Journal of Chromatography, 82 (1973) 59–74

CHROM. 6611

CHEMICAL POST-LABELING METHODS FOR THE BASE COMPOSITION AND SEQUENCE ANALYSIS OF RNA

KURT RANDERATH* and ERIKA RANDERATH

Department of Pharmacology, Baylor College of Medicine, Texas Medical Center, Houston, Texas 77025 (U.S.A.)

SUMMARY

A method for determining the major and minor base composition of ribopolynucleotides by tritium derivatization is presented, which consists of the following steps:

(a) enzymatic digestion of the ribopolynucleotide to a mixture of nucleosides;
(b) oxidation of the digest with periodate;
(c) reduction with tritiated borohydride to labeled nucleoside derivatives;
(d) two-dimensional thin-layer chromatography on cellulose;
(e) detection of tritium-labeled derivatives by fluorography;
(f) liquid scintillation counting.

The method is extremely sensitive: tritium-labeled digest derived from less than 1 μg of polynucleotide is required for evaluation of the base composition. It is particularly well suited for assaying the modified bases in transfer RNA.

Several schemes for sequence analysis by post-labeling of non-radioactive RNA on an ultramicro-analytical scale are discussed.

For sensitive detection of tritium-labeled spots on chromatograms, a fluorographic film procedure has been developed.

INTRODUCTION

The advantages of combining radioactive derivative methods with thin-layer chromatography were first demonstrated in the field of lipid analysis by Mangold and his associates[1]. We shall discuss in this paper the possibilities of utilizing isotope (especially tritium) derivative methods for the base composition and sequence analysis of RNA**.

* American Cancer Society Faculty Research Awardee.

** Abbreviations: RNA = ribonucleic acid; tRNA = transfer ribonucleic acid. Abbreviations used for nucleosides are as recommended in 1970 by the IUPAC–IUB Commission on Biochemical Nomenclature (*Biochim. Biophys. Acta*, 247 (1971) 1, or *J. Mol. Biol.*, 55 (1971) 299). A′, C′, U′, G′, m^1A′, etc. = nucleoside trialcohols of adenosine, cytidine, uridine, guanosine, 1-methyladenosine, etc. PPO = 2,5-diphenyloxazole.

Such methods can be characterized by the following common features:

(1) it is not required that, prior to analysis, the RNA be radioactively labeled *in vivo*, *e.g.*, with radioactive phosphate;

(2) as an initial step, unlabeled RNA is degraded enzymatically, or sometimes chemically, to fragments such as oligonucleotides or nucleosides;

(3) radioactive label is then incorporated into these fragments by chemical or enzymatic methods;

(4) the radioactive derivatives thus formed are separated by chromatography and/or electrophoresis;

(5) the resolved products are finally assayed by counting.

While steps (2) through (5) usually suffice for RNA base composition analysis, sequence analysis may require additional steps involving further digestion, isotope derivatization and/or separation procedures (see below).

Present methods for the chemical characterization of RNA can be divided into two groups: (a) those in which the final analysis is based largely on the optical properties of its constituent nucleotides and (b) those in which the final analysis depends mainly on the radioactivity of labeled RNA constituents. In the case of RNA derived from mammalian sources, the small amounts of pure material available in most instances (less than 1–5 μg per gram of tissue) preclude the utilization of spectrophotometry for analysis, thus calling for analytical methods based on isotope techniques.

Extensive use has been made in several laboratories of procedures originally developed by Sanger and coworkers[2–4], in which uniform biological pre-labeling of RNA is achieved by the *in vivo* incorporation of [^{32}P]orthophosphate. After isolation, the purified radioactive RNA is degraded to nucleoside monophosphates and oligonucleotides, usually by suitable enzymatic methods. Following two-dimensional "fingerprinting" by high-voltage electrophoresis or a combination of electrophoresis and chromatography, the various compounds are isolated and analyzed further by well established procedures.

This approach has been successfully applied in sequence studies on three out of the four RNAs derived from mammalian sources whose primary structure has been elucidated thus far[5–7]. The fourth RNA, a tRNA^{Ser} from rat liver, is the only mammalian RNA whose sequence has been determined[8] without the aid of radioactive labeling, as in this instance sufficient amounts of starting material were available for such studies. In mammalian systems, the biological pre-labeling approach has been applied mainly to *in vitro* situations, *i.e.*, in tissue culture or cell suspensions. RNA of the very high specific activity (of the order of 10^9 c.p.m. ^{32}P/mg RNA), which is required for structural studies, can be obtained from Novikoff hepatoma cells incubated in a relatively concentrated suspension with a phosphate-free medium containing a large amount of [^{32}P]orthophosphate[9]. Unfortunately, this approach is usually not successful *in vivo* because the required high specific radioactivity of RNA precursors is not readily obtained in the "open" system of the intact animal as compared with the "closed" *in vitro* situation. It is, of course, not possible to pre-label nucleic acids in human cells biologically for purposes of structural analysis unless the cells can be grown *in vitro*, which is not feasible in a great number of interesting situations. For cell lines that grow in culture, DNA transcription into RNA sequences may differ from that in the parent cell clone *in vivo*, so that types of RNA as well

as the primary structures of individual RNA species, isolated from cells *in vitro*, are not necessarily the same as those in the parent cell population from which the culture line had been originally derived.

To summarize, presently available methods may be inadequate for analyzing the structure of RNA species in tissues of higher organisms for three main reasons:

(1) the amount of isolable material is frequently too small for spectrophotometric assays;

(2) biological pre-labeling is not feasible in many situations where the required high specific radioactivity cannot be achieved, or where this approach is in principle not applicable;

(3) data on RNA structure derived on the basis of biological labeling of tissue culture cells are not necessarily representative of the *in vivo* situation.

Considering such limitations of the existing methods, the need is thus clear for alternative approaches to the characterization of RNA structure. In the following, we shall discuss novel methods for ribonucleotide and RNA analysis that have recently been developed in our laboratory[10–14]. These methods are characterized by three salient features:

(1) great sensitivity, which is achieved without the requirement of biological labeling;

(2) high resolving power for RNA constituents and derivatives;

(3) feasibility of accurate and precise quantitative analysis.

These features can be achieved by a combination of chemical post-labeling of non-radioactive RNA derivatives obtained by suitable degradation procedures, and high-resolution thin-layer chromatographic or thin-layer electrophoretic techniques. As an initial step, an ultramicro-analytical scheme for the base composition analysis of RNA and RNA derivatives was developed, which is based on the stoichiometric incorporation of tritium label into the nucleosides obtained by enzymatic digestion of a polynucleotide (see Fig. 1)[11–14]. Sensitive base composition analysis is a prerequisite for further sequence studies. In the following, we shall outline the scope of this approach and discuss some applications. For the interested reader, literature references are quoted in which experimental details and further data can be found.

BASE COMPOSITION ANALYSIS BY TRITIUM DERIVATIZATION

Principles

Consider two compounds, A and B, which react quantitatively with a radioactive reagent, R*, to form derivatives:

$$\begin{aligned} A + R^* &\rightarrow A\text{–}R^* \\ B + R^* &\rightarrow B\text{–}R^* \end{aligned} \qquad (1)$$

A mixture of A (m moles) and B (n moles) will then react according to

$$mA + nB + (m+n)R^* \rightarrow mA\text{–}R^* + nB\text{–}R^* \qquad (2)$$

Following separation of A–R* and B–R*, the count-rates of the two derivatives

$$\frac{\text{c.p.m.}_{A\text{–}R^*}}{\text{c.p.m.}_{B\text{–}R^*}} = \frac{m}{n} \qquad (3)$$

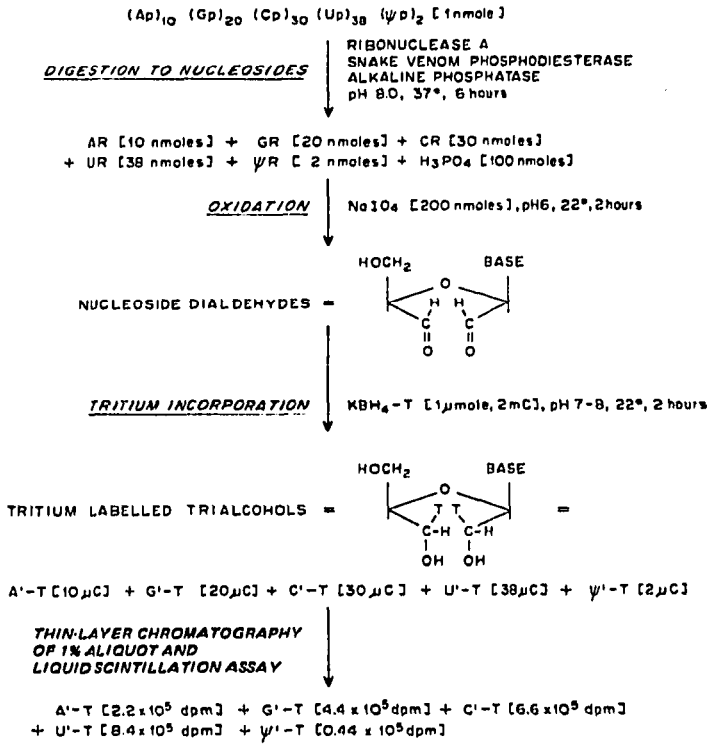

Fig. 1. Base composition analysis of a ribopolynucleotide by enzymatic digestion and stoichiometric tritium incorporation.

permit the calculation of the composition of the original mixture in terms of the ratio m/n. If there are more than two compounds in the mixture, which are all derivatized quantitatively in this way, the result can be expressed in terms of the percentage of each component in the original mixture.

As shown in Fig. 1, this principle can be used for the analysis of the composition of a polymer, such as RNA, or possibly protein, provided that the polymer has been cleaved to the monomer level prior to isotope derivatization. Partition thin-layer chromatography on cellulose was found to be an ideal method for separating tritium-labeled trialcohols[11,13,14] obtained from unlabeled RNA by post-labeling according to this scheme.

Enzymatic digestion[13,14]

For the initial digestion of the RNA to nucleosides, a mixture of three enzymes, ribonuclease A, snake venom phosphodiesterase, and *Escherichia coli* alkaline phosphomonoesterase, is used. Incubation is carried out for 6 h at 37°. The presence of ribonuclease A results in endonucleolytic cleavage at pyrimidine sites within the RNA chain. The obtained nucleoside-3'-phosphates and oligonucleotides are dephosphorylated by the action of phosphomonoesterase to yield pyrimidine nucleosides and dephosphorylated oligonucleotides. Further degradation of oligonucleotides from the dephosphorylated 3'-terminus is catalyzed by the exonuclease, snake venom phospho-

diesterase. The resulting nucleoside-5'-monophosphates are finally converted into nucleosides by enzymatic dephosphorylation.

It was found to be important to carry out the enzymatic digestion under strictly controlled conditions with respect to concentrations, temperature, time, pH and the buffer used. Under the conditions specified[14], complete digestion is obtained with a minimum of side-effects such as hydrolysis of alkali-labile nucleosides (*e.g.*, 7-methylguanosine). A suitable buffer for the reaction is N,N-bis(2-hydroxyethyl)-glycine (Bicine). It is essential to prepare a fresh solution of this buffer immediately before commencing the enzymatic digestion, because storage of solutions of this compound at low temperature leads to the formation of aggregates, which may interfere with the subsequent labeling reaction (see below). The enzymes, on the other hand, can be stored frozen (—18°) for many months without noticeable deterioration. Substitution of other endonucleases, such as ribonuclease T_1, for ribonuclease A may result in incomplete digestion, for example owing to the formation of oligonucleotides terminating in cyclic phosphates, which are resistant to attack by phosphomonoesterase. It appears, however, to be possible to use ribonuclease T_2 at acidic pH[15] for initial degradation to nucleoside monophosphates and then to complete the reaction by adding alkaline phosphomonoesterase after appropriate adjustment of the pH. This approach appears to be possible but its usefulness in combination with the labeling reaction has not yet been evaluated in detail.

Periodate oxidation and borotritide reduction[14]

Periodate oxidation of ribonucleosides and their 5'-phosphates is quantitative at low concentrations of compounds and reagent (Fig. 2). However, nucleotides react more slowly than the parent nucleosides (Fig. 2a), and the reaction rate is also influenced by the base moiety, purine ribonucleosides reacting faster than pyrimidine ribonucleosides (Fig. 2b).

In practice, an aliquot of the enzymatic digest is subjected directly to treatment with a two-fold molar excess of $NaIO_4$ without prior removal of enzymes and buffer, the total nucleoside concentration being 0.05–0.1 m*M*. The reaction is usually carried out for 2 h at room temperature in the dark. It is important not to destroy the excess of periodate at the end of the oxidation reaction by adding glycol or similar compounds, because residual periodate removes labeled impurities present in commercial borotritide preparations (see below).

Before reduction with tritium-labeled potassium borohydride (KB^3H_4), the solution is buffered to pH 7–8 by adding a small amount of phosphate buffer[14] so as to protect alkali-labile compounds. An aliquot of a 0.1 *M* KB^3H_4 solution, corresponding to a 10-fold molar excess of the reagent over nucleoside, is added and the reaction is allowed to proceed for 2 h at room temperature in the dark. After residual KB^3H_4 has been destroyed with an excess of acetic acid (converting KB^3H_4 into tritium gas and borate), the final residue, after evaporation, is dissolved in 0.1 *N* formic acid and the radioactivity of the solution is determined by liquid scintillation counting[13,14]. This solution can be stored at —10 to —20° for several weeks, but after prolonged storage self-radiolysis of some of the labeled RNA derivatives occurs.

The following observations pertain to this reaction sequence:

(1) The solution at the end of the oxidation reaction should contain $NaIO_4$ at a prospective ratio of $NaIO_4/KB^3H_4$ of approximately 1:10 because this residual

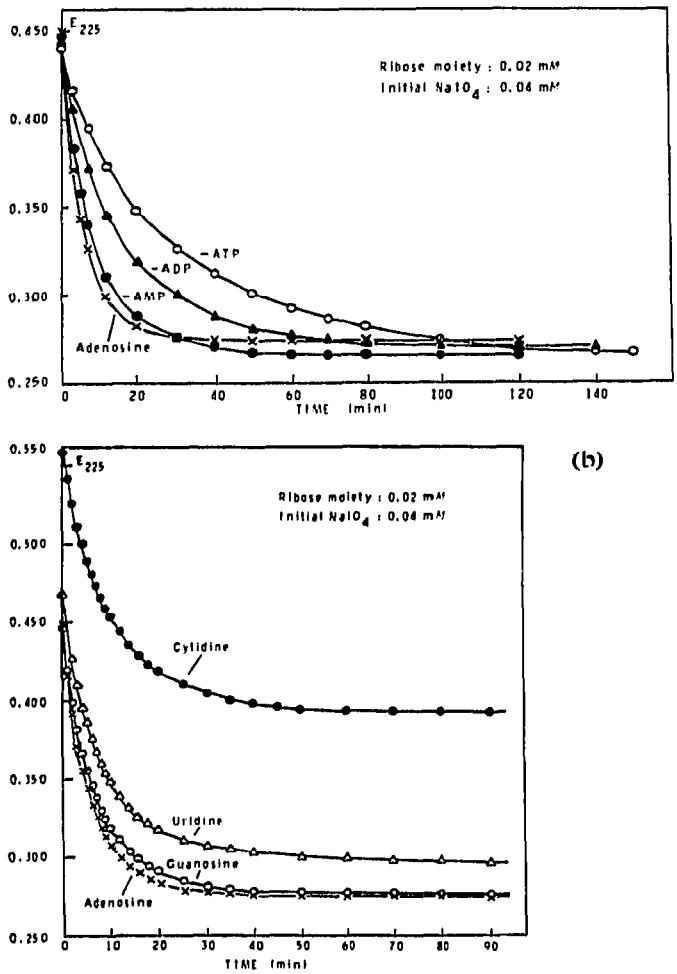

Fig. 2. Change in $E_{225\ nm}$ during reaction of adenosine and adenosine-5'-phosphates (a) and various ribonucleosides (b) with $NaIO_4$. A 1-cm path divided cell containing a volume of 1.25 ml in each side was used. One side contained the nucleoside or nucleotide (0.02 m*M*), the other side $NaIO_4$ (0.04 m*M*). $E_{225\ nm}$ was measured against a water blank. The zero time value represents the sample cell in which nucleoside or nucleotide and $NaIO_4$ were not mixed. After mixing, the extinction was measured at the time intervals indicated.

periodate destroys radioactive impurities frequently present in commercial KB^3H_4 preparations. Otherwise such impurities may overlap certain nucleoside derivatives on two-dimensional thin-layer chromatograms.

(2) An excess of periodate is also essential for quantitative oxidation of the ribose derivatives. Because of the different reaction rates, incomplete oxidation will lead to incorrect quantitative results. For the same reason, the RNA or the enzymatic digest should be free of contaminants that are susceptible to periodate oxidation. Periodate and borotritide consumption by the enzymes and buffers used is negligible.

(3) Commercial NaB^3H_4 was frequently found to contain large amounts of

radioactive impurities, which interfere with the subsequent assays. Small amounts of impurities present in commercial KB^3H_4 of high specific activity (Amersham-Searle TRK-293) are usually readily removed by reaction with residual periodate, see point (1).

(4) The choice of suitable buffers is a crucial part of the analytical procedure. Buffers may cause several types of artifacts, such as reaction with the reagents used, phosphate and base elimination (of particular importance in terminal labeling of oligonucleotides, see below), or inhibition of the reduction of nucleoside dialdehydes. Too large amounts of salts or buffers may also interfere with subsequent chromatographic separation. Certain buffers were found to inhibit the complete reduction of nucleoside dialdehydes to tritiated trialcohols, thus leading to the formation of tritiated nucleoside monoaldehydes[13,14]. The reduction of dialdehydes of adenosine, guanosine and pseudouridine was found to be more easily inhibited in this way than that of other nucleoside dialdehydes. At the concentration used[14], Bicine does not lead to the formation of monoaldehydes if a fresh solution is used each time for the digestion of the polynucleotide. As mentioned earlier, Bicine solutions that contain aggregates owing to prolonged storage tend to inhibit the nucleoside dialdehyde reduction. As this applies also to enzymatic digests containing this buffer, the labeling reaction has to be carried out soon after digestion. The replacement of Bicine or phosphate with other buffers, for example, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer of the same concentration and pH, favors the formation of nucleoside monoaldehydes. Tris(hydroxymethyl)aminomethane (Tris) buffer should also be absent from the reaction mixtures, including the original polynucleotide solution. (This and other buffers can be easily removed by precipitating the RNA with ethanol and washing the precipitate with 80% or absolute ethanol.)

Partition thin-layer chromatography of labeled compounds[12–14,16]

Separations are carried out on cellulose thin layers without fluorescent indicator (Eastman "Chromagram" sheets). For two-dimensional mapping, the tritium-labeled enzymatic digest (usually 1–5 μCi) is applied to the layer 2.5 cm from both the left-hand and the bottom edges in 1-μl portions with intermediate drying in a stream of cool air. No precautions are taken to ensure a defined moisture content of the layer as we have observed no influence of this factor on the overall spot pattern resulting from partition chromatography of nucleosides and nucleoside derivatives. However, the degree of tank saturation with solvent vapor was found to influence the separations considerably. Ascending development is started no later than 2–3 min after the freshly prepared solvent has been poured into a standard thin-layer chromatography tank (inside dimensions 27 × 25 × 7.5 cm). During chromatography, the tank is covered with a plane glass plate whose edges are not ground or sealed with grease. The walls of the tank are not lined with filter-paper. The temperature during the run should be 22–25°. The solvent is used only once and only one chromatogram is run per tank.

Solvents for the resolution of complex mixtures of nucleoside trialcohols and labeled RNA digests are: (A) acetonitrile–ethyl acetate–*n*-butanol–isopropanol–6 *N* aqueous ammonia (7:2:1:1:2.7) and (B) *tert.*-amyl alcohol–methyl ethyl ketone–acetonitrile–ethyl acetate–water–formic acid, sp.gr. 1.2 (4:2:1.5:2:1.5:0.18). Development in the first dimension is carried out with solvent A to 17 cm from the origin.

After drying (see below), the chromatogram is developed in the second dimension with solvent B to 5 cm on a Whatman No. 1 wick attached to the top (the original right-hand side) of the sheet with metal staples. After termination of the development in the first dimension, the layers are thoroughly dried, first in a stream of cool air and then in a stream of warm air (50–60°) for 2–3 min each. The sheets are trimmed about 1 cm below the front of the first dimension and 1–1.5 cm above the bottom edge. After development in the second dimension, the sheets are thoroughly dried as above and the paper wicks are removed. Because many nucleoside trialcohols are labile in concentrated acids[17], it is important to remove most of the formic acid in cool air before drying in warm air.

Nucleoside trialcohols can also be resolved by partition chromatography on silica gel layers (see Fig. 7 below) or silica gel/Kieselguhr layers[13,16]. Additional solvents for the resolution of these compounds on cellulose thin layers have been reported in the literature[10–14].

^{3}H-labeled compounds are rendered visible on such chromatograms by fluorography following impregnation of the layer with scintillator (PPO)[18,19] (see also Appendix). Fig. 3 depicts such a fluorogram of a model map of ^{3}H-labeled nucleoside trialcohols. The excellent resolution is due mainly to specific effects that result from the presence of acetonitrile in the solvents. One-dimensional chromatography on cellulose with acetonitrile–water or acetonitrile–aqueous ammonia solvents (Table I) permits the separation of several trialcohols (*e.g.*, A′, m^{1}G′, hU′, and m$_2^2$G′), which could not be readily resolved otherwise[14]. This acetonitrile effect may be due to a disruption of hydrogen bonds, which are responsible for the high degree of association of classical partition chromatography solvents, such as alcohol–acid (or base)–water mixtures. Simple aqueous acetonitrile systems, however, do not afford sufficient resolution of the major nucleoside trialcohols (U′, A′, C′, G′), or of certain modified derivatives (Table I). In order to obtain complete separation of complex nucleoside trialcohol mixtures on one chromatogram, alcohols and some

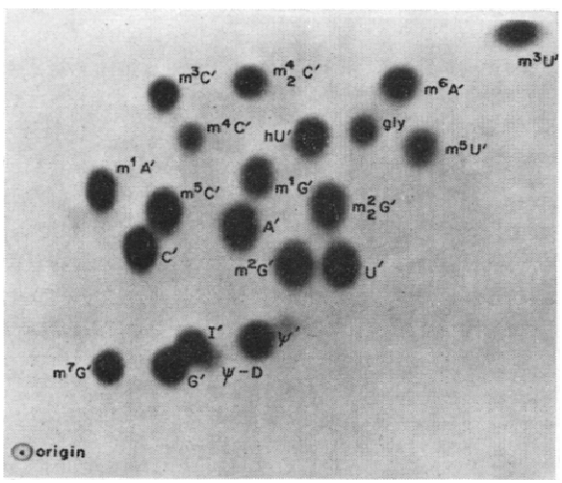

Fig. 3. Two-dimensional separation of model mixture of nucleoside trialcohols (2 μCi) on cellulose. Development with solvents A and B (see text). First dimension from bottom to top; second dimension from left to right. Detection by fluorography at −80°C (see text). Exposure of film for 3 days.

TABLE I

R_F VALUES OF NUCLEOSIDE TRIALCOHOLS ON CELLULOSE THIN LAYERS IN TWO SOLVENTS

Solvent 1 = acetonitrile–water (8:2.25); solvent 2 = acetonitrile–2.5 *N* ammonia solution (4:1).

Nucleoside trialcohol	R_F	
	Solvent 1	*Solvent 2*
U′	0.56	0.33
A′	0.37	0.30
C′	0.34	0.29
G′	0.32	0.16
m^3U'	0.73	0.66
m^5U'	0.63	0.47
hU′	0.55	0.46
ψ′	0.38	0.20
m^1A'	0.05	0.34
m^6A'	0.60	0.52
m_2^6A'	0.71	—
i^6A'	0.76*	—
m^3C'	0.13	0.51
m^4C'	0.51	0.45
m_2^4C'	0.62	0.56
m^5C'	0.38	0.33
m^1G'	0.48	0.39
m^2G'	0.47	0.30
m_2^2G'	0.58	0.39
m^7G'	0.04	0.14
I′	0.37	0.20
m^1I'	0.58	0.48
m^7I'	0.07	—
Glycerol	0.56	0.46

* Frequently elongated; R_F not well reproducible.

other ingredients have to be present. The effects of the various components were found to be largely additive. The two-dimensional combination of solvents A and B was derived by taking advantage of such additive solvent effects. The R_F values of these compounds are also strongly dependent on their respective net charge, as discussed elsewhere[14].

Assay of tritiated trialcohols by liquid scintillation counting[13,14]

Following fluorography[13,19], the compounds are cut from the cellulose sheets, transferred to flat-bottomed beakers and extracted (layer side down), usually with 1–2 ml of 2 *N* aqueous ammonia, for one to several hours at room temperature. After transferring 500-μl aliquots of the eluates to polyethylene liquid scintillation vials, assay of the labeled trialcohols is carried out as described elsewhere[13,14].

Because, as shown previously[14], for the four major and most modified constituents of RNA the base composition f_i (expressed as nanomoles of an individual nucleoside divided by nanomoles of total nucleoside in the RNA) is reflected by the relative radioactivities of the corresponding labeled derivatives (expressed as the count-rate of each individual derivative divided by count-rate of all derivatives),

the base composition is calculated according to the equation

$$f_i = \frac{\text{c.p.m.}_i}{\sum_{i=1}^{N} \text{c.p.m.}_i}$$

where N is the number of radioactive nucleoside derivatives separated and c.p.m.$_i$ is the count-rate of an individual compound.

The base composition can be expressed as a percentage of the total according to the equation

$$f_i = \frac{\text{c.p.m.}_i}{\sum_{i=1}^{N} \text{c.p.m.}_i} \times 100\,(\%)$$

The base composition of RNAs of known chain-length can be calculated according to the equation

$$f_i = \frac{\text{c.p.m.}_i}{\sum_{i=1}^{N} \text{c.p.m.}_i} \times \text{chain-length (mole)}$$

Applications

The novel tritium derivative method for base composition analysis has been used recently for analysis of various RNAs, *e.g.*, tRNA from mammalian brain, brain tumors[20], liver, liver tumors (Morris hepatomas)[21] and other normal and neoplastic mammalian tissues[22], tRNA from avian liver, avian leukemic cells and an RNA tumor virus[23], tRNA and rRNA from *Mycoplasma hominis*[24], various nuclear and nucleolar RNAs from Novikoff hepatoma cells[25], as well as for 3'-terminal labeling of high-molecular-weight RNA from RNA tumor viruses[26]. The method has been applied also in investigations relating to evolutionary aspects of tRNA base composition[27]. Modified and major nucleosides in purified *E. coli* tRNAs have been assayed by this method[14].

Fig. 4 represents a fluorogram of a thin-layer chromatogram obtained from a ^{3}H-labeled digest of human brain tumor tRNA (*cf.* also refs. 20 and 28). Comparative investigations in our laboratory have shown the base composition of tRNA in tumor cells to be similar to but not identical with that of normal control cell tRNA, and the base constituency of tRNA of individual tumors, such as different Morris hepatomas, appears to be characteristic for each tumor[21].

In Table II, data are represented pertaining to the base composition of valine-specific tRNA from *E. coli* K12 (ref. 14). There is close agreement between these results and base composition values expected from the known sequence of this tRNA[29]. The low value for N^6-methyladenosine (Table II), which is not due to the low recovery of the labeled derivative of this nucleoside, has been discussed elsewhere[14]. The data in Table II demonstrate that the tritium derivative method is a tool for measuring the purity of tRNA preparations[14].

In combination with sequential analysis of polyribonucleotides, this method appears to be indispensable as a final step required to establish the sequence (*cf.* the

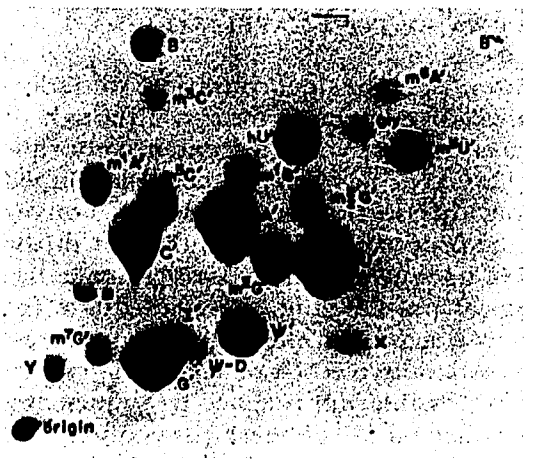

Fig. 4. Two-dimensional separation of ^{3}H-labeled digest of unfractionated human brain tumor (glioblastoma multiforme) tRNA. About 4 μCi of labeled digest was applied to a cellulose thin layer. Development was with solvents A and B (see text). First dimension from bottom to top; second dimension from left to right. Detection by fluorography at −80°C (see text). Exposure of film for 4 days. For abbreviations of nucleoside trialcohols, see text. B = background, not derived from RNA; X = unidentified compound; gly = [^{14}C]glycerol; ψ-D = decomposition product derived from pseudo-uridine; y = [^{3}H]trialcohol of N-[9-(β-D-ribofuranosyl)purin-6-yl carbamoyl] threonine.

TABLE II

BASE COMPOSITION OF *E. coli* K12 $tRNA_1^{Val}$ AS DETERMINED BY CHEMICAL TRITIUM INCORPORATION

Nucleoside	*Means of 4 determinations*			*Expected*[29]
	In 100	*In 76*	S_{rel}* (%)	*In 76*
U	13.00	9.88	0.26	9 + 1**
A	18.22	13.84	0.44	14
C	31.26	23.76	0.31	23
G	29.66	22.54	0.35	23
m^6A	0.97	0.74	1.91	1
m^5U	1.37	1.04	3.01	1
hU	1.53	1.16	1.46	1
ψ	1.43	1.09	0.87	1
m^7G***	1.34	1.02	4.10	1
V§	1.20	0.91	1.25	1

* Relative standard deviation, S_{rel} = S.D./mean × 100(%).

** 4-Thiouridine (1 mole of which is present per mole of this RNA) is recovered mainly as [^{3}H]U'.

*** The radioactivity of [^{3}H]m^7G' was corrected for 65% recovery[14].

§ Uridine-5-oxyacetic acid.

following section). Its sensitivity, requiring, in a scaled-down version[27], only 1–5 μg of polynucleotide for analysis, makes it a promising tool for use in future studies on the base constituency of natural ribopolynucleotides, including RNAs of possibly altered structure occurring in tumor cells[27].

SEQUENCE ANALYSIS OF NON-RADIOACTIVE RNA BY POST-LABELING

Terminal post-labeling of oligonucleotides, *i.e.*, the incorporation of radioactive label into the 3′- or 5′-termini by chemical or enzymatic means, although of great potential value in sequence studies[30,31], has not been utilized extensively thus far (*cf.* a recent review[27]). As methodology in this area has not yet been developed to a stage which permits its general application, this section is intended to convey general principles rather than to describe specific procedures. As pointed out in the introduction, presently available methods for RNA sequence studies, which make use of ultraviolet spectrophotometry and/or biological pre-labeling, are inadequate for many RNA species in higher organisms. There is a need for alternative procedures, which are based on isotope post-labeling of non-radioactive RNA or RNA fragments.

Figs. 5 and 6 illustrate schematically two possible approaches to sequencing RNA by end-group labeling[27]. A more detailed discussion of these procedures will be presented elsewhere[27]. In Figs. 5 and 6, radioactive groups are indicated by aster-

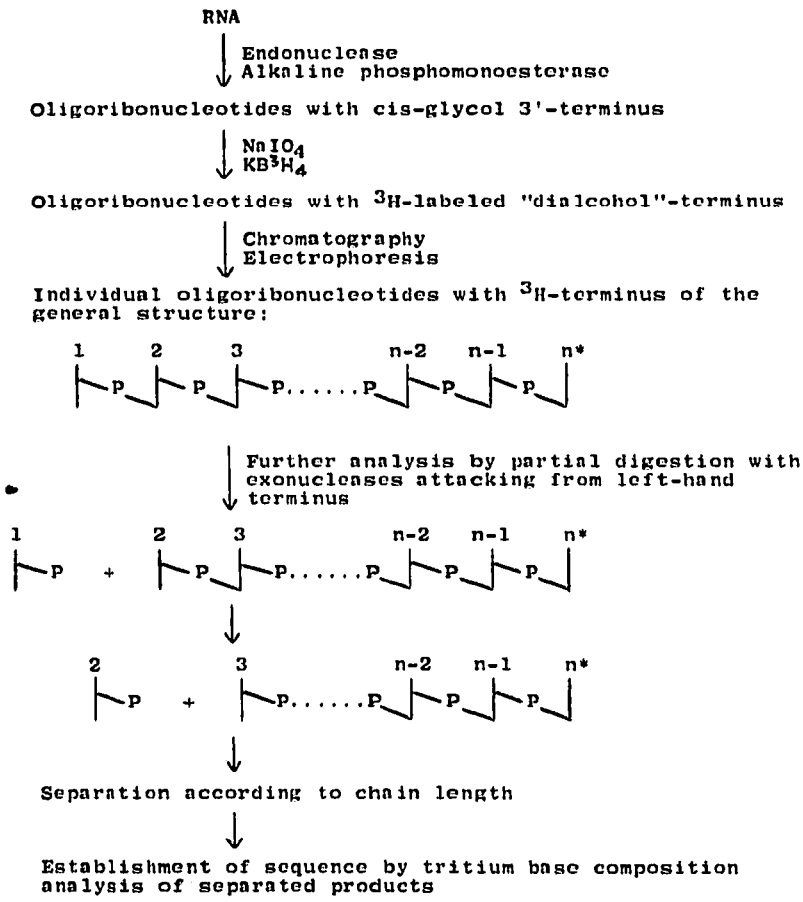

Fig. 5. A scheme for sequential analysis of RNA by chemical tritium post-labeling. n* represents the ^{3}H-labeled 3′-terminus. The bases are numbered 1 to n from the left-hand (5′-) to the right-hand (3′-) terminus.

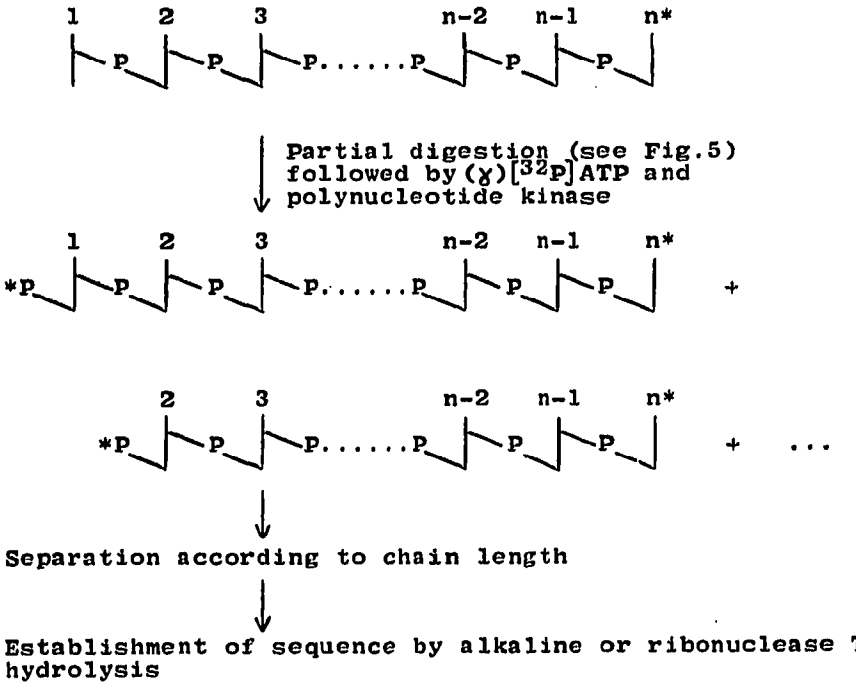

Fig. 6. A scheme for sequential analysis of RNA by a combination of chemical tritium post-labeling and enzymatic ^{32}P post-labeling. n* represents the ^{3}H-labeled 3′-terminus and *P the ^{32}P-labeled 5′-terminus.

isks. The procedures illustrated, as well as related procedures[27], incorporate modifications and refinements of existing methods, for example, enzymatic digestion and separation procedures are similar to those used for sequencing of RNA following biological labeling[2–4].

Both procedures (Figs. 5 and 6) comprise an initial degradation of the RNA to oligonucleotides carrying a free *cis*-glycol 3′-terminus. As a second step, this mixture of oligonucleotides is subjected to terminal labeling with $NaIO_4$–KB^3H_4 followed by a suitable separation procedure. The products thus obtained can then be further analyzed by controlled digestion with exonucleases attacking from the left-hand (5′-) terminus, such as purified spleen phosphodiesterase (spleen exonuclease)[32], to mixtures of oligonucleotides of decreasing chain-length, all carrying the same labeled right-hand (3′-) terminus. These oligonucleotides are subsequently resolved and the sequence of each original 3′-terminal labeled oligonucleotide is established by tritium base composition analysis (Fig. 5). Provided that all partial digestion products have been recovered following their separation, the sequence of the parent oligonucleotide can be deduced by tritium base composition analysis (Fig. 5).

A few comments concerning this procedure appear appropriate. The initial digestion of the RNA can be accomplished by standard enzymatic techniques[4,27], freshly dissolved Bicine being the buffer of choice for the reasons stated above. Subsequent labeling of the 3′-terminus is usually quantitative at 2–4 m*M* $NaIO_4$ (pH 6) and 10–30 m*M* KB^3H_4 (pH 7.2–8.0) if incubations are carried out for 2–3 h each at

room temperature in the dark[27]. Resolution of such a labeled digest can then be carried out by standard techniques[2-4,33], and the compounds are rendered visible by fluorographic film detection[18,19] (see below). Controlled partial digestion, resolution according to chain-length and film detection will usually present no problems. The last step of this analytical scheme appears to be more difficult as it requires $NaIO_4$–KB^3H_4 treatment of extremely small amounts of oligonucleotides after isolation from the chromatographic or electrophoretic medium. A major problem is the co-extraction of periodate-susceptible background material from the usually cellulose-based separation medium[27]. It appears to be possible to eliminate such technical difficulties by suitable purification of the separation medium or by development of $NaIO_4$–KB^3H_4-resistant materials that are suitable for chromatography and/or electrophoresis of polyanions such as oligonucleotides. These problems are currently being investigated in our laboratory.

As shown in Fig. 6, the introduction of a second 5'-terminal isotope into the individual 3'-terminal labeled oligonucleotides of the initial RNA digest (Fig. 5) would enable one to circumvent the final tritium base composition analysis for establishing the oligonucleotide sequence. In this procedure, the 3'-terminal labeled oligonucleotide mixture, obtained by partial exonuclease digestion as indicated in Fig. 5, is 5'-terminal labeled by treatment with [γ-^{32}P]ATP in the presence of the enzyme polynucleotide kinase[34,35]. After separation of the double-labeled partial digest, the sequence of the individual oligonucleotides can then be determined by alkaline or ribonuclease T_2 hydrolysis, as these treatments will release the 5'-termini as ^{32}P-labeled nucleoside-3',5'-diphosphates.

The principles of a novel post-labeling method for sequence analysis of polyribonucleotides, in which derivatives from internal as well as terminal positions are radioactively labeled, will be published shortly[36].

In summary, the procedures outlined in Figs. 5 and 6, as well as others[27], will provide means of sequencing non-radioactive RNA or RNA fragments by post-labeling with similar precision and sensitivity as is now possible by methods that take advantage of biological pre-labeling. Such post-labeling methods may represent the only feasible approach to elucidate the structural characteristics of many RNA species from higher organisms, including man. As alterations of RNA structure are suspected to exist in human disease, particularly cancer, further development and application of these methods may be significant steps towards an understanding of pathological processes at the molecular level.

APPENDIX: FILM DETECTION OF TRITIUM-LABELED SPOTS ON CHROMATOGRAMS AND ELECTROPHEROGRAMS

For locating accurately tritium-labeled compounds on sheet chromatograms and electropherograms, a sensitive fluorographic film detection procedure was developed in our laboratory[18,19]. The method enables a spot containing 1–4 nCi of ^{3}H to be rendered visible in 24 h, the sensitivity depending mainly on the spot size and, to a lesser extent, on other factors discussed elsewhere[19]. Three factors were found to contribute most to the sensitivity[19]: (1) incorporation of an optimum amount of scintillator into the separation medium, (2) film exposure at low temperature (−70 to −90°C), and (3) choice of sensitive film material.

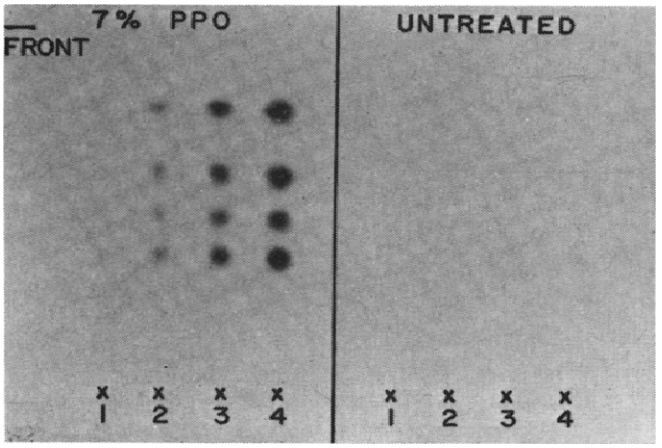

Fig. 7. Detection of ^{3}H-labeled nucleoside trialcohols by fluorography on Silica Gel F_{254} layer. The following volumes of a mixture of ^{3}H-labeled nucleoside trialcohols, containing 1.8 nCi of A′, 2.1 nCi of C′, 2.1 nCi of U′ and 1.4 nCi of G′ per microlitre, were applied to the origins on either half of the chromatogram: 0.5 μl each to the left-hand and right-hand origin 1, 2 μl each to 2, 5 μl each to 3 and 10 μl each to 4. Development was as described in the text for cellulose layers; the solvent (*tert.*-amyl alcohol–methyl ethyl ketone–water, 3:3:1) was allowed to ascend to 12 cm above the origin. Only the left-hand side of the chromatogram was treated with a 7% solution of PPO in ether. The entire chromatogram was covered with Kodak Royal Blue X-ray film. Exposure was for 24 h at −80°C. The spots visible on the PPO-treated half are, from bottom to top, C′, G′, A′ and U′. Note: no compounds are rendered visible on that part of the film which was in contact with the untreated half of the chromatogram.

Fig. 7 depicts a fluorogram of a separation of nucleoside trialcohols on a Silica Gel F_{254} layer. As shown in Fig. 7, treatment of a chromatogram with 7% (w/v) PPO in ether [18,19] makes possible the detection of as little as 1 nCi of a tritium-labeled compound after exposure for 1 day, whereas without such treatment the limit of detection is 30–150 nCi per spot, depending on the film[19]. In contrast to organic scintillators such as PPO, the green fluorescent indicator present in the Merck F_{254} type layers, an Mn-activated zinc silicate, was found not to influence the sensitivity of the film detection method (*cf.* Fig. 7). Compared with standard autoradiography of tritium-labeled compounds, fluorography is approximately 100 times more sensitive[19]. As Kodak Royal Blue X-ray film, originally recommended for the fluorographic procedure[18,19], is no longer available, we now use Kodak RP/R54 Royal X-Omat X-ray film, which appears to be slightly less sensitive than the former. Details of the technique are described elsewhere[18,19]. This general method appears to be applicable to virtually all chromatographic and electrophoretic media that are currently in use, and its applicability includes non-polar as well as polar compounds.

ACKNOWLEDGEMENTS

Work in the authors' laboratory described in this paper was supported by American Cancer Society Grants P516 and NP-37, U.S. Public Health Service Grant CA-13591, U.S. Public Health Service Cancer Research Center Grant CA-10893, U. S. Public Health Service Research Career Development Award CA-42570 and American Cancer Society Faculty Research Award PRA-108.

REFERENCES

1 H. K. Mangold, in E. Stahl (Editor), *Dünnschicht-Chromatographie*, Springer Verlag, Heidelberg, 1962, p. 62.
2 F. Sanger, G. G. Brownlee and B. G. Barrell, *J. Mol. Biol.*, 13 (1965) 373.
3 F. Sanger and G. G. Brownlee, *Methods Enzymol.*, 12A (1967) 361.
4 B. G. Barrell, *Procedures Nucleic Acid Res.*, 2 (1971) 751.
5 B. G. Forget and S. M. Weissman, *J. Biol. Chem.*, 244 (1969) 3148.
6 K. Ohe and S. M. Weissman, *J. Biol. Chem.*, 246 (1971) 6991.
7 T.-S. Ro-Choi, R. Reddy, D. Henning, T. Takano, C. W. Taylor and H. Busch, *J. Biol. Chem.*, 247 (1972) 3205.
8 T. Ginsberg, H. Rogg and M. Staehelin, *Eur. J. Biochem.*, 21 (1971) 249.
9 C. M. Mauritzen, Y. C. Choi and H. Busch, *Methods Cancer Res.*, 6 (1971) 253.
10 K. Randerath and E. Randerath, *Experientia*, 24 (1968) 1192.
11 E. Randerath, J. W. ten Brocke and K. Randerath, *FEBS Lett.*, 2 (1968) 10.
12 K. Randerath and E. Randerath, *Anal. Biochem.*, 28 (1969) 110.
13 K. Randerath and E. Randerath, *Procedures Nucleic Acid Res.*, 2 (1971) 796.
14 E. Randerath, C.-T. Yu and K. Randerath, *Anal. Biochem.*, 48 (1972) 172.
15 T. Uchida and F. Egami, *Methods Enzymol.*, 12A (1967) 239.
16 K. Randerath, K. M. Flood and E. Randerath, *FEBS Lett.*, 5 (1969) 31.
17 J. X. Khym and W. E. Cohn, *J. Amer. Chem. Soc.* 82 (1960) 6380.
18 K. Randerath, *Anal. Chem.*, 41 (1969) 961.
19 K. Randerath, *Anal. Biochem.*, 34 (1970) 188.
20 K. Randerath, S. K. MacKinnon and E. Randerath, *FEBS Lett.*, 15 (1971) 81.
21 E. Randerath, L. S. Y. Chia, J. B. Williams, H. P. Morris and K. Randerath, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, 32 (1973) 653 Abs.
22 K. Randerath, S. K. MacKinnon and E. Randerath, unpublished results 1972.
23 K. Randerath, L. J. Rosenthal and P. C. Zamecnik, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 3233.
24 J. D. Johnson and J. Horowitz, *Biochim. Biophys. Acta*, 247 (1971) 262.
25 R. Reddy, T.-S. Ro-Choi, D. Henning, H. Shibata, Y. C. Choi and H. Busch, *J. Biol. Chem.*, 247 (1972) 7245.
26 H. B. Maruayama, M. Hatanaka and R. V. Gilden, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 1999.
27 K. Randerath and E. Randerath, *Methods Cancer Res.*, 9 (1973) in press.
28 K. Randerath, *Cancer Res.*, 31 (1971) 658.
29 M. Yaniv and B. G. Barrell, *Nature (London)*, 222 (1971) 278.
30 U. L. RajBhandary, R. J. Young and H. G. Khorana, *J. Biol. Chem.*, 239 (1964) 3875.
31 J. A. Hunt, *Biochem. J.*, 120 (1970) 353.
32 A. Bernardi and G. Bernardi, *Biochim. Biophys. Acta*, 155 (1968) 360.
33 B. E. Griffin, *FEBS Lett.*, 15 (1971) 165.
34 C. C. Richardson, *Proc. Nat. Acad. Sci. U.S.*, 54 (1965) 158.
35 A. Novogrodsky and J. Hurwitz, *J. Biol. Chem.*, 241 (1966) 2923.
36 K. Randerath, *FEBS Lett.*, 32 (1973) in press.