept constant throughout the year, either in a specialized area of the laboratory or a specially constructed chamber.

2. "Maturing" solvents to temperature of the run: this should be carried out consistently. Further, re-use of solvents alters composition and should be checked empirically by standard R_F (we do not re-use solvent after each run).
3. Checking standards: must maintain less than 5% variability.
5. Equilibrating paper: pre-equilibration increases length of run and is typically not necessary in an adsorbent system. Partition chromatography requires this.
5. Seal development chamber: increment in ambient humidity of solvents will diminish run time.
6. Solvent travels X cm beyond origin: typically run a minimum of 18 cm beyond origin and slightly below front edge. Caution may require ceasing run constantly at 0.5 cm before edge. Initially, we did this, but ultimately found it unnecessary in this system.
7. All manipulations and drying constant.
8. TLC sheet from a single source, stored under same conditions, with batch checks.
9. R_F standard deviation less than 1–3%.
10. Spot geometry typically round or oval. Other shapes imply impurities. Purification of samples must be consistent.
11. Center of zone for R_F (peak) done by computer.
12. Care to measure adducts at margins: this is better carried out by laser densitometry which allows visual dissection of zones.
13. Complete reproducibility from lab to lab is almost impossible. Net results, though, are reproducible.
14. Though R_F are uncorrected (similar to melting points), rather, R_F in literature should be viewed as a starting point.

Role of solvent and mobile phase

The present separation technique has emphasized the differential migration of the dNMPs with selective retention. The solvent effects all moieties equally as a non-selective driving force. Further resolution can be accomplished by selective obliteration of moieties, *e.g.* to emphasize a specific dNMP we would consider competing with an analogue, *e.g.* deaza-dGMP for dGMP adducts, or depurinating to emphasize pyrimidines. This strategy allows us to maintain simplicity in the solvent system. We have adopted a "Maxam-Gilbert" approach to adduct chemistry, maintaining consistency of the separation, yet taking advantage of modifying nucleic acid chemistry.

Stationary phase

Standard paper chromatographic principles apply. We use adsorption principle, not partition. Solutes remain in solvent until they reach fresh sorbent (sheet). Spot remains when capacity for sorbent exceeds solvent. The solute undergoes repeated sorption/desorption, and migration is based on mean occupancy time. Weakly adsorbing moieties migrate farther, and the degree of sorption is large enough to effect separation. The cellulose binds –OH, =O, H_2O, yet the chemistry is ill-defined within the amorphous water–cellulose complex (grain size 2–20 μm). Weak physical interactions in TLC include Van der Waal's forces, dipole–dipole forces, and hydrogen bonding. Cellulose ion-exchange further employs PEI ($-CH_2CH_2NH$; 0.7 mval/g) for more specific separations. Typically, polar solvents are employed for polar solutes, and reversed phase for hydrophobicity. Solvents are based on eluotropic profile, and elution increases with polarity. Speed of solvents also depend on viscosity. Saturated hydrocarbons are poorly adsorbed. Adsorption is highest for –COOH > –OH > $-NH_2$ > C=O > O–alkyl > $-CH_3$ for the lowest.

Troubleshooting quality and quantification of zones

Some initial considerations include the following (17).

1. Diffuse: sharp, small initial spot at origin, fewer theoretical separation plates, so sharper spots.
2. Isotherm (head, trailing, or streak-zone): a. overloaded solute; b. solvent too fast, no equilibrium; c. irreversible change in solute; d. too strong adsorption.
3. double tailing: competition with non-labeled contaminating substances.
4. Projections from the solvent front: impurities.

5. Flattened zones: close to front.
6. Lateral spreading: impregnated with varying different solvents.
7. Swerved, heart, V, arrowhead: distorted solvent flow through initial zone.
8. Multiple: a. gradient (pH or concentration); b. various ionic forms of solute; c. polymerization; d. oxidation; e. *cis-*, *trans-*; f. overloading and ppt with cellulose complex; g. salt complexes; h. heating origin.
9. Typically ammonium produces single spots, salts produce doublets.
10. Spot spreading: by molecular diffusion, or by gradient eddy diffusion through random multiple paths. Concomitantly, non-equilibrium is produced by uneveness of flow *versus* slowness of attainment at front.
11. Estimation of spots: computerized laser densitometry and direct scintillation counting from plates defines geometry, exactly calculates R_F (peak), and quantifies zone.

Chemical markers of DNA damage

The basis for a choice of DNA adducts as a marker of chronic xenobiotic or endogenous oxidative damage is based on the following [10–12].

1. DNA is a molecule significantly at risk for the ill effects of many drug therapies, a number of disease states, toxic environmental exposure, long-term dietary exposures, tetratogenic agents, or sequelae of aging.
2. The formation of adducts is a chemical mechanism and may therefore be stoichiometric with dose.
3. Many of these DNA adducts are stable and therefore measurable.
4. An interindividual balance of DNA repair enzymes exist [7,8] and may or may not adequately protect DNA from the long-term effects of these adducts, therefore, fluctuating adduct content may be measurable (Table III).

The analysis of stable DNA adducts can act as markers for DNA damage, the ability of DNA enzymes to repair and provide long-term sequential information on tissue damage. This information may be important in therapies including cancer chemotherapy, *e.g. cis*-platinum or adriamycin, anti-viral therapies that employ altered nucleosides that may incorporate in DNA, radiation or oxygen stress injuries, aging, and glycation [10–12,21,22].

TABLE III

DAILY BASE DAMAGE EVENTS [22]

	Events per cell per day
Base loss	
Depurination	25 000
Depyrimidination	1300
Base deamination	
Cytosine	350
Base alkylation	
N-7-Me-guanosine	84 000
N-3-Me-adenosine	840
Base dimerization	
Cyclobutane	37 500
Pyr (6-4) pro type	12 500
Base oxidation	
Thymine glycol	400
5-Hydroxymethyluracil	600
Single-strand breaks	100 000

Chemical alterations in DNA have biological consequences. These chemical alterations (adducts or apurinic–apyridimic sites) are the substrates of repair enzymes. Some of the ways the nitrogenous bases in DNA may incur damage is in the form of tautomeric shifts, deamination and alkylation. Tautomeric shifts cause damage by a

TABLE IV

HUMAN DISEASES OF ACCELERATED DNA DAMAGE

Ageing and progria or progeriod syndromes[a]
Alcoholism[b]
Alzheimer's disease (including familial)[a]
Ataxia telangiectasia[a]
Bloom's syndrome[a]
Cockayne syndrome[a]
Diabetes mellitus (glycation)[b]
Down's syndrome[a]
Environmental: atmosphere (pollution, *e.g.*, ozone), chemicals, diet, radiation[b]
Fanconi's anemia[a]
Hutchinson-Guilford progeria[a]
Therapy-nucleoside or DNA target: anti-neoplastic or anti-viral[b]
Werner's syndrome[a]
Xeroderma pigmentosum[a]

[a] Diseases of diminished DNA repair.
[b] Diseases of accelerated DNA damage.

transient rearrangement of bonding in which the base may shift from its usual *keto*-configuration to an *enol*-configuration, with subsequent addition reaction or rearrangement. Deamination occurs in the three bases consisting of exocyclic amino groups (cytosine, adenine, and guanine) and results in the conversion of these bases into uracil, hypoxanthine, and xanthine, respectively. Deamination can result in base mispairing during replication, resulting in transitions (G:C→A:T), abnormalities in protein (histone) binding, and abnormalities in transcription, and hence, translation [23,24] which ultimately effect transcription/translation errors. Alkylation occurs when electrophilic compounds bind to the various bases in DNA. This may result in DNA strand cross-links which may also interfere with transcription fidelity.

Artefactual DNA

The quality of the base composition in genomic DNA is a prime focus of these studies. Though cellular nucleic acid biochemistry is one thrust, another is the quality of analyzeable DNA: is what one extracts actually the similar chemical composition of DNA within the genome? What changes by extraction, buffers, solvents, oxygen, and ions occur prior to or due to the application of modern molecular biology? A necessary benchmark for this would be nucleotide quality and adduct formation as soon as DNA is extracted, which this TLC technique offers, and prior to further analyses. This has application to cloning, restriction enzyme recognition sequences, and polymerase chair reaction amplification of DNA.

DNA damage, adduct formation and clinical application (Table IV)

Chromatin is constantly being damaged by environmental agents such as UV irradiation, ionizing radiation, and chemical compounds found in air, food, and water. Similarly, products of normal cell gene regulation, *e.g.* methylation [23,25], and metabolic processes, such as non-enzymatic glycation and free radicals [26], or xenobiotics causing alkylation [19,27] also inflict DNA damage. If not for DNA repair mechanisms, the extent of damage would seriously impair DNA-regulated activities in a short period of time. Further, DNA damage occurs at a fixed rate throughout an organisms lifespan, with diminished repair capability and altered transcription possible [28,29]. Studies have shown an accumulation of single-stranded breaks in late-passage fibroblasts in culture [30,31], an increased incidence of DNA cross-links [32,33], a greater degree of spontaneous chromosomal aberrations [34,35], and an overall decrease in DNA synthetic capacity [36,37]. Single-stranded breaks were found to increase in an age-related manner in the livers [38], neurons [38,39], astrocytes, Kupffer cells, and heart muscle fibers [40] derived from mammalian tissue. Cross-linkages were also found to occur with increasing incidence with age [41,42], while studies which looked at levels of modified nucleotides found a nine-fold increase in aged mouse myocardium [43] and an equally significant decline in DNA methylation in mouse hepatocytes [44]. Furthermore, the increased frequency of spontaneous chromosomal aberrations occurring in aged cells has long been an accepted finding [45,46] and probably plays a role in the increased incidence of malignancies seen with aging. Base damage has been difficult to substantiate, due to lack of technique, but may play a part in many of these findings.

CONCLUSION

The importance of maintaining normal DNA repair and the consequences of DNA damage cannot be dealt with here, and numerous reviews exist [22]. The possibility of the application of this assay as a marker of damage from the metabolic consequences of disease or chemical injury to tissue samples and biopsy specimens is feasible. This is due to the requirement of only milligram or microgram amounts of DNA and tissue, and quantifying abnormal adduct formation. Ultimately one can correlate deleterious exposure effects on DNA, and measure nucleotide markers of disease processes and organ injury. We may be able to recommend metabolic guidelines to the host which could alter the course of those at high risk of experiencing organ complications and teratogenic risk in many disease states. Further, analyzeable DNA requires a compositional baseline prior to extensive molecular biology.

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