ANALYSIS OF NUCLEIC ACID DERIVATIVES AT THE SUBNANOMOLE LEVEL. (V) HIGH RESOLUTION MAPPING OF TRITIUM LABELLED RNA DERIVATIVES

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1. Introduction

A previous communication in this series described an isotope derivative method for the analysis of ribopolynucleotides [1]. The method involves enzymatic degradation of the polynucleotides to nucleosides, which are converted to ^{3}H-labelled trialcohols by treatment with periodate and tritiated borohydride. The labelled derivatives are separated by thin-layer chromatography and assayed by scintillation counting. The method has thus far been applied only to polynucleotides containing the major bases adenine, guanine, cytosine, and uracil. We report in this communication separation procedures that make possible the application of this scheme to nucleoside mixtures obtained by digestion of nucleic acids or oligonucleotides containing minor constituents. An essential requirement of the method is the visualization of low activities of tritium on chromatograms [2].

2. Materials and methods

(^{3}H)-$NaBH_4$ (Amersham-Searle Corp., 16 curie/mmole) was dissolved in 0.1 N NaOH; unlabelled $NaBH_4$ was added to give a final radioactivity of 2 curie/mmole at 0.1 M total borohydride concentration. The solution was stored at −90°. Tritium was introduced into individual nucleosides as follows. To a solution of 3 nanomoles nucleoside in 30 μl water was added 5 μl of an aqueous solution containing 6 nanomoles $NaIO_4$. The reaction was allowed to proceed for 60–100 min at 22° in the dark. 0.5 μl 0.1 M (^{3}H)-$NaBH_4$ (100 microcurie) was added and the solution was incubated for 60–100 min at 22° in the dark. 25 μl 1 N acetic acid was added to convert excess (^{3}H)-$NaBH_4$ to H_3BO_3 and $^3H_2$ gas. After 25 min in the open air the solution was evaporated completely at 22°. The residue was taken up in 60 μl water. The final radioactivity was about 0.05 microcurie/μl indicating quantitative conversion to ^{3}H-trialcohol.

RNA digests were prepared as described previously [1] but incubation usually was for 24 hr at 37°. For the incorporation of ^{3}H, the digest from 1.5 μg RNA was treated with 10 nanomoles $NaIO_4$ in aqueous solution (total volume 20 μl). After 2 hr at 22° in the dark 1 μl 0.1 M (^{3}H)-$NaBH_4$ (200 microcurie) was added and the reaction was allowed to proceed for 2 hr at 22° in the dark. 25 μl 1 N acetic acid was added. Further treatment was as described for ^{3}H incorporation into individual nucleosides. The residue was dissolved in 10 μl 0.1 N formic acid. Blanks containing no RNA were subjected to the same procedure.

Mapping was carried out on cellulose (Eastman Kodak 6064) and silica gel-kieselguhr (Merck 5523/0025) thin layers. The solvents were: A, *n*-butanol/isopropranol/7.5 N ammonia (3:3:2, by vol.); B, *t*-amyl alcohol/methyl ethyl ketone/water/formic acid, sp. gr. 1.2 (2:2:1:0.1); C, *n*-butanol/isopropanol/7.5 N ammonia (4:3:1); D, *n*-butanol/methyl ethyl ketone/water/formic acid, sp. gr. 1.2 (6:6:1:0.3). Between developments the layers were thoroughly dried. Development on cellulose was with solvent A to 16 cm from the origin (1st dimension) and twice with solvent B to 15 cm from the origin (2nd dimension). Development on silica gel-kieselguhr

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was with solvent C to 16 cm from the origin (1st dimension) and with solvent D to 15 cm from the origin (2nd dimension).

^{3}H-labelled compounds were visualized by solid scintillation fluorography at −78.5° [2], eluted directly in scintillation vials and assayed by liquid scintillation counting [3]. Larger areas were extracted in beakers covered with Parafilm and aliquots of the extracts were assayed.

3. Results and discussion

Figs. 1 and 2 depict fluorograms of maps of ^{3}H-labelled nucleoside derivatives. Although the patterns are similar on cellulose (fig. 1) and silica gel-kieselguhr (fig. 2), there are distinct differences. For example, 1MeG′ * overlaps with 1MeI′ on silica gel-kieselguhr but not on cellulose where it coincides with A′. I′ and G′ are well separated on cellulose but overlap on silica gel-kieselguhr. 4MeC′ and 4DiMeC′ separate on cellulose but not on silica gel-kieselguhr. 2MeG′ travels ahead of 2DiMeG′ in the ammoniacal solvent on silica gel-kieselguhr but in the reverse order on cellulose. Spots are generally less diffuse on cellulose. However, due to the excellent separation of A′, DiHU′, and 1MeG′ silica gel-kieselguhr layers have proven to be superior in our hands for the analysis of digests containing A, DiHU, and 1MeG.

For complete conversion of the dialdehydes to trialcohols, a sufficiently large excess of (^{3}H)-$NaBH_4$ over $NaIO_4$ (>5:1) is required; otherwise partial reduction to ^{3}H-monoaldehydes (cf. [4]) may occur leading to spurious spots on the maps.

* *Abbreviations used:*
A, adenosine; G, guanosine; C, cytidine; U, uridine; I, inosine; 1MeA, 1-methyladenosine; 6MeA, N^6-methyladenosine; 6DiMeA, N^6,N^6-dimethyladenosine; IPA, N^6-(Δ^2-isopentenyl)-adenosine; 1MeG, 1-methylguanosine; 2MeG, N^2-methylguanosine; 2DiMeG, N^2,N^2-dimethylguanosine; 7MeG, 7-methylguanosine; 1,7DiMeG, 1,7-dimethylguanosine; 3MeC, 3-methylcytidine; 5MeC, 5-methylcytidine; 4MeC, N^4-methylcytidine; 4DiMeC, N^4,N^4-dimethylcytidine; 3MeU, 3-methyluridine; rT, thymine riboside; ψ, pseudouridine; 4TU, 4-thiouridine; DiHU, 5,6-dihydrouridine; 1MeI, 1-methylinosine; 7MeI, 7-methylinosine, A′, etc., the trialcohol of adenosine, etc. 7MeI$^+$, 1,7DiMeG$^+$, an unidentified radioactive compound formed when 7MeI or 1,7DiMeG is subjected to $NaIO_4$-(^{3}H)-$NaBH_4$ treatment.

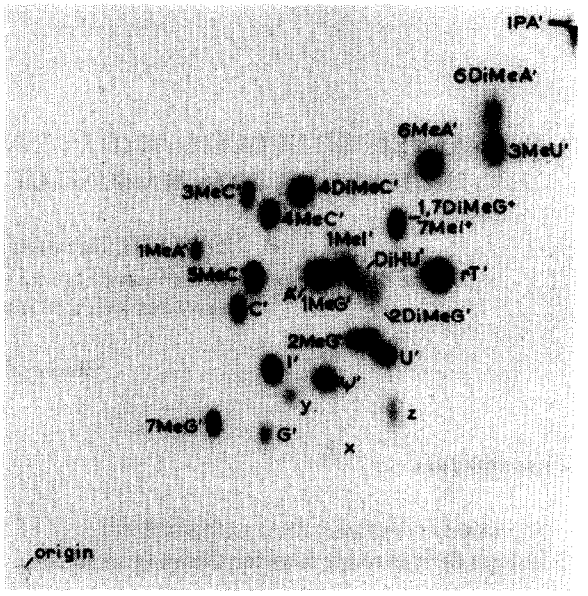

Fig. 1. Cellulose map of ^{3}H-labelled nucleoside derivatives. Total radioactivity applied, about 1 microcurie. First dimension from bottom to top. Second dimension from left to right. Detection by solid scintillation fluorography at −78.5° [2]. Kodak Royal Blue X-ray Film. Exposure for 27 hr. *x, y, z* unidentified compounds probably arising from self-radiolysis. (The solution had been stored frozen for several months prior to chromatography.)

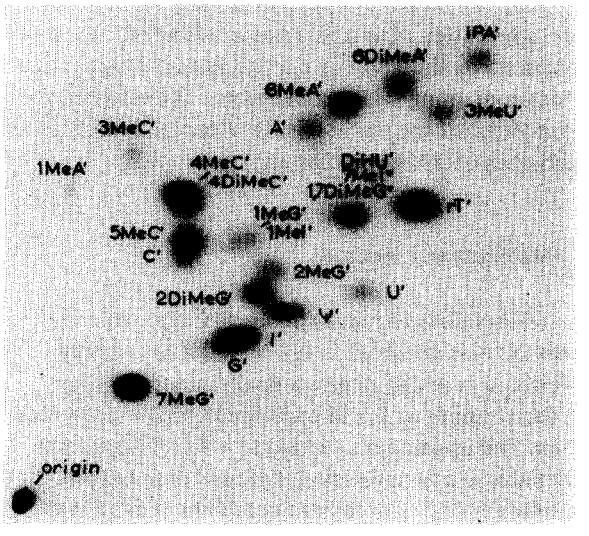

Fig. 2. Silica gel-kieselguhr map of ^{3}H-labelled nucleoside derivatives. Total radioactivity applied, about 1 microcurie. First dimension from bottom to top. Second dimension from left to right. Detection as in fig. 1. Exposure for 34 hr.

The use of the isotope derivative method for quantitative analysis is based on the observation [3] that when a mixture of ribonucleosides is subjected to the labelling procedure its composition (expressed as mole of individual nucleoside/mole of total nucleoside) is accurately reflected by the relative radioactivities of the corresponding derivatives (expressed as count rate of individual derivative/count rate of all derivatives). This is true also for mixtures containing minor nucleosides, and a quantitative analysis of digests of RNA and oligonucleotides containing such compounds can therefore be carried out according to this scheme. So far we have observed only a few exceptions. 4TU is converted to (^{3}H)-U′ and two as yet unidentified radioactive products. 7MeG is converted to 2-amino-4-hydroxy-5-methylformamido-6-ribosylamino-pyrimidine [5,6] during the enzymatic digestion. On subsequent labelling the latter compound is converted to a radioactive product coinciding on both maps with the product obtained by labelling 1,7DiMeG and 7MeI. However, when subjected to $NaIO_4$-(^{3}H)-$NaBH_4$ treatment alone, the main product of 7MeG is the "normal" trialcohol, the location of which is indicated in figs. 1 and 2. 2′-*O*-methylnucleosides, not being susceptible to $NaIO_4$ oxidation, cannot be analyzed by tritium labelling.

The lower limit of the method depends upon the specific activity of the tritiated borohydride. The 2 curie/mmole preparation used by us for labelling RNA digests enables one to assay amounts as low as 0.1 picomole, so that the method under these conditions is over 10 000 times more sensitive than conventional spectrophotometry; it should be particularly valuable, therefore, whenever homogeneous biological labelling of the RNA [7] is difficult or impossible to achieve and only minute quantities of nucleic acid are available.

In addition to being a method for quantitative analysis the conversion of polynucleotides to labelled derivatives followed by high resolution mapping makes possible the detection of trace components. A digest obtained from 5 μg RNA yields a radioactivity of about 10 microcurie when labelled with a 2 curie/mmole preparation of (^{3}H)-$NaBH_4$; because fluorography enables one to detect 6–8 nanocurie ^{3}H/cm^2/day [2] it is possible to visualize on a single chromatogram one individual minor base in a chain of over 3000 nucleotides after an exposure for only 2–3 days. The sensitivity can still be increased about three-fold [8] by chemical intensification of the final image on the film [9]. By carrying out the labelling step with (^{3}H)-$NaBH_4$ of 15–20 curie/mmole specific activity (not diluted with (^{1}H)-$NaBH_4$) and subjecting the film to the intensification procedure [9] one should be in a position to detect one base in a chain of 100 000 nucleotides after an exposure for 5 days. The tritium derivative method appears particularly suited, therefore, for the detection and quantitative analysis of trace components in ribosomal, high molecular weight nuclear, and viral RNA species.

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