A Method for Determination of the Methylated Constituents of Transfer Ribonucleic Acid†

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ABSTRACT: A method that permits both a rapid and reliable estimate of the methylated constituents of tRNA is presented. The procedures consist of (a) isolating and purifying tRNA preparations from cells or tissues previously labeled with L-[*methyl*-^{3}H or -^{14}C]methionine, (b) hydrolysis of the tRNA with 88% formic acid at 180° for 2 hr and (c) separation of the resultant methylated base constituents in the hydrolysate by two-dimensional thin-layer chromatography for measurement of radioactivity. In addition, a procedure which permits the determination of 2′-*O*-methylribose moieties of RNA is presented. These procedures were employed to determine the methylated-base patterns of tRNA of rat uterine tissues, human KB cells, and *Escherichia coli* B cells. Approximately 95% of the total radioactivity incorporated into purified tRNA preparations was accounted for and identified as 1-methylhypoxanthine, 1-, 7-, N^2- and N^2,N^2-methylguanine, 1-, N^6- and 2-methyladenine, 3- and 5-methylcytosine, 5-methyluracil, and 2′-*O*-methylribose moieties. The patterns of methylated constituents of tRNA from the rat uterus and KB cells were quite similar, but differed considerably from those obtained with *E. coli* B cells. Comparative data are presented regarding the patterns of methylated constituents of (a) total-unfractionated RNA of KB cells and of tRNA preparations obtained following successive fractionation treatments of total RNA with 1.2 M NaCl and 2-propanol and (b) of tRNA following hydrolysis with 1.0 N HCl and 98% trifluoroacetic acid.

The transfer of the methyl group of methionine to newly synthesized RNA molecules *via* *S*-adenosylmethionine and RNA methylases to produce base- and/or ribose-specific methylated products has been well documented (reviews by Borek, 1963; Starr and Sells, 1969; Söll, 1972). Subsequent investigations have attempted to characterize RNA methylase activities in biological systems experiencing various degrees of growth and differentiation. For the most part, such studies have concentrated on determining the rate and extent of methyl group acceptance by heterologous tRNA substrates *via* *S*-adenosylmethionine in the presence of various methylase preparations. More recently, these studies have been extended to include analysis of the types of methylated products that occur on tRNA substrates derived from both *in vitro* and *in vivo* methylase reactions (reviews by Borek and Kerr, 1972; Kerr and Borek, 1972). Of critical importance in the above investigations is the reliability of the procedures employed to hydrolyze tRNA into methylated products that can be successfully separated and measured quantitatively. In addition, the progress of these studies is dependent upon the simplicity and speed with which such determinations can be performed. Unfortunately, many of the available procedures lack one or both of these requisites.

Among the procedures available for hydrolyzing RNA, those employed most frequently for characterization of methylated constituents are the hydrolysis of RNA with enzymes to yield either nucleotides or nucleosides (Hall, 1967; Randerath, 1971; Nishimura, 1972), with 1.0 N HCl to yield purine bases and pyrimidine nucleotides (Vischer and Chargaff, 1948; Bjork and Svensson, 1967) and with 70% perchloric acid to yield purine and pyrimidine bases (Iwanami and Brown, 1968a). Each of these procedures, however, results in the decomposition and/or alteration of one or more of the principal methylated bases of tRNA. For example, Randerath (1971) and Lawley and Shah (1972) have reported that under the alkaline conditions required to hydrolyze RNA enzymatically, 7-methylguanosine undergoes partial decomposition while 1-methyladenosine is partially converted to N^6-methyladenosine. In HCl, 1-methyladenine was found to be converted into a product tentatively identified as 7-methyladenine (Lawley and Shah, 1972). Iwanami and Brown (1968a) have also reported the destruction of 3-methyluracil and 4-methylcytosine during hydrolysis with 70% perchloric acid. A second problem inherent in these hydrolysis procedures pertains to the difficulties associated with completely separating the resultant products by a single, rapid chromatographic technique (Iwanami and Brown, 1968a; Hall, 1967; Lawley and Shah, 1972; Nishimura, 1972).

In the present investigation we have attempted to overcome these difficulties by reexamining the suitability of formic acid as an agent for the hydrolysis of RNA (Vischer and Chargaff, 1948) with respect to the ability of this acid to hydrolyze tRNA into its respective purine- and pyridine-methylated bases. We present data which demonstrate the suitability of formic acid in this regard, which, coupled with a two-dimensional thin-layer chromatographic system for separating methylated bases, provides measurements of the patterns of methylated bases of L-[*methyl*-^{3}H or ^{14}C]methionine-labeled tRNAs in a rapid and reproducible manner.

Experimental Section

Materials. The sources and R_F values of standard bases and nucleosides employed in this investigation are listed in Table I. All solvents, including formic acid (88%) and ammonium hydroxide (58%), were of analytical grade (Mallinckrodt) and used without further purification. Silica gel

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TABLE I: Absolute R_F Values of Nucleosides and Bases of RNA in Thin-Layer Chromatographic Systems.[a]

Nucleosides and Bases (Source)[b]	Solvent Systems[c]			
	I	II	III	IV
Adenosine (S)	0.32	0.14	0.24	0.45[d]
Adenine (S)	0.37	0.36	0.46	0.43
1-Methyladenine (S, E)	0.10	0.40	0.35	0.50
2-Methyladenine (S, H)	0.37	0.49	0.55	0.35
N^6-Methyladenine (S, C)	0.54	0.57	0.58	0.37
N^6,N^6-Dimethyladenine (S, C)	0.65	0.69	0.67	0.31[d]
1-Methylhypoxanthine (S, C)	0.42	0.37	0.39	0.61
Guanosine (S)	0.22	0.14	0.19	0.85
Guanine (S)	0.16	0.14	0.23	0.74[d]
1-Methylguanine (S, E)	0.26	0.38	0.37	0.49
7-Methylguanine (S, E)	0.28	0.24	0.32	0.45[d]
N^2-Methylguanine (S, C)	0.36	0.31	0.37	0.45[d]
N^2,N^2-Dimethylguanine (S, E)	0.41	0.35	0.42	0.42
Cytidine (S)	0.20	0.31	0.23	0.74
Cytosine (S)	0.21	0.40	0.33	0.55
3-Methylcytosine (S, C)	0.15	0.54	0.48	0.67
5-Methylcytosine (S)	0.21	0.47	0.41	0.62
Thymidine (S)	0.55	0.56		
Ribothymine (S, C)	0.47	0.37	0.48	0.87
Uridine (S)	0.41	0.23	0.33	0.93
Uracil (S)	0.48	0.35	0.48	0.83
3-Methyluracil (C)	0.78	0.70	0.63	0.85
5-Methyluracil (S)	0.69	0.58	0.53	0.78

[a] Chromatography was conducted with glass plates (20 × 20 cm) coated to a thickness of 0.5 mm with a composite adsorbent consisting of 42% silica gel GF and 58% Avicel microcrystalline cellulose. Other details of these procedures are briefly described in the Experimental Section and in detail elsewhere (Munns *et al.*, 1973). [b] Source of nucleosides and bases; S = Sigma Chem. Co., C = Cyclo Chem. Co., E = Dr. G. Elion, Burroughs Wellcome Co., H = Dr. R. Hall, McMaster University. [c] Solvent systems: I, 100:25:20:1 (ethyl acetate–methanol–water–88% formic acid); II, 40:30:20:10:5:22 (acetonitrile–ethyl acetate–2-propanol–1-butanol–water–58% ammonium hydroxide); III, 50:40:15:3 (1-butanol–acetone–water–58% ammonium hydroxide); IV, water. [d] Moderate to extensive trailing.

GF 254 and Avicel Microcrystalline Cellulose were purchased from Brinkmann and the latter was washed with versene buffer according to Grippo *et al.* (1965). L-[*methyl*-^{14}C]- and [*methyl*-^{3}H]methionine were purchased from Amersham-Searle.

Immature female rats (NLR strain, Wistar origin), 21–22 days old and weighing 40–50 g, were purchased from National Laboratory Animal Company, Creve Coeur, Missouri. KB cells, a line of cultured human carcinoma cells, were provided by Dr. Maurice Green of the Institute of Molecular Virology, St. Louis University School of Medicine and *Escherichia coli* B cells were supplied by Dr. Stanley Roodman of the Biochemistry Department, St. Louis University School of Medicine.

Labeling of Tissues and Cells. Groups of 5–10 intact, surviving uteri were removed from immature rats and labeled with L-[*methyl*-^{3}H or -^{14}C]methionine under conditions previously described by Munns and Katzman (1971a). KB cells at a density of 1×10^6 cells/ml in MEM medium (for suspension culture, Grand Island Biological Co.) were incubated at 37° in 250-ml spinner flasks. Following a 30-min preincubation period, an appropriate quantity of either L-[*methyl*-^{3}H or -^{14}C]methionine (25–500 μCi) was added and the incubation continued for the desired period of time (usually 2–4 hr). Cells were harvested by low-speed centrifugation (800*g*, 10 min) and the pellets stored at −20°. *E. coli* B cells were grown under forced aeration at 37° in M9 + 0.2% glucose medium (Klagsbrun and Rich, 1970) and cell growth was monitored at an optical density of 650 nm (1-cm light path). Either [*methyl*-^{3}H or -^{14}C]methionine (100–500 μCi) was added to cultures of exponentially growing cells and 2 hr later the cells were collected by centrifugation, washed twice in 0.01 M MgOAc–0.01 M Tris (pH 8.0) (medium A) and stored as a pellet at −20°.

All preparations of RNA isolated from cells or tissues previously labeled with L-[*methyl*-^{3}H or -^{14}C]methionine will be referred to as [*methyl*-^{3}H]- or [*methyl*-^{14}C]RNA.

Isolation and Characterization of RNA. E. coli B cells were originally suspended in 5 ml of medium A and successively frozen and thawed three times in the presence of lysozyme (Sigma) and purified DNase (Worthington) as described by Bjork and Neidhardt (1973) prior to homogenization as described below for uterine tissues and KB cells. KB cells and uterine tissue were thawed and homogenized (Duall ground glass) in 5 ml of ice-cold buffer B (0.05 M NaOAc–0.001 M EDTA–0.014 M NaCl (pH 5.1)) in the presence of appropriate quantities of bentonite (Fraenkel-Conrat *et al.*, 1961). The RNA of homogenates of uterine tissues (5–10), KB cells (2–5 × 10^7), and *Escherichia coli* B cells (6–12 × 10^{10}] was then extracted with phenol–sodium dodecyl sulfate at 65° (Scherrer and Darnell, 1962) and the tRNA isolated and purified from the bulk of other RNAs by successive fractionation treatments of 1.2 M NaCl (Crestfield *et al.*, 1955) and 2-propanol (Zubay, 1962).

Each fraction of [*methyl*-^{3}H or -^{14}C]RNA isolated in the above manner (total RNA, salt-fractionated tRNA and 2-propanol-fractionated tRNA) was characterized by its radioactivity profile following acrylamide gel electrophoresis (Figure 1) as previously described by Munns and Katzman (1971b).

Formic Acid Hydrolysis of tRNA. Aliquots of [*methyl*-^{3}H or -^{14}C]tRNA (initially dissolved in water and containing upwards of 200 μg of tRNA with specific activities ranging from 200 to 400 cpm/μg) were evaporated under N_2 at 40°, redissolved in 0.5 ml of 88% formic acid and hydrolyzed at various temperatures (100, 150, and 180°) for 2 hr in sealed Pyrex tubes (300 × 11 mm, i.d. of 9 mm). After hydrolysis, the tubes were briefly cooled and opened behind a protective shield, since a positive pressure was developed in tubes heated at 180°. The hydrolysates were evaporated under N_2 and the residues stored at −20° for later chromatographic processing. In comparative hydrolysis studies with HCl and trifluoroacetic acid, identical conditions with those reported by Iwanami and Brown (1968a) and Klagsbrun (1973) were employed as described in Table IV.

Chromatographic Procedures and Determination of Radioactive Methylated Constituents. The separation of a mixture of 13 methylated bases by two-dimensional thin-layer chromatography has been described previously

(Munns *et al.*, 1973). Modifications of this procedure in the present study included a continuous development in the second dimension (as described below) and omission of heat to aid in the drying or activation of thin-layer plates before and between chromatographic developments.

Residues, obtained by evaporating acid hydrolysates of RNA, were redissolved in 30 μl of formic acid containing 2–5 μg of each of the standard methylated bases (Table I) and 15 μl of this solution applied to the corner of chromatographic plates (20 × 20 cm) which were then developed in preequilibrated tanks containing 100 ml of solvent system I (Table I) until the solvent front had migrated to the top of the plates. The plates were then removed and allowed to air-dry at room temperature for 2 hr before development in the second direction. For satisfactory resolution of all methylated bases, continuous development in the second dimension was conducted for approximately 3–4 hr by absorbing the solvent from the plate into coarse filter paper clamped across the top (and hanging over the back side) of the plate until the N^2- and N^2,N^2-methylguanines were completely resolved from each other. The position of each base was routinely monitored by momentarily replacing the lid of the tank with a uv lamp (254 nm). Cochromatography of the radioactive products of hydrolysates with standard bases permitted the positions of the authentic substances to be identified and the radioactivity contained within these regions served to measure each radioactive product. For this purpose the adsorbent in each of the identified areas was scraped directly into scintillation vials (Bjork and Svensson, 1967) for radioactivity determination with a Packard Tri-Carb liquid scintillation spectrophotometer after the addition of 10 ml of aqueous PCS scintillant. As much as threefold the amount of adsorbent usually removed for such measurements had no observable effect on the efficiencies of this scintillation system which ranged from 20 to 70% depending upon the type and/or mixture of isotope(s) present.

Loss of Radioactivity (Volatilization) Accompanying Formic Acid and Perchloric Acid Hydrolysis of tRNA. The work of Baskin and Dekker (1967) and more recently that of Abbate and Rottman (1972) has indicated that the methyl group attached to the 2′ position of nucleosides (but not those attached to the base portion of nucleosides) was quantitatively converted to methanol during digestion with 70% perchloric acid at 100° for 90 min. With modifications, we have utilized this perchloric acid treatment to assess the content of 2′-*O*-methylribose constituents in [*methyl*-^{14}C]RNA preparations. Essentially, this modified procedure consists of determining the loss of radioactivity incurred to [*methyl*-^{14}C]RNA during perchloric acid digestion by measurements of radioactivity before and after such treatment. These measurements were simplified by the addition of an internal isotopic standard ([^{32}P]P_i) that was not volatilized during digestion.

Aliquots of aqueous solutions of [*methyl*-^{14}C]RNA containing added [^{32}P]P_i were taken to dryness and redissolved in 0.5 ml of 70% perchloric acid. For measurement of ^{14}C/^{32}P ratios of these solutions (nondigested), 0.1-ml aliquots were transferred to scintillation vials containing 1.0 ml of 0.55 N perchloric acid and stored overnight at 37° to hydrolyze the RNA prior to the addition of 9.0 ml of aqueous scintillator (PCS, Amersham Searle). For measurement of ^{14}C/^{32}P ratios following digestion in 70% perchloric acid for 2 hr at 100°, the digest was placed under a stream of N_2 at 60° to remove the [^{14}C]methanol. Two successive additions of 1.0 ml of unlabeled methanol were used to ensure the removal of [^{14}C]methanol from the perchloric acid (which did not evaporate under these conditions) prior to removing 0.1-ml aliquots for determination of ^{14}C/^{32}P ratios as described above for nondigested samples. The differences in the ^{14}C/^{32}P ratios (corrected for crossover) before and following digestion reflected the extent of volatilized radioactivity and was taken as a measure of the content of 2′-*O*-methylribose moieties of RNA. Preparations of [*methyl*-^{3}H]RNA were not employed for measurement of this parameter since an appreciable quantity of tritium from [*methyl*-^{3}H]thymidine digests was converted into a volatile form (exchanged) under the perchloric acid conditions employed.

Comparable studies with 88% formic acid for 2 hr at 100, 150, and 180° were conducted to assess the ability of this acid to generate volatile radioactivity. In these studies, measurements of radioactivity before and following digestion were made in the same manner as those digested with perchloric acid with the exception that formic acid digests were taken to dryness and the residue was redissolved in a small volume of formic acid before taking aliquots for radioactivity measurements. The possibility of ^{3}H-exchange reactions accompanying formic acid hydrolysis was negated by the finding that the patterns of methylated bases derived from

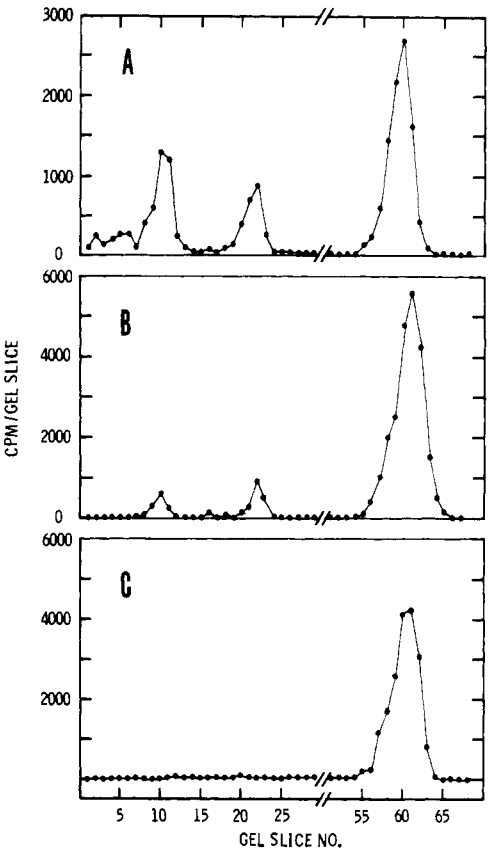

FIGURE 1: Electrophoretic characterization of various preparations of RNA obtained from KB cells previously labeled in suspension culture for 3 hr with L-[*methyl*-^{3}H]methionine. The RNA was extracted and purified to yield preparations defined in the Experimental Section as: total RNA (A), salt-fractionated tRNA (B), and 2-propanol-fractionated tRNA (C). Procedures employed for the isolation of these RNA preparations, their electrophoresis on 2.5% composite gels, and the determination of the radioactivity profiles are described in the Experimental Section. Similar profiles of radioactivity were obtained from preparations of RNA of rat uterine tissues and *E. coli* B cells except that the rRNA from the latter migrated as 23S and 16S species.

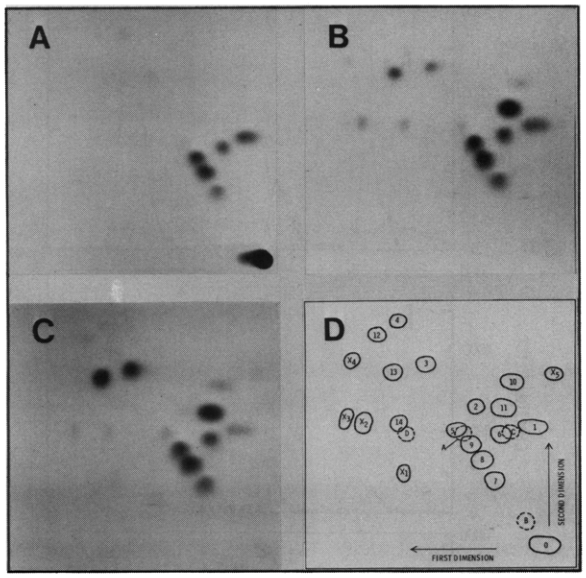

FIGURE 2: Autoradiographs of formic acid hydrolysates of [*methyl*-^{14}C]tRNA prepared from KB cells previously labeled for 4 hr with L-[*methyl*-^{14}C]methionine. The tRNA was purified by salt fractionation (see Figure 2B) and hydrolyzed in 88% formic acid for 2 hr at temperatures of 100° (plate A), 150° (plate B), and 180° (plate C). The methylated constituents of these hydrolysates were separated *via* two-dimensional thin-layer chromatography, and the chromatographic plates were exposed to X-ray film for 2 weeks. The above procedures are described in detail in the Experimental Section and in the legend of Table II. Direction of chromatography and identification of methylated constituents are as indicated in plate D. A, adenine; B, guanine; C, cytosine; D, uracil; O, origin; 1, 1-methyladenine; 2, 2-methyladenine; 3, N^6-methyladenine; 4, N^6,N^6-dimethyladenine; 5, 1-methylhypoxanthine; 6, 1-methylguanine; 7, 7-methylguanine; 8, N^2-methylguanine; 9, N^2,N^2-dimethylguanine; 10, 3-methylcytosine; 11, 5-methylcytosine; 12, 3-methyluracil; 13, 5-methyluracil; 14, ribothymine; and X_1–X_5 are unidentified constituents detected in the autoradiographs.

[*methyl*-^{3}H]- and [*methyl*-^{14}C]tRNA digests were identical (compare Tables II and V).

Autoradiography of Chromatograms. Autoradiographs of thin-layer plates were prepared on 8 × 10 in. medical X-ray film (NS 54 T, Kodak). After two-dimensional development of hydrolysates of [*methyl*-^{14}C]tRNA, the plates were dried in a desiccator overnight before exposure to X-ray film for 10–20 days at −20°. Other details of this procedure are described in the legend of Figure 2.

Results and Discussion

Electrophoretic Characterization of Methyl-Labeled RNA Preparations. Preparations of [*methyl*-^{3}H]RNA from KB cells were characterized electrophoretically to ascertain the types of RNA species that become methylated during a 3-hr *in vivo* pulse, as well as, to establish the extent of purification of tRNA following successive fractionation treatments of total RNA preparations with 1.2 M NaCl and 2-propanol. Analysis of the radioactivity profile of total RNA preparations (Figure 1A) indicated that the principal methylated species of RNA from KB cells were rRNA (28 S and 18 S) and tRNA (4 S). The distribution of radioactive methyl groups incorporated into these RNAs was 25, 15, and 60%, respectively, for the 28S, 18S, and 4S RNA and corresponded with the values reported by Brown and Attardi (1965) and Klagsbrun (1973) for other mammalian systems. As shown in Figure 1B, the bulk of radioactive rRNA present in the total RNA preparation was removed following salt fractionation, while the small amount of rRNA remaining in the salt-fractionated tRNA preparation was effectively removed by 2-propanol fractionation (Figure 1C). With regard to the purification of tRNA achieved by these fractionation procedures, greater than 90 and 98% of the radioactivity incorporated into the salt and 2-propanol preparations, respectively, migrated within the tRNA region of the gel. A similar degree of purification was obtained with uterine and *E. coli* B tRNAs.

The ineffectiveness of treatment with DNase or incubation of pH 8.5 (30 min at 35°) to alter the distribution of radioactivity associated with the above RNA preparations indicated that the incorporated radioactivity reflected RNA methylation, rather than DNA methylation and/or acylated tRNA labeling (*i.e.,* methionyl-tRNA). Furthermore, since 5S RNA is not methylated (Forget and Weissman, 1968), the absence of radioactivity in this region of the gel indicated that incorporation of radioactive methyl groups into RNA *via* purine biosynthesis was not significant. This was confirmed directly by measurement of the radioactivity incorporated into the guanine residues of [*methyl*-^{14}C]tRNA preparations (Figure 2 and Table II).

Estimates of the recovery of RNA from KB cells following phenol extraction and salt and 2-propanol fractionations were 60, 15, and 5%, respectively. On the basis that tRNA represents 20% of the total cellular RNA, the recovery of tRNA from KB cells was approximately 50 and 25%, respectively, following purification with 1.2 M NaCl and 2-propanol.

Assessment of Chromatographic Procedures. The pertinent information regarding the thin-layer chromatographic systems employed in this investigation is presented in Table I. Not presented are the 3′- and 5′-monophosphates of adenosine, guanosine, cytidine, uridine, and thymidine which did not migrate in any of the above solvent systems (R_F 0). Although repeated analysis indicated that the absolute R_F values listed in Table I were subject to approximately a 15% variation, the relative migration of each standard constituent with respect to the other standards was uniform in any given solvent system. All of these solvent systems were used routinely to assess the purity of each standard base and nucleoside, although systems I and II were used exclusively for separating mixtures of methylated bases. It is important to note that separation of the 13 methylated bases listed in Table I was best achieved by initial development with solvent system I, followed by continuous development in the second dimension with system II. This sequence of solvent systems as well as the continuous development in the second dimension were particularly helpful in separating the N^2- and N^2,N^2-methylguanines and 1-methylhypoxanthine from each other (Figure 2). Additional characteristics of these and related chromatographic systems are described elsewhere (Munns *et al.,* 1973).

Formic Acid Hydrolysis of tRNA: Effects of Temperature on Methylated-Base Patterns. The effect of temperature on the hydrolysis of tRNA with formic acid is presented (1) by the autoradiographs in Figure 2 which exhibit the distribution of radioactivity on chromatograms of hydrolysates prepared at different temperatures, and (2) by the data in Table II which indicate the percentage of total radioactivity accounted for by each methylated constituent. It is important to note that the values listed in Table II were calculated on the basis of the total radioactivity incorporated into tRNA and not as the per cent recovered as methylated bases.

These findings (Figure 2 and Table II) indicate that the

methylated purines of tRNA (1-,7-,N^2- and N^2,N^2-methylguanine, 1-methylhypoxanthine, and 1-methyladenine) were completely liberated by formic acid at 100°, since the radioactivity associated with these methylated bases remained unchanged following hydrolysis at higher temperatures. Similar analysis of the methylated pyrimidines revealed that 3- and 5-methylcytosine were liberated at 150°, while the radioactivity associated with 5-methyluracil continued to increase with temperature to 180°. Based on the supplementary data regarding the hydrolysis of unlabeled ribothymine to 5-methyluracil, it was assumed that the liberation of 5-methyluracil was completed at the latter temperature. Hydrolysis of tRNA at temperatures of 200° and greater led to partial destruction of the methylated purines and conversion of the methylcytosines to the corresponding methyluracils.

Further studies regarding the chromatographic behavior of various unlabeled methylated nucleosides (1-,7-,N^2- and N^2,N^2-methylguanosine, 1-methylinosine, 1-,2-,N^6- and N^6,N^6-methyladenosine, 3- and 5-methylcytidine, and ribothymine; Sigma and/or Cyclo Chem. Co.) before and after formic acid hydrolysis (2 hr, 180°) indicated that these constituents were completely hydrolyzed to their respective bases without apparent destruction, with the exception of 1-methyladenosine. In regards to 1-methyladenine and its nucleoside, both undergo a rearrangement in formic acid to yield a second component that spectrally (240–310 nm) and chromatographically (R_F values, Table I) appears to be identical with N^6-methyladenine. This rearrangement, which has previously been described to occur under alkaline conditions (Brooks and Lawley, 1960), does not obviate the estimation of 1-methyladenine in mammalian tRNA preparations, since these tRNAs do not contain significant amounts of N^6-methyladenine (Iwanami and Brown, 1968a; Reddy *et al.*, 1972). In reference to other RNA preparations that may contain N^6-methyladenine, use can be made of the finding that the net conversion of this rearrangement is less than 5% during hydrolysis with formic acid at 100° (2 hr), whereas at 180° it is approximately 65% (Table II).

Approximately 95% of the radioactivity originally incorporated into tRNA was accounted for by recovery of radioactive methylated bases on chromatograms and that lost by volatilization during hydrolysis with formic acid at 180°. Hydrolysis at 100 and 150°, which was satisfactory for recovering the methylated purines, was not suitable in this regard for the methylated pyrimidines because of incomplete liberation of the latter at these temperatures (Table II). The majority of the large amounts of radioactivity remaining at the origin of the chromatograms from 100° hydrolysates represented pyrimidine nucleotides, since nucleotides do not migrate in either of the chromatographic systems employed. However, the radioactivity at the origin in the case of 100° hydrolysates (42.9% of the total) was greater than that recovered as methylated pyrimidines from 180° digests (30.1% of the total). This difference (12.8%) approximated the amount of radioactivity that was volatilized during formic acid hydrolysis at 180° (12.4%) and suggested that it was associated with 2′-*O*-methylribose derivatives. Additional support for this assumption was provided by the finding that the *difference* in the per cent of radioactivity volatilized from aliquots of [*methyl*-^{14}C]tRNA following digestion with formic and perchloric acid was recovered from the formic acid digest by further treatment with perchloric acid (unpublished data). It is significant that nearly 100% of the radioactivity of [*methyl*-^{14}C]tRNA preparations can be accounted for following hydrolysis at 180° if the 2′-*O*-methylribose content (as determined by perchloric acid treatment) is taken into consideration.

The identity of the small amounts of radioactivity distributed in areas of the chromatograms not occupied by the standard methylated bases, and designated as X_1–X_5 in the autoradiographs in Figure 2, has not been investigated.

The Distribution of Methylated Constituents of Total

TABLE II: Formic Acid Hydrolysis of *t*RNA. Effects of Temperature.[a]

Per Cent of [*methyl*-^{14}C]tRNA Radioactivity Accounted for as[b]	Temp 100°	150°	180°
O. Origin	42.9	3.2	0.2
B. Guanine	0.1	0.2	0.1
1. 1-Methyladenine (1A)	10.6	7.3	4.2
2. 2-Methyladenine	0.2	0.3	0.2
3. N^6-Methyladenine (MA)	0.5	4.6	8.1
1A + MA = (1A)[c]	(11.1)	(11.9)	(12.3)
4. N^6,N^6-Dimethyladenine	0.4	0.4	0.4
5. 1-Methylhypoxanthine	0.9	1.0	1.2
6. 1-Methylguanine	6.6	6.4	6.4
7. 7-Methylguanine	5.3	5.4	5.6
8. N^2-Methylguanine	13.2	13.3	13.4
9. N^2,N^2-Dimethylguanine	10.8	10.4	10.7
10. 3-Methylcytosine	0.1	2.6	2.5
11. 5-Methylcytosine	0.3	16.4	16.0
12. 3-Methyluracil	0.2	0.5	0.3
13. 5-Methyluracil	0.2	6.9	11.3
14. 2′-*O*-Methylribose[d]	18.4	18.4	18.4
15. Volatilized *via* formic acid[e]	0.8	11.2	12.4
Methylated purines (1–9)	48.5	49.1	50.2
Methylated pyrimidines (10–13)	0.8	26.4	30.1
Methylated bases (1–13)	49.3	75.5	80.3
Methylated constituents (1–14)	67.7	93.9	98.7

[a] Identical aliquots of salt-fractionated tRNA of KB cells totaling 180 μg of RNA with a specific activity of 460 cpm/μg were hydrolyzed with formic acid for 2 hr at temperatures of 100, 150, and 180°. Following hydrolysis and evaporation, the residues were redissolved in formic acid (50 μl) containing the standard methylated bases listed above and 20-μl aliquots applied to two chromatographic plates for development in solvent systems I and II (see Table I). One plate was taken for measurement of radioactive methylated bases directly, while the other was processed for autoradiographs (Figure 2). The above methods are described in detail in the Experimental Section. All values represent the mean of at least three experiments with a standard deviation of less than ±9% of the mean (excluding all values less than 1.0). [b] The per cent of radioactivity represents the cpm initially hydrolyzed and is equivalent to the cpm applied to the chromatographic plate plus that lost (volatilized) during evaporation of formic acid hydrolysates. [c] The sum of 1- and N^6-methyladenine was taken as a measure of 1-methyladenine as described in the text. [d] The 2′-*O*-methylribose content was determined by perchloric acid digestion as described in the Experimental Section. [e] Determined as in the Experimental Section and used in the calculation of per cent of total radioactivity (see b).

TABLE III: Distribution of Methylated Constituents of Total RNA (Unfractionated) and Salt- and 2-Propanol-Fractionated tRNA from KB Cells.[a]

Per Cent of [*methyl*-^{14}C]RNA Radioactivity Accounted for as	Preparation of RNA		
	Total	Salt	2-Propanol
O. Origin	3.6	0.2	0.1
B. Guanine	0.4	0.1	0.1
1. 1-Methyladenine (1A)	3.5	4.2	4.5
2. 2-Methyladenine	0.2	0.2	0.1
3. N^6-Methyladenine (MA)	6.7	8.1	8.1
1A + MA = (1A)[b]	(10.2)	(12.3)	(12.6)
4. N^6,N^6-Dimethyladenine	2.0	0.4	0.1
5. 1-Methylhypoxanthine	0.5	1.2	1.3
6. 1-Methylguanine	4.8	6.4	6.6
7. 7-Methylguanine	4.9	5.6	5.8
8. N^2-Methylguanine	7.8	13.4	13.6
9. N^2,N^2-Dimethylguanine	6.4	10.7	10.8
10. 3-Methylcytosine	1.3	2.5	2.7
11. 5-Methylcytosine	10.7	16.0	16.7
12. 3-Methyluracil	0.8	0.3	0.1
13. 5-Methyluracil	5.6	11.3	11.6
14. 2′-*O*-Methylribose[c]	30.1	18.4	13.4
15. Volatilized *via* formic acid[d]	18.5	12.4	8.4
Methylated purines (1–9)	36.8	50.2	50.9
Methylated pyrimidines (10–13)	18.4	30.1	31.1
Methylated bases (1–13)	55.2	80.3	82.0
Methylated constituents (1–14)	85.3	98.7	95.4

[a] The isolation of each RNA preparation is described in the Experimental Section and characterized electrophoretically in Figure 1. Aliquots from each [*methyl*-^{14}C]RNA preparation, containing not more than 300 μg of RNA with specific activities ranging from 150 to 300 cpm/μg, were hydrolyzed in 0.5 ml of 88% formic acid at 180° for 2 hr. Chromatography and measurements of radioactive methylated constituents as described in the Experimental Section and in Table II. All values represent the mean of three experiments. Standard deviations were less than ±12% of the mean values (excludes values less than 1.0). [b] (1A), only valid for tRNA preparations. The value listed for total RNA reflects only the sum of 1-methyladenine and N^6-methyladenine. [c,d] Determined as in Table II.

RNA and Salt- and 2-Propanol-Fractionated tRNA of KB cells. Table III compares the resulting patterns of methylated constituents of each of the RNA preparations characterized in Figure 1. Whereas the percentages of radioactive methylated constituents of the salt- and 2-propanol-fractionated tRNA preparations were similar, they were significantly different from total RNA preparations. These differences were attributed to the amount of rRNA present in each preparation. Purification of tRNA resulted in an increased percentage of radioactive methylated bases from 55% for total RNA to 80 and 82% after salt and 2-propanol fractionation, respectively, while the 2′-*O*-methylribose content (volatile radioactivity resulting from perchloric acid digestion) decreased from 30% to 18 and 13%, respectively. These results are in accord with those obtained by other investigators (Brown and Attardi, 1965; Iwanami and Brown, 1968b; Klagsbrun, 1973) and indicate that rRNA species contain a significantly higher percentage of ribose methylations than those of tRNA. The lower recoveries of radioactivity from chromatograms of total RNA hydrolysates may reflect the presence of methylated bases not employed as standards in this study. In this regard, Iwanami and Brown (1968b) have reported relatively high levels of 4-methylcytosine in the rRNA of HeLa cells.

The small differences that were observed in the patterns of methylation of salt- and 2-propanol-fractionated tRNA preparations were attributed primarily to rRNA contamination of the former (approximately 10%, as in Figure 1B). On several occasions, unduly large amounts of radioactivity associated with N^2,N^2-dimethyladenine and 2′-*O*-methylribose derivatives in salt-fractionated RNA preparations were traced to high levels of rRNA contamination.

Hydrolysis of tRNA with HCl and Trifluoroacetic Acid. The patterns of methylated constituents following hydrolysis of identical aliquots of [*methyl*-^{14}C]-tRNA with 1.0 N HCl and 98% trifluoroacetic acid were compared with those obtained with formic acid and are presented in Table IV. Except for 1-methyladenine, the distribution of radioactive methylated-bases of HCl hydrolysates was indistinguishable from that of formic acid hydrolysates (hydrolyzed at 100° for 2 hr). The difference observed for 1-methyladenine was not surprising in view of the findings that 1-methyladenine is partially converted to 7-methyladenine in HCl (Lawley and Shah, 1972) and to N^6-methyladenine in formic acid (Munns *et al.* 1973). The extent of such conversions under the conditions of hydrolysis employed in Table IV was estimated to be 50 and 5% for HCl and formic acid, respectively. The inability of both of these acids to hydrolyze the methylated pyrimidine constituents of tRNA at 100°, however, limited their usefulness at this temperature to measurement of methylated purines in the chromatographic systems described in Table I. At higher temperatures, HCl destroyed most of the methylated constituents of tRNA (unpublished observations).

Analysis of the methylated constituents of tRNA in trifluoroacetic acid hydrolysates revealed significantly lower percentages of radioactivity for 7- and N^2-methylguanine, 3- and 5-methylcytosine, and 5-methyluracil. The lower values observed for the methylpyrimidines may be due to incomplete hydrolysis of their parent nucleotides, since relatively large quantities of radioactivity from these hydrolysates remained at the origin of the chromatograms. The reason for the reduction in the per cent of radioactivity recovered as 7- and N^2-methylguanine is not readily apparent. These findings, however, discouraged further use of trifluoroacetic acid, but are presented here for the information of other investigators in this field.

Examination of autoradiographs of chromatograms of both HCl and trifluoroacetic acid hydrolysates (not illustrated) corroborated the data presented in Table IV.

The Distribution of Methylated Constituents in the tRNA of Rat Uterus, Human KB Cells, and E. coli B Cells. The patterns of methylated constituents of tRNA from two mammalian systems (one neoplastic) and one bacterial system are listed in Table V. Whereas the patterns obtained from the tRNA of rat uterine tissues and KB cells were quite similar, they differed considerably from those obtained with *E. coli* B cells. Most significant in the *E. coli* B pattern was the absence of radioactivity in N^2- and N^2,N^2-methylguanine, 1-methyladenine, and 5-methylcytosine which accounted for approximately 50% of the total radioactivity in mammalian tRNA preparations. With

TABLE IV: Distribution of Methylated Constituents of tRNA Following Hydrolysis with HCl and Trifluoroacetic Acid.[a]

Per Cent of [*methyl*-^{14}C]tRNA Radioactivity Accounted for as	HCl[b]	CF_3CO_2H[c]
O. Origin	41.6 (42.9)	18.2 (0.2)
B. Guanine	0.1 (0.1)	0.1 (0.1)
1. 1-Methyladenine (1A)	4.7 (10.6)	11.4 (4.2)
2. 2-Methyladenine	0.3 (0.2)	0.2 (0.2)
3. N^6-Methyladenine (MA)	0.4 (0.5)	0.9 (8.1)
1A + MA = 1A[d]	(11.1)	(12.3)
4. N^6,N^6-Dimethylhypoxanthine	0.4 (0.4)	0.3 (0.4)
5. 1-Methylhypoxanthine	1.1 (0.9)	1.3 (1.2)
6. 1-Methylguanine	6.7 (6.6)	6.6 (6.4)
7. 7-Methylguanine	5.5 (5.3)	2.5 (5.6)
8. N^2-Methylguanine	13.1 (13.2)	6.4 (13.4)
9. N^2,N^2-Dimethylguanine	10.4 (10.8)	11.0 (10.7)
10. 3-Methylcytosine	0.2 (0.1)	1.3 (2.5)
11. 5-Methylcytosine	0.3 (0.3)	8.6 (16.0)
12. 3-Methyluracil	0.1 (0.2)	0.2 (0.3)
13. 5-Methyluracil	0.5 (0.2)	2.9 (11.3)
14. 2′-*O*-Methylribose[e]	18.4 (18.4)	18.4 (18.4)
15. Volatilized *via* HCl or CF_3CO_2H[f]	1.0 (0.8)	3.4 (12.4)
Methylated purines (1–9)	42.6 (48.5)	40.6 (50.2)
Methylated pyrimidines (10–13)	1.1 (0.8)	13.0 (30.1)
Methylated bases (1–13)	43.7 (49.3)	53.6 (80.3)
Methylated constituents (1–14)	62.1 (67.7)	72.0 (98.7)

[a] Aliquots of [*methyl*-^{14}C]tRNA, identical with those described in Table II, were hydrolyzed at 100° for 1 hr with 1.0 N HCl in glass-stoppered tubes as described by Iwanami and Brown (1968a) or with 98% trifluoroacetic acid at 175° for 30 min as described by Klagsbrun (1973). Hydrolysates were taken to dryness under N_2 at 40° and processed for chromatography, etc., as described in the Experimental Section. Values represent the average of triplicate determinations with a standard deviation less than ±8% of the mean for any given measurement (excludes values less than 2.0). [b,c] Values in parentheses were obtained from 100° (*b*) and 180° (*c*) hydrolysates of formic acid in Table II. [d] 1A, this relationship is not valid for HCl hydrolysates as described in the text and has not been characterized with trifluoroacetic acid. [e] 2′-*O*-Methylribose was determined as in Table II. [f] Determined in an identical manner with that of formic acid volatilized radioactivity as described in the Experimental Section.

TABLE V: Distribution of Methylated Constituents of tRNA from Rat Uterus, Human KB Cells, and *Escherichia coli* B Cells.[a]

Per Cent of [*methyl*-^{3}H]tRNA Radioactivity Accounted for as	Uterine	KB	*E. coli* B
O. Origin	0.4	0.3	0.4
B. Guanine	ND[b]	ND	ND
1. 1-Methyladenine (1A)	4.8	4.2	0.3[c]
2. 2-Methyladenine	0.2	0.2	8.3
3. N^6-Methyladenine (MA)	8.0	8.0	3.6[c]
1A + MA = (1A)	(12.8)	(12.2)	
4. N^6,N^6-Dimethyladenine	0.5	0.2	0.2
5. 1-Methylhypoxanthine	1.3	1.1	ND
6. 1-Methylguanine	6.9	6.7	5.7
7. 7-Methylguanine	6.3	6.0	22.4
8. N^2-Methylguanine	10.4	13.2	ND
9. N^2,N^2-Dimethylguanine	11.5	10.4	ND
10. 3-Methylcytosine	3.1	2.8	ND
11. 5-Methylcytosine	18.2	14.2	ND
12. 3-Methyluracil	ND	0.2	0.5
13. 5-Methyluracil	10.3	10.1	39.5
14. 2′-*O*-Methylribose[d]	19.0	18.4	18.2
15. Volatilized *via* formic acid	14.0	11.3	8.2
Methylated purines (1–9)	49.9	50.0	40.5
Methylated pyrimidines (10–13)	31.6	27.3	40.0
Methylated bases (1–13)	86.5	77.3	80.5
Methylated constituents (1–14)	100.5	95.7	98.7

[a] Aliquots of salt-fractionated tRNA preparations previously labeled with [*methyl*-^{3}H]methionine, containing approximately 150 μg of RNA with specific activities ranging from 200 to 600 cpm/μg, were hydrolyzed with 88% formic acid for 2 hr at 180°. Chromatography of hydrolysates and determination of radioactivity are described in the Experimental Section. These values represent the mean of at least two separate experiments and are in excellent agreement with data obtained from methyl-^{14}C preparations. [b] ND, not detected or less than 0.1%. [c] These values were determined from 100° hydrolysates and approximate the true values of each constituent, since under these hydrolysis conditions less than 5% of 1-methyladenine is converted to N^6-methyladenine (Table II). [d] The 2′-*O*-methylribose content was determined from ^{14}C preparations.

these differences, it is easily understood why the tRNAs from *E. coli* B are excellent heterologous substrates for mammalian tRNA methylases (Srinivasan and Borek, 1964; Turkington, 1969; Liau *et al.*, 1972).

The tRNA used to determine the pattern of methylated constituents of KB cells in Table V was isolated from cells previously labeled with L-[*methyl*-^{3}H]methionine. A comparison of the percentages of radioactivity incorporated into ^{3}H-methylated bases of this preparation with similarly pulsed methyl-^{14}C preparations of tRNA (Table II) revealed identical patterns of labeled methylated bases, and indicated that the radioactivity of *methyl*-^{3}H-methylated bases was not exchanged during formic acid hydrolysis at 180°.

The results in Table V, with regard to the distribution and type of methylated constituents found in tRNA, are in excellent agreement with previously reported findings for other mammalian and bacterial systems (Iwanami and Brown, 1968a; Randerath, 1971; Bjork and Neidhardt, 1973). The validity of this agreement is enhanced by the fact that the methods employed in the present study were completely different from those employed by the above investigators.

Summary and Conclusions

The hydrolysis of RNA with formic acid was assessed

from the standpoint of generating methylated constituents that were amenable to rapid chromatographic separation and characterization. Under the conditions of 180° for 2 hr, 88% formic acid hydrolyzed RNA into its respective methylated and nonmethylated bases without apparent destruction (or exchange of the tritium of methyl-^{3}H-methylated bases). Coupled with a two-dimensional thin-layer chromatographic system for separating the methylated bases, these procedures offer a simplified, rapid, and reproducible method for determining the methylated base patterns of tRNAs previously labeled *in vivo* with [*methyl*-^{3}H or -^{14}C]methionine (or *in vitro* with [*methyl*-^{3}H or -^{14}C]-*S*-adenosylmethionine).

For complete analysis of all methylated constituents, use was made of the finding that 70% perchloric acid quantitatively converted the 2′-*O*-methylribose moieties of RNA to methanol. By measurements of the radioactivity of [*methyl*-^{14}C]RNA preparations before and following perchloric acid digestion, the content of 2′-*O*-methylribose derivatives was determined.

Utilization of these procedures accounted for approximately 95% of the methylated constituents of tRNA from three distinctly different biological systems, namely, the rat uterus, human KB cells, and *E. coli* B cells.

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