DETERMINATION OF 2'-O-METHYL LEVELS AND DISTRIBUTION IN RIBOSOMAL RNA FROM RAT TISSUES

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ABSTRACT

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rRNA was prepared from purified ribosomes from normal rat tissues and neoplastic rat livers. The purity of the rRNA was established by gel electrophoresis and UV spectral analysis.

2'-O-Methyl levels were determined by gas chromatography of methanol released upon perchloric acid hydrolysis of the RNA. The gas chromatography method used for these studies was optimized to permit detection of small variations in methylation.

For distributional analysis the rRNA was hydrolyzed to nucleosides by the combined action of pancreatic ribonuclease, snake venom phosphodiesterase, and bacterial alkaline phosphatase. The 2'-O-methyl-nucleosides were fractionated from the nucleoside mixture by DE-22 borate chromatography and analyzed by high speed liquid chromatography.

The resulting levels and distributions were subjected to statistical analysis using a nested analysis of variance, to determine differences between tissues and confidence intervals for the values obtained. No differences in 2'-O-methyl levels or distribution were found between the differentiated tissues or between neoplastic and normal rat liver.

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Dedicated to my parents and my husband.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CTAB	Cetyltrimethylammonium bromide
d.f.	Degrees of freedom
DOC	Sodium deoxycholate
E[M.S.]	Estimated mean squares
GC	Gas chromatography
HSLC	High speed liquid chromatography
M.S.	Mean squares
OD	Optical density unit
Poly (Am,U)	Heteropolymer of 2'-O-methyl adenosine and uridine
SDS	Sodium laurel sulfate
T1	Takadiastase
TKM	Buffer; 0.01 M Tris HCl pH 7.6, 0.01 M KCI, 0.002 M $MgCl_2$
UV	Ultraviolet

LITERATURE REVIEW

Ribosomes contain three size classes of RNA; 23-28S, 16-18S and 5S. The two high molecular weight components are transcribed from their DNA as a single 45S precursor molecule (Scherrer and Darnell, 1962). This precursor is modified and cleaved during processing to yield, in mammals, 18 and 28S cytoplasmic rRNA (Weinberg and Penman, 1970; Perry, 1962). In Hela cells, for example, only about half of the transcriptional unit codes for stable rRNA. The other, nonconserved, fractions of the ribosomal RNA precursor molecules are transcribed from DNA segments lying between and to the side of the 28S and 18S cistrons (Weinberg and Penman, 1970; Wellanauer and Dawid, 1973).

The genes for the 45S RNA are found in the nucleolus of the cell (Busch and Smetana, 1970), and are highly redundant. The extent of gene redundancy of the ribosomal cistrons has been determined by saturating DNA with homologous rRNA. The redundancy of Hela cells was found to be about 1000/cell for each of the two high molecular weight components; 28 and 18S (Jeanteur and Attardi, 1969). The genes are located serially on the nucleolar DNA with non-transcribed "spacer" regions separating them (Ford, 1972).

5S RNA is very highly redundant with about 7600 copies in Hela cells (Hatlen and Attardi, 1971). This DNA is not found in the nucleolus but is apparently distributed on most

of the chromosomes (Brown, 1974). It is synthesized as a larger RNA molecule and trimmed to 5S. This DNA also occurs as a serially repeating sequence with nontranscribed spacer regions separating the cistrons.

On an evolutionary scale the extent of redundancy of the ribosomal genes roughly parallels the increase in overall genomic content (Birnstiel et al, 1971). Since the cistrons show such a high degree of multiplicity, especially in higher organisms, the question arises as to whether this large number is constant throughout the life cycle of the cell, in differentiated tissues of an organism. Alternatively, they could be synthesized de novo either by amplification of a few "master genes" or by some similar mechanism that is sensitive to certain metabolic demands. Amplification of rDNA has been shown in oocytes of amphibians and insects. However, this amplification appears to be a response to demand for more rRNA. There is no evidence of rDNA amplification in any somatic tissue from vertebrates (Birnsteil, 1971).

From partial digests with T_1 RNase it is evident that nucleotide sequences of rRNA have changed considerably over the course of evolution. Therefore rDNA is subject to evolutionary changes. This raises the question whether traces of past evolutionary development can still be seen in the form of heterogeneity within the highly redundant ribosomal cistrons within one genome. RNA-DNA hybridization experiments show that the multiple cistrons are very similar (DiGirolamo et al., 1969; Retel and Planta, 1968) but there is no evidence that they are

identical (Birnstiel, 1971).

If variability of the genes does exist, heterogeneity of the population of each of the rRNA components within the same cell could result. If all the rRNA cistrons are not equally expressed in different cell types of the same organism, differences between rRNA preparations from different tissues or developmental stages of the same organism could exist. This would have far reaching implications in determining variations in function between ribosomes. Of especial interest is the possibility of differences between neoplastic and normal tissues. Another possibility is that the various rRNA genes are expressed in a coordinate fashion, though at a different rate under all conditions. In such a case all rRNA samples prepared from the same organism will be equally heterogeneous.

The problem of heterogeneity of rRNA in a population of ribosomes has been approached on several levels. Loening (1968) examined rRNA from several organisms. He found a change in size of the rRNA components as shown on acrylamide gels in different species. He did not find heterogeneity in size between tissues from the same organism. However, this method would not detect changes in chain length of less than 4%.

Gould (1966, 1967) looked for differences in the large rRNA component between organisms and tissues from the same organism on 5% gels after limited digestion with T1 RNase. His method produced large fragments with a molecular weight of 10,000-40,000. While he showed large differences between

organisms, especially between eucaryotes and procaryotes, he could detect little difference between different tissues from the mouse.

Analysis of base composition of the rRNA has produced varying results. Hirsh (1966) found no significant base differences in rRNA from rabbit reticulocytes and liver. With this method a 40-60 nucleotide change would be required to produce a detectable difference. Amaldi and Attardi (1971) likewise reported no difference between HeLa cells and several normal human tissues. Higashi et al. (1971) have not found base compositional differences in mature ribosomes from rat tumor and normal tissues. Nevertheless, differences in the base composition of 45S precursors in these tissues have been shown (Yadzi, et al., 1969).

A more sensitive technique for determining differences between base sequences involves finger printing of oligonucleotide fragments. In this procedure radioactively labeled RNA is degraded with Pancreatic RNase A in combination with T1 RNase. The resulting oligonucleotide mixture is then chromatographed by two dimensional high voltage ionophoresis (Sanger, et al. 1965). Most workers using this technique do not recover the oligonucleotides of rRNA in molar yields. Depending on the bias of the investigator, this can be explained by a heterogeneity of the base sequence expressed, or could be the result of inaccuracies in the method.

Using this technique, Fellner, et al. (1972) found heterogeneity of the 16S RNA primary sequence in E. coli. One of the

fragments which was recovered in less than molar yield was methylated while another fragment with the same base sequence was unmethylated.

Attardi and Amaldi (1971) using labeled HeLa cell rRNA compared the oligonucleotides produced by T1 digestion with unlabeled rRNA from normal human tissues. They observed no difference in relative specific activity of the fragments between HeLa cell rRNA and any normal tissues.

Other investigators, however, have reported differences based on the oligonucleotide distributions of rRNA. Busch's group (Seeber and Busch, 1971; Wikman, et al., 1970; Nazar and Busch, 1972) has found a significant difference in 28S rRNA from Novikoff ascites cells and rat liver. They have shown a difference in distribution in GpU and GpC dinucleotides produced by pancreatic RNase digestion.

Hashimoto and Muranatsu (1973) studied rRNA from mouse liver and mouse MH 134 hepatoma. They identified two nucleotide sequences (GpG$\overset{*}{p}$Cp and ApApApApUp) in 18S RNA of liver and kidney which were always absent from the hepatoma. Higashi, et al. (1971) reported different distributions of the various size classes of oligonucleotides produced by pancreatic and T1 RNase digestion of rat liver and AH-130 tumor cells. In addition he studied kidney and embryonic liver tissues. It was shown that the oligonucleotide distributions of the adult liver and kidney were similar, but not necessarily identical. A similarity in the frequencies of individual components of dinucleotides was also found on comparison of tumor and fetal

liver cells, but the rRNAs of these less differentiated tissues differed from those of the less actively growing adult liver and kidney (Higashi, et al., 1972).

Small degrees of variation of base sequence in the 5S rRNA has also been noted. In each of two strains of E. coli, two forms have been found (with one in common), in about equal amounts which differ from each other in sequence in one position. (Brownlee, et al., 1968). Ford (1972) has indicated that in Xenopus there are at least three and possibly five different 5S RNA sequences. There is tissue-specific expression of the different sequences, in that only one appears to be expressed in kidney cells but all are found in total RNA from ovary. On the other hand, the 5S species from man (Forget and Weismann, 1969) and mouse were shown to be identical (Williamson and Brownlee, 1969).

Another possible source of variation in ribosome population lies in modification of the basic nucleotide sequence by methylation of the rRNA. The role of methylation of rRNA is not completely clear. Addition of methyl groups to the base or ribose moieties of RNA is known to be a very specific, non-random process (Salim and Maden, 1973; Klagsbrun, 1973). Methylation appears to be determined by specificites of the methylating enzymes for sequence and structure (Salim and Maden, 1973). It has also been shown that methylation occurs in all the major RNA species of the eucaroytic cell: rRNA, tRNA and mRNA (Perry, 1974; Desrosiers, et al, 1974).

Methylation of the high molecular weight components of eucaryotic ribosomal RNA is about 90% 2'-O-methyl (Brown and Attardi, 1968; Lane and Tamaoki, 1969; Vaughn, et al., 1967; Salim and Maden, 1973). This methylation occurs while the rRNA 45S precursor is being synthesized in the nucleolus (Greenberg and Penman, 1966; Zimmerman and Holler, 1967). All or most of the methyl groups which are incorporated into the 45S precursor are conserved during its processing to 18 and 28S rRNA, despite the fact that about one-half of the precursor is lost (Wagner, et al., 1967; Choi and Busch, 1970; Wellanauer and Dawid, 1973). 2'-O-Methylation is necessary for the proper processing of the precursor to its constituent 28 and 18S rRNA products. In HeLa cells, Vaughn, et al., (1967) have shown 45S precursor synthesized in the absence of methionine is not methylated and is not processed to cytoplasmic rRNA, but is turned over in the nucleus.

While all the methyl groups found in the ribose moeities are added before cleavage of the 45S precursor this is not true for all the base modifications (Salim and Maden, 1973). A late base methylation in ribosomal RNA which has been studied recently, occurs on adenosine in the nucleotide sequence $Am^6_2Am^6_2AACUG$. This particular sequence has been found in the smaller high molecular weight component (16S - 18S) of *E. coli* (Ehresman, et al., 1971), yeast (Klootwijk, et al., 1972) and HeLa cells (Salim and Maden, 1973). It has been suggested that this methylation plays some role in the final maturation of the small subunit (Ehresman, et al., 1972) or functions

during protein synthesis itself (Salim and Maden, 1973). It is of interest that a mutant of E. coli which is resistant to the antibiotic kasugamycin is not methylated on this sequence (Hesler, et al., 1971).

Heterogeneity of methylation of rRNA has been investigated. Lau and Lane (1971) found upon alkaline hydrolysis of yeast rRNA that certain dinucleotides (CmA, CmU, UmC and UmU) could not be present in every 17S polynucleotide chain. Lane and Tamaoki (1969) reported that not all the sugar and base methylations could occur on every chain in 16S and 28S RNA from HeLa cells. On the other hand Higashi, et al., (1972) using similar methods could find no evidence for differences in total 2'-O-methylation between mouse liver and AH-130 tumor. Salim and Maden (1973) showed that the majority of methylated products from T1 digestion of HeLa cell rRNA occurred in molar yields. Most of the conclusions concerning heterogeneity or lack of heterogeneity of methylation are open to question since similar results are interpreted differently, depending on the investigator.

In view of the fact that methylation of rRNA is of importance in its processing, and possibly in its function, it is of interest to look for differences between the normal and cancerous tissues. Abnormalities in the morphology of the nucleolus (the site of rRNA synthesis) generally accompany the neoplastic state. This raises the question of involvement between carcinogenesis and rRNA synthesis and processing. Possible differences in expressed sequences have already been

discussed.

Certain carcinogens are known to affect the base and ribose modifications of rRNA. Ethionine induction of liver tumors in rats has been studied by Farber's group (Axel, et al., 1967). Administration of L-ethionine to rats leads to the ethylation of proteins and nucleic acids in the liver. The ethylation of nucleic acids after ethionine administration could be produced by S-adenosyl-L-ethionine substituting for S-adenosyl-L-methionine in the reactions catalyzed by nucleic acid methylases. Pegg (1972) found incorporation of H^3-L-ethionine into tRNA of rats after one dose of 100 mg. to male rats. Of the ethylation all occurred on bases where methylation usually occurs. He also reported that 23% of the ethylation was on the sugar ribose.

Diethylnitrosamine is an acute hepatotoxin and is carcinogenetic in many animals. Prolonged feeding to rats at a high dosage in the diet produces liver tumors. Dimethyl and diethylnitrosamines alkylate nucleic acids of the target organ (Swann and Magee, 1971). Presumably, the mode of action is direct alkylation rather than S-adenosyl mediated alkylation. Swann and Magee (1971) studied alkylation of the N-7 position of guanine in animals injected with C^{14}-diethylnitrosoamine. Ethylation of microsomal, soluble, and nuclear RNA was found.

INTRODUCTION

Little is known about the role of methylation of rRNA. It is known to be a very specific, non-random process and important in the processing of rRNA. The nucleolus is the intranuclear site of sugar methylation of rRNA. Since many carcinogens cause significant changes in nucleolar structure or function, it is of importance to determine whether these agents specifically affect ribose methylation of RNA. It has been suggested that certain chemical carcinogens may interfere with the normal mechanisms for processing newly synthesized nuclear RNA (Al Arif and Sporn, 1972).

There is some evidence for variability in basic nucleotide sequence of rRNA in different cell types. This heterogeneity could extend to variability in methylation of the cytoplasmic rRNA of these cells.

In this study 2'-O-methylation levels and distributions were studied for rRNA prepared from a variety of rat tissues. Several normal differentiated tissues were investigated. Rats were also treated with carcinogens which were known to affect methylation of tRNA. Liver tissue from these rats was studied and compared to normal rat liver. As ascites hepatoma which is very deviated from its parent liver tissue was also investigated.

MATERIALS AND METHODS

Carcinogen treatment of rats

Diethylnitroamine was administered to eight week male Fisher rats in their drinking water at a concentration of 40 mg/l. The rats were maintained on this water for two months and then transferred to tap water for one to two months before they were sacrificed.

Ethionine was administered to 150 g female Sprague-Dawley rats by intraperitoneal injection. Four to five ml of a solution of 25 mg/ml L-ethionine in saline was injected once daily for four days. The rats were starved overnight and sacrificed on the fifth day.

Novikoff ascites cells were maintained in 160 to 200 g male Sprague-Dawley rats by intraperitoneal injection of 1.5 to 2.0 ml of fluid. Cells were transferred every seven days.

Collection of tissues

Animals used for liver preparations were fasted overnight before sacrificing by decapitation. The abdominal cavity and the thorax were opened and the liver was perfused with cold saline through the hepatic vein. The kidneys and testes were removed from the abdominal cavity. The brain was obtained by cutting open the skull with heavy scissors. The tissues were trimmed of connective tissue, rinsed in cold saline, blotted dry and weighed. Livers from diethylnitrosamine treated rats

were trimmed of normal tissue before tumorous tissue was weighed.

The Novikoff ascites cells were drained from the abdominal cavity of the rat after six to seven days of growth. The cells were centrifuged from the ascites fluid at 10,000 x g for ten minutes. The red cells contaminating the fluid form a layer below the ascites cells in the centrifuge tube. The ascites cells were scraped from above the red cells and resuspended in 0.25 M sucrose in TKM buffer (0.01 M Tris-HCl pH 7.6, 0.01 M KCl, o.002 M $MgCl_2$) in a volume equal to the original fluid volume. The cells were recentrifuged at 10,000 x g for ten minutes.

Preparation of ribosomes

The rat tissues were minced with a scissors and suspended in 0.25 M sucrose in TKM buffer at a concentration of 0.2 g tissue per ml buffer. Homogenization was accomplished by five to six passes in a motor driven glass-teflon homogenizer (0.013" clearance). The suspension was rehomogenized in another glass-teflon homogenizer with 0.007" clearance. The homogenate was filtered through two layers of cheesecloth, and centrifuged at 8,000 x g for ten minutes to remove cell debris, mitochondria and nuclei. One tenth volume of 10% DOC was added to the supernatant and this was centrifuged at 28,000 rpm for ninety minutes in a Spinco thirty rotor, to pellet ribosomes. The ribosomal pellet was resuspended in a hand held glass-teflon homogenizer in 0.25 M sucrose in TKM buffer in a volume equal to 10% that of the 8,000 x g

supernatant and one tenth volume of 10% DOC was added. Three ml was then layered over six ml 1 M sucrose in TKM buffer and centrifuged at 38,000 rpm for two hours in a Spinco forty rotor. Alternatively, if the volume was large, five ml of the ribosome suspension was layered over ten ml of 1 M sucrose in TKM buffer and centrifuged at 28,000 rpm for three hours in a Spinco thirty rotor.

Purified ribosomes were suspended in 0.1 M Na acetate pH 5.1 in a hand held glass-teflon homogenizer and 1/20 volume of 10% SDS was added. The concentration of ribosomes was approximately 50 A_{260} units of ribosomes per ml buffer. At this point the preparation was usually frozen at -20°C overnight.

For preparation of ribosomes from Novikoff cells, the washed cells were suspended in six to seven ml TKM buffer per g cells and allowed to stand in an ice bath for ten minutes. The swollen cells were centrifuged at 2,000 x g for ten minutes. The supernatant was carefully decanted and the volume of the cells recorded. TKM buffer was added to make the concentration 0.35 g of cells per ml buffer. The cells were broken by eight passes in a Dounce homogenizer with the tight fitting pestle. Breakage was monitored by a phase-contrast microscope. When cells were well broken, the homogenate was added to an equal volume of 0.5 M sucrose in TKM buffer. This was centrifuged at 30,000 x g for thirty minutes to remove cell debris, mitochondria and nuclei. The rest of the ribosome purification was then performed as for the rat tissues.

Preparation of RNA

Ribosomes suspended in 0.1 M Na acetate and SDS were extracted two times with redistilled phenol (H_2O saturated) at room temperature. The RNA was precipitated overnight from the aqueous phase with two volumes of absolute ethanol. RNA which was to be used for nucleoside ratio determinations was resuspended in 0.1 M Na acetate pH 5.1 and reprecipitated with ethanol. RNA to be used to determine 2'-O-methyl levels was dissolved in water and extracted 2x with ether to remove phenol. The RNA solution was made 0.1 M in Na acetate pH 5.1 at a concentration of about thirty A_{260} units/ml. The RNA was then precipitated with CTAB (Bellemy and Ralph, 1968) followed by two washes with 70% ethanol/0.1 M Na acetate pH 5.1. (CTAB does not precipitate neutral or negatively charged polysaccharides). The precipitate was dissolved in 0.1 M Na acetate pH 5.1 and precipitated with two volumes of ethanol. The RNA was then dissolved in H_2O with a drop of NH_4OH added to facilitate solubilization of the RNA. The RNA solution was stored at -20°C until further analyses were performed.

tRNA was prepared from the supernatant of the first high speed ribosomal sedimentation. The supernatant was extracted three times with an equal volume of phenol at room temperature. The aqueous phase was extracted with ether one time and applied to a water washed, 1.5 x 10 cm DEAE Cl^- column at the rate of one ml/min. The column was washed with about 500 ml of water until no UV material eluted from the column. Any small molecular weight material was eluted with 250 ml of 0.2

M NaCl. tRNA was removed from the column with approximately 75 ml of 1.0 M NaCl. The tRNA was dialyzed against water to remove the NaCl, and lyophilized.

Synthetic polynucleotides used as standards for GC analysis were polymerized from nucleoside diphosphates with polynucleotide phosphorylase (Rottman and Heinlein, 1968). Polynucleotides were checked for contaminating nucleoside diphosphates by chromatography of one OD on Whatman No. 1 paper in Heppel's solvent (n-propanol:NH_4OH:H_2O, 55:10:35). The base ratio of Poly (Am,U) was determined by HSLC.

Characterization of RNA

The purity of the rRNA and tRNA preparations was checked by 2% acrylamide/0.5% agarose gel electrophoresis prepared by the method of Dingman and Peacock, (1968). A sample of RNA (0.2 A_{260} units) was applied to the gel slot and electrophoresis was carried out at 200 v for 2 to 2.5 hours at 4°C. The gel was stained in 'Stains all' (0.005% in 50% formamide) overnight. After destaining, the gel was cut into 0.5 cm x 10 cm strips and scanned in a Gilford linear transport gel scanner at 570 nm.

For ultraviolet spectral analysis the RNA solution was diluted to about 50ug/ml in TKM buffer. The absorbances at 260 and 280 nm were recorded and ultraviolet spectra were obtained from 210 to 310 nm with a Gilford Scanning spectrophotometer.

To determine the nmoles of phosphate per A_{260} unit of RNA a total phosphate assay was performed (Ames and Dubin, 1960).

Pyrex or Kimax test tubes (10 cm x 1.2 cm) were soaked overnight in aqua regia to remove any residual phosphate from detergent used to wash glassware. RNA was diluted to a concentration of 0.2 mg/ml and 50 ul aliquots were placed in the sample tubes. Using the same 50 ul pipette, aliquots were added to 950 ul TKM and the absorbance at 260 nm was recorded. Standard and blank tubes were also prepared using a phosphorous standard solution purchased from Sigma.

RNA was ashed by adding 25 ul of 10% $MgNO_3$ in ethanol and heating in the flame of a bunsen burner until brown fumes were no longer produced. After cooling the tubes, 0.6 ml of 0.5 N HCl was added, and the tubes were mixed on the vortex mixer until the white ash was completely dissolved. The tubes were topped with marbles and heated for twenty minutes in a boiling water bath. The samples were cooled and briefly centrifuged in the clinical centrifuge to remove condensed liquid from the sides of the tubes.

Inorganic phosphate was assayed using the method of Chen et al. (1956). To each tube 1.4 ml of Reagent C (10% ascorbic acid: 0.42% NH_3molybdate in 1 N H_2SO_4, 1:6) was added. The samples were incubated at 45°C for twenty minutes. Absorbance was measured at 820 nm. The standard curve was linear from 0 - 90 nmoles of phosphate.

Adequacy of the ashing procedure and accuracy of the phosphate standard was verified using AMP. The concentration of an AMP solution was determined by measuring the absorbance of an aliquot at 257 nm in 0.01 N HCl. An extinction coefficient

of 15.1×10^{-3} was used to calculate the concentration. Varying amounts of this solution were carried through the ashing procedure and the inorganic phosphate was measured as above.

Gas Chromatography

The determination of 2'-O-methyl levels by GC was adapted from the method of Abbate and Rottman, (1972). Hydrolysis tubes were prepared by cutting 0.5 cm pyrex tubing into ten cm. lengths. The tubes were soaked in aqua regia overnight, rinsed, boiled in detergent for fifteen minutes, then in distilled water for fifteen minutes, rinsed well with distilled water and sealed at one end.

Six foot glass columns for the gas chromatograph were cleaned in chromic acid cleaning solution overnight. The column was rinsed well with distilled water and washed with nanograde methanol. The column was then washed with 5% dimethyldichlorosilane in hexane and rinsed with methanol and dried. This silanization was necessary to reduce trailing of water due to interaction with active sites on the glass.

The column was packed with Porapak Q 100-120 mesh (Waters assoc.). Packing was accomplished by attaching a vacuum hose from a water aspirator to one end of the column and pouring the packing in the other while vibrating the column with an electric vibrator. Careful packing was necessary to produce a column with adequate resolution and reasonably symmetrical peaks. If this was not accomplished the column was discarded and a new one made. Occasionally a particular lot of Porapak Q was found to be inadequate for the resolution of methanol and

acetaldehyde. The top three to six inches of the column was replaced every few runs. This was necessary to remove the accumulation of tar and degradation products from the hydrolysis reaction. The column was conditioned at a flow rate of 0.3 cc/sec at 240°C for five to six hours, then at 200°C at least twenty-four hours prior to use.

For hydrolysis, a 40 to 100 ul aliquot of the RNA solution was introduced into the hydrolysis tube. The solution was centrifuged to the bottom of the tube, frozen in dry ice and lyophilized. Thirty ul of 70% $HClO_4$ was added to each tube, the tube was sealed and centrifuged in a clinical centrifuge. Hydrolysis was carried out at 100°C, for sixty minutes, in a sand bath kept in an oven. After hydrolysis the tubes were cooled slightly and placed in powdered dry ice. While the contents remained frozen, the tubes were opened and 40 ul of 10 N KOH was added to the upper portion of the tube and resealed. The KOH solution was carefully titrated against the $HClO_4$ so that the final solution was slightly basic (pH 7 to 9), since acidic solutions rapidly destroyed the packing. After sealing, the KOH and $HClO_4$ were mixed by vortexing. Care was taken not to allow the mixture to become too hot and the tubes were replaced in dry ice to facilitate precipitation of $KClO_4$. The tubes were centrifuged at 20,000 x g for twenty minutes. Thirty-five ul of the aqueous supernatant was carefully withdrawn with a Hamilton syringe and transferred to a cold 6 x 50 mm pyrex tube. To this, 10 ul of a 0.4 ug/ul solution of n-propanol was added, as an internal standard.

The tubes were sealed immediately and stored at -20°C until further use.

Gas chromatography was carried out on a Hewlett-Packard F and M 402 gas chromatograph with a flame ionization detector. The column was run at 140°C with a nitrogen flow of 75 cc/min. The optimum hydrogen flow rate varied considerably and had to be determined for each column.

The pH of each sample was checked by spotting a small amount on pH paper prior to injection. If the pH was below 6, a small amount of 10 N NaOH was added to the sample before injecting into the column. Sample size was generally 4 ul.

The methanol/propanol ratio was obtained by cutting out the area under each peak and weighing the paper on an analytical balance. In each run 2'-O-methyladenosine was carried through the hydrolysis as a standard. The amount of 2'-O-methyladenosine was determined by measuring the absorbance at 257 nm and using an extinction coefficient of 15.1×10^{-3}. The methanol/propanol ratio produced by this known standard was set at 100% methylation and the values for the other samples were calculated accordingly. The mole percent of methanol was determined by dividing the moles of methanol produced by the moles of polymer phosphate in the sample.

Determination of nucleoside ratios in RNA

The determination of ribonucleoside and 2'-O-methylnucleoside ratios was primarily that described by Pike and Rottman, (1974). RNA was hydrolyzed to its component nucleosides by a combination of enzymes. The reaction mixture

contained 5 mg of RNA in 1.2 ml 50 mM NH_4formate, pH 9.0, and 2 mM MgAc. Bacterial Alkaline phosphatase (Worthington Biochemical Corp., BAPF) was dialyzed six hours against 0.05 M NH_4HCO_3 and 5 units was used for the reaction. Bovine pancreatic ribonuclease A (Sigma Chemical Co. Type III A) was added at 10 ug per mg RNA. Phosphodiesterase I from Crotalus adamanteus venom (Worthington Biochemical Corp., VPH) was dissolved in water with a resulting concentration of 5 mg/ml and 0.3 mg was used for the reaction. Hydrolysis was carried out at 37°C for thirty-six hours.

Hydrolysis of RNA to nucleosides was assayed by paper electrophoresis of a 10 ul aliquot of the reaction mixture on Whatman No. 1 paper. Electrophoresis was performed in 0.05 M Tris Cl^- pH 7.8, at 400 v for forty-five minutes in a Gelman electrophoresis chamber. The reaction was considered complete if all visible UV absorbing material (estimated to be greater than 97%) remained near the origin.

2'-O-Methyl nucleosides were separated from the normal nucleosides in the reaction by DE-22 borate chromatography. Fibrous DE-22 (Whatman) was recycled with 0.5 M HCl and 0.5 M NaOH, then converted to the borate form with 0.7 M boric acid. A 1.1 x 13 cm column was poured, washed with 250 ml 0.7 boric acid and equilibrated with 150 ml 0.15 M boric acid, prior to use. One ml of the reaction mixture was applied and the column was eluted with 0.15 M boric acid. Three to four ml fractions were collected at the rate of 1 ml/min. 2'-O-Methyl nucleosides were eluted in the first twenty ml following the

void volume. Normal nucleosides were eluted with 0.7 M boric acid. The 2'-O-methyl nucleoside fraction was taken to dryness by flash evaporation and the boric acid was removed as its methylester by two flash evaporations with methanol. The nucleosides were dissolved in water, transferred to a small test tube and lyophilized. The column could be regenerated and reused about ten times before loss of resolution occurred.

Ribonucleoside compositions were determined by High Speed Liquid Chromatography (HSLC) on a Chromatronix SS-2-500 column. The column was packed at 3000 psi with Bio-Rad Aminex A-5 cation exchange resin which had been defined and washed with 50% acetone, 50% ethanol and 3 M NH_4formate. The buffer used was 0.4 M NH_4formate adjusted to pH 4.55 with concentrated formic acid. The ribonucleosides were eluted at 26°C with a flow rate of 33 ml/hr and a column pressure of 2300 to 2500 psi.

2'-O-Methylnucleoside compositions were determined on a ninety cm column of stainless steel tubing, 1/8 in. O.D. by 0.02 in. I.D. packed with Aminex A-5 treated as above. The buffer used was 0.4 M NH_4formate in 40% ethylene glycol adjusted to pH 4.15. The 2'-O-methyl nucleosides were eluted at 40°C with a flow rate of fifteen ml/hr and the column pressure was 3000 psi.

The buffers for both columns were filtered through a millipore filter (0.25 u) and degassed. The buffers were pumped through the columns with a Milton Roy Mini-pump capable of 5000 psi and elution was monitored with a Gilford

spectrophotometer using a 40 ul flow cell with a 1 cm light path. Full scale on the chart was generally 0.10 OD. Most nucleosides were monitored at 260 nm, but 2'-O-methylcytidine was usually monitored at 271 nm.

Areas under the peaks were calculated by measuring heights x width at half-height. The areas were converted to relative molar amounts using extinction coefficients determined by measuring a known concentration of each nucleoside at pH 4.55 at the appropriate wavelength. (A and A_m, $a_M \times 10^{-3} = 15.2$ at 260 nm; G and Gm, $a_M \times 10^{-3} = 11.7$ at 260 nm, U and Um, $a_M \times 10^{-3} = 10.0$ at 260 nm; C and Cm, $a_M \times 10^{-3} = 6.9$ at 260 nm or $a_M \times 10^{-3} = 10.0$ at 271 nm). These values agreed with those calculated from Circular OR-10, P. L. Biochemicals Inc. Compositions were determined as the portion of each nucleoside relative to the sum of all four nucleosides.

RESULTS

Purity and yield of rRNA

Purified ribosomes were used to prepare the rRNA for these studies to insure that a well defined population of RNA, free of DNA, was obtained. The procedure used was optimized to produce relatively large yields of ribosomes with high purity. The preparation of ribosomes was carried out in the cold as quickly as possible to reduce degradation of the rRNA.

Generally, tissues used for ribosome purification were fresh. Occasionally testes or brain were frozen at -80 C for use later. Freezing reduced the yield of ribosomes considerably. Since the rRNA extracted from frozen tissues showed little degradation the reduction in yield was presumably due to difficulty in breaking frozen cells.

The yield of rRNA varied between tissues (Table 1). This is probably a reflection of the efficiency of cell breakage as well as differences in actual ribosome number. Rat tissue homogenates were centrifuged at 8,000 x g to remove nuclei and cell debris, instead of 10,000 or 15,000 x g as is generally done. It was found that microsomes tended to be lost in the higher speed centrifugation. Due to the lower speed spin the ribosomes may be slightly contaminated with mitochondria.

UV absorbance spectra were taken at various stages during the rRNA preparation. As can be seen in Figure 1, centrifugation

through 1M sucrose greatly reduced the amount of contaminating proteins in the ribosome preparation. Phenol extraction to remove ribosomal proteins, and ethanol precipitation, results in a 260 nm/ 280 nm absorbance ratio of 2.03 to 2.09.

Purity of the rRNA was also analyzed by 2% polyacrylamide, 0.5% agarose gel electrophoresis. Figure 2 shows the profiles obtained for various tissues. Only kidney rRNA showed considerable degradation bands. These could be reduced or increased depending on the rapidity with which the ribosomes were purified.

To calculate mole percent of 2'-O-methyl nucleosides for the RNA it was necessary to quantitate the number of nucleotides in a given RNA sample. This was done using the total phosphate assay to determine polymer phosphate per A_{260} unit of RNA. Typically, rRNA from rat tissue was about 130 nmoles/ A_{260} (Table 2).

CTAB precipitation of RNA used for the GC analysis is important in that it elimates neutral and basic polysaccharides. Polysaccharides frequently contain methoxy residues which would contribute extraneous methanol during the methyl analysis of RNA. Plant tissues present a real problem in this respect, since they contain high levels of polysaccharides (Jakob and Tal, 1973). Polysaccharides with a negative charge will co-purify with RNA in the CTAB precipitation.

Table 1. Yield of ribosomal RNA from rat tissues

	Grams tissue / Rat	A_{260} units / g tissue Ribosomes	rRNA
Rat liver	15.0	23	12.0
Rat brain	1.7	10	8.0
Rat testes	3.2	21	12.8
Rat kidney	2.0	24	14.5
Rat liver Ethionine treated	8.0[a]	16	13.0
Rat liver Nitrosodiethylamine	12.0[b]	25	15.2
Novikoff ascites cells	6.5	35	18.0

[a]While rats generally used in these studies were young adult males (250-300 g), ethionine treated rats were smaller females (150-200 g).

[b]Weight represents areas of liver which were tumerous. Healthy tissue was discarded.

Figure 1. UV scan of rat brain ribosomes and rRNA. All measurements were at pH 7.8 in 0.01 M Tris Cl^-, 0.01 M KCl, and 0.003 M Mg Cl_2. A) Ribosomes sedimented from 0.25 M sucrose in TKM; A_{260}/A_{280} = 1.42. B) Ribosomes purified by centrifugation through 1.0 M sucrose; A_{260}/A_{280} = 1.77. C) Purified ribosomal RNA after ethanol precipitation; A_{260}/A_{280} = 2.04.

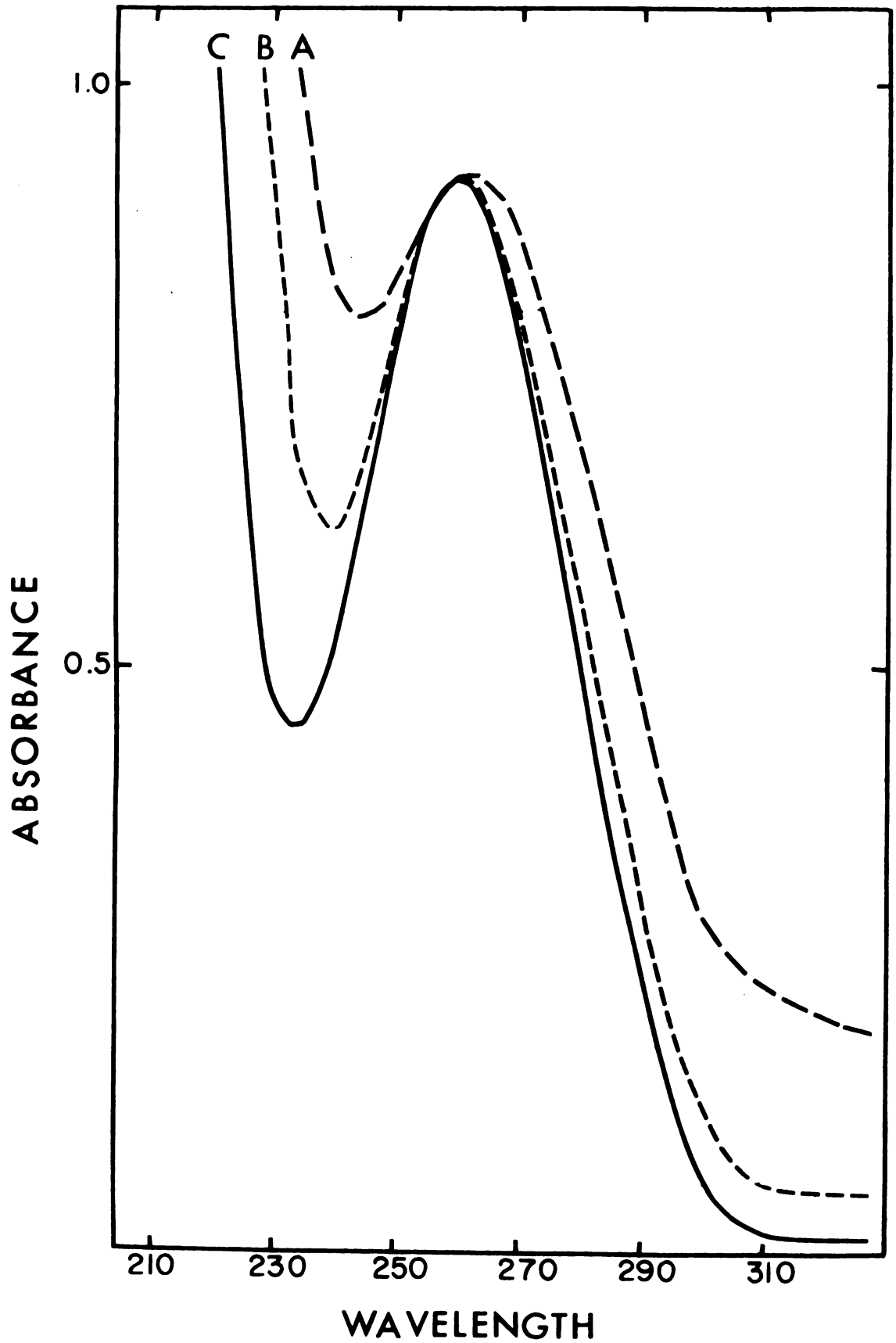

Figure 1

RNA characterization by GC assay

For this study it was important to be able to recognize small differences in 2'-O-methyl levels between different rRNA samples. Therefore, the GC method used for these determinations was carefully studied and optimized to give good resolution of the methanol from the other RNA hydrolysis products.

Polar compounds in water present a problem in gas chromatography. Most GC packing materials are designed for use with samples in organic solvents. Porapak was used as it is relatively resistant to water and reduces the trailing of polar compounds.

The GC column was packed as described in Materials and Methods. An improperly packed column produced assymetrical peaks and long tails. Tailing interfered with quantitation of the peaks due to overlap with other peaks and nonlinear response in the tail region. Sample size was kept small (4 ul) to minimize cooling of the flame when water eluted from the column. The pH of the sample was very important and each sample was checked before injection. If an acidic sample (pH 6.5) was applied, the column resin broke down and gave a late eluting peak, as well as an increased baseline in the methanol region. A basic sample (pH 9.0) produced similar but not as drastic results. While an acidic sample could be titrated with base immediately before injection, a basic could not be titrated with acid as this produced a cloudy precipitate. A GC profile is shown in Figure 3.

Figure 2. Polyacrylamide-agarose gel electrophoresis of RNA from rat tissues. Gel consisted of 2% polyacrylamide, 0.5% agarose buffered with Peacock's buffer pH 8.3. 0.25 A_{260} units of RNA was applied to each gel. Migration was from left to right at 200v, 0°c for 2 to 2.5 hours. A) Novikoff rRNA, B) Rat liver rRNA, C) Rat kidney rRNA, D) Rat testes rRNA, F) Ethionine treated rat liver tRNA.

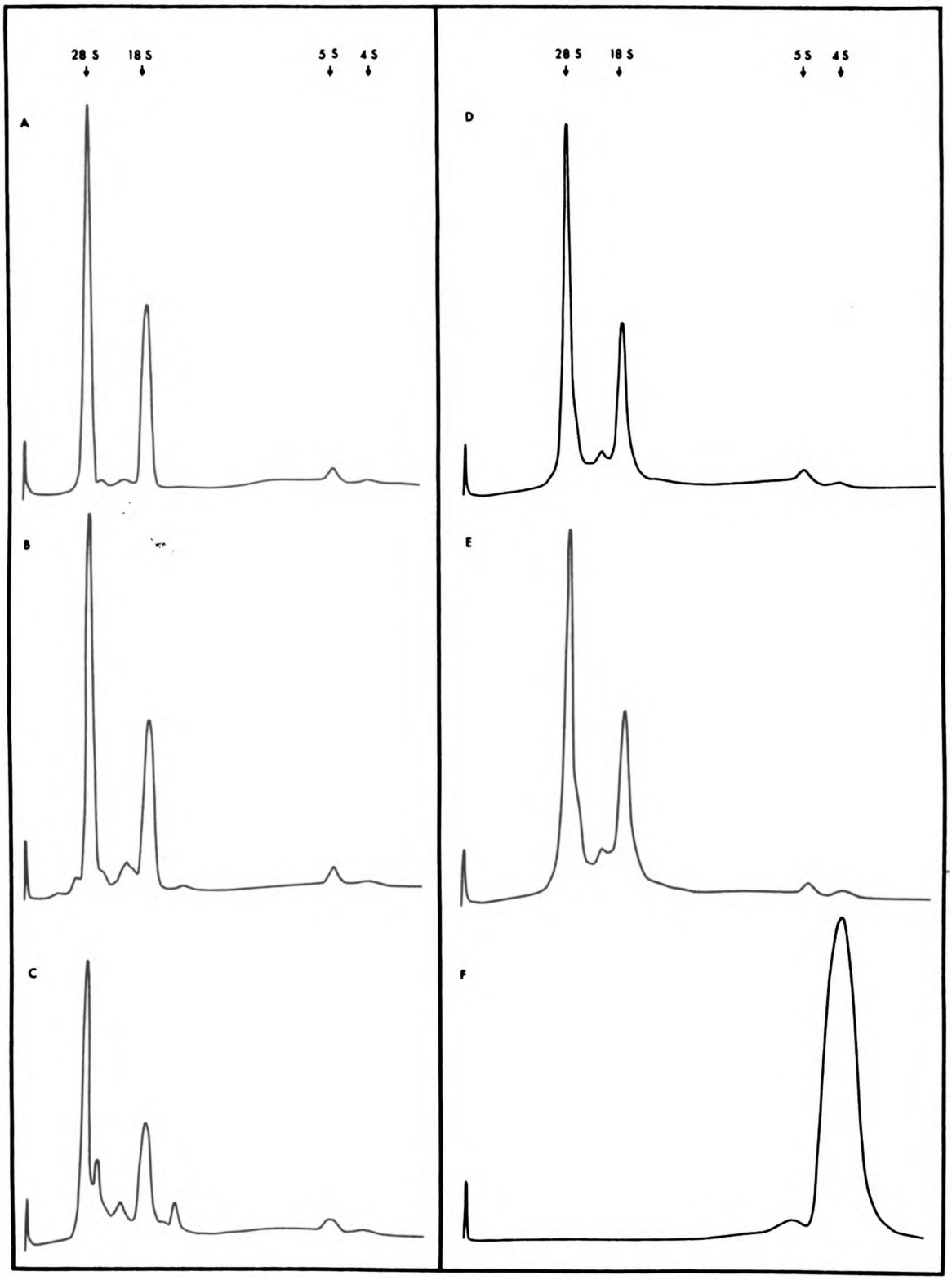

Figure 2

Table 2. Determination of polymer phosphate.

	$\frac{\text{nmoles } PO_4^{\equiv}}{A_{260} \text{ unit RNA}}$	Number of determinations
Rat Tissues		
Rat liver rRNA	126-133	6
Rat brain rRNA	130-133	2
Rat kidney rRNA	117-130	4
Rat testes rRNA	126-140	3
Novikoff rRNA	126-136	6
Rat liver rRNA Ethionine treated	124-126	2
Rat liver rRNA Nitrosodiethylamine	129-131	2
Standards for GC assay		
Poly (Am,U)	107	2
Poly (Am)	109	1
Poly (Um)	107	1
E. coli rRNA	112	1

Hydrolysis of RNA by $HClO_4$ produces free bases, acetaldehyde, acetone and methanol. Furfural appears as an intermediate reaction product. The time courses of hydrolysis are shown in Figure 4 and Figure 5. Methanol was completely released within ten minutes. The reaction was allowed to proceed for sixty minutes to reduce the size of the acetaldehyde peak which could interfere with the methanol peak. This also eliminated the furfural peak which eluted late from the GC column.

The effect of varying the amount of RNA in the samples used for determination of 2'-O-methyl content was studied for Novikoff and E. coli rRNA. At low levels of RNA there is an apparent elevation of released methanol (Figure 6). The cause of this is unknown but is probably not due to the small size of the methanol peak at these low RNA amounts, since the peaks in the linear region for the E. coli rRNA were smaller than those in the elevated region of Novikoff rRNA. The amount of RNA necessary to reach the linear part of the curve depends partly on the level of 2'-O-methyl in the sample. For rat tissue rRNA 1000 nmoles were usually used in each sample. More RNA was necessary for samples containing lower 2'-O-methyl levels.

The tubes containing 2'-O-methyladenosine used for standards had 20 ug of Poly(A) added to prevent loss of the lyophilized sample. When Poly (A) was not used the nucleoside formed a very fine powder during lyophilization which was

Figure 3. Gas chromatography elution profile. Area within bracket indicates change in chart speed from one inch per minute to five inches per minute. Arrow (A) indicates change in attenuation from Range 1, Attenuation 8, to Range 10, Attenuation 2. Sample was 4 ul rat liver rRNA. Identification of peaks: 1) methanol, 2) acetaldehyde, 3) ethanol, 4) acetone and 5) propanol.

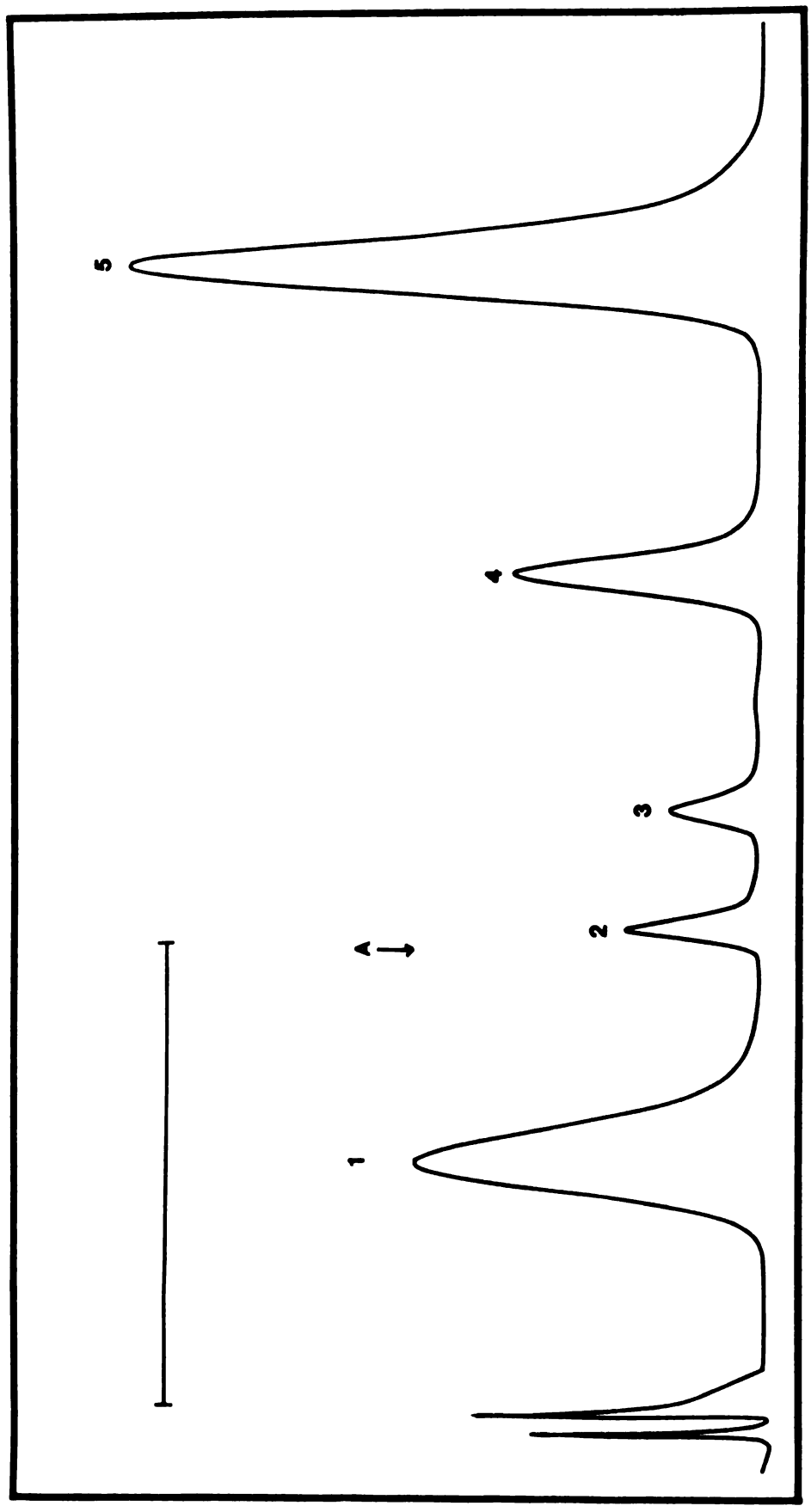

Figure 3

Figure 4. Time course of hydrolysis; Methanol production.

Duplicate determination performed on 1 umole Novikoff ascites rRNA.

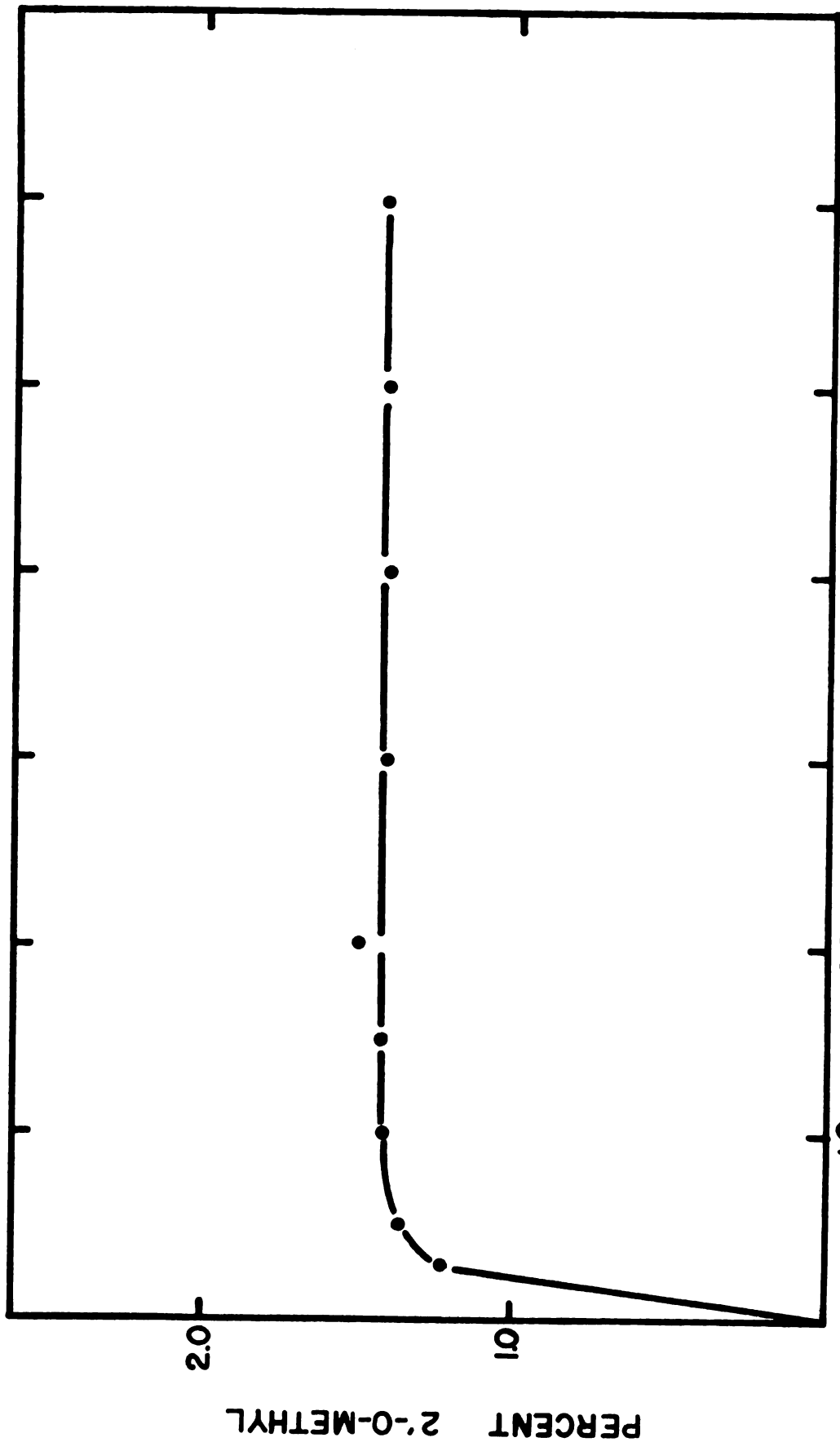
PERCENT 2'-O-METHYL
2.0
10
10
20
30
40
50
60
TIME OF HYDROLYSIS (min)

Figure 4

Figure 5. Time course of hydrolysis; production of reaction products. A) Furfural, B) Acetone, C) Acetaldehyde.

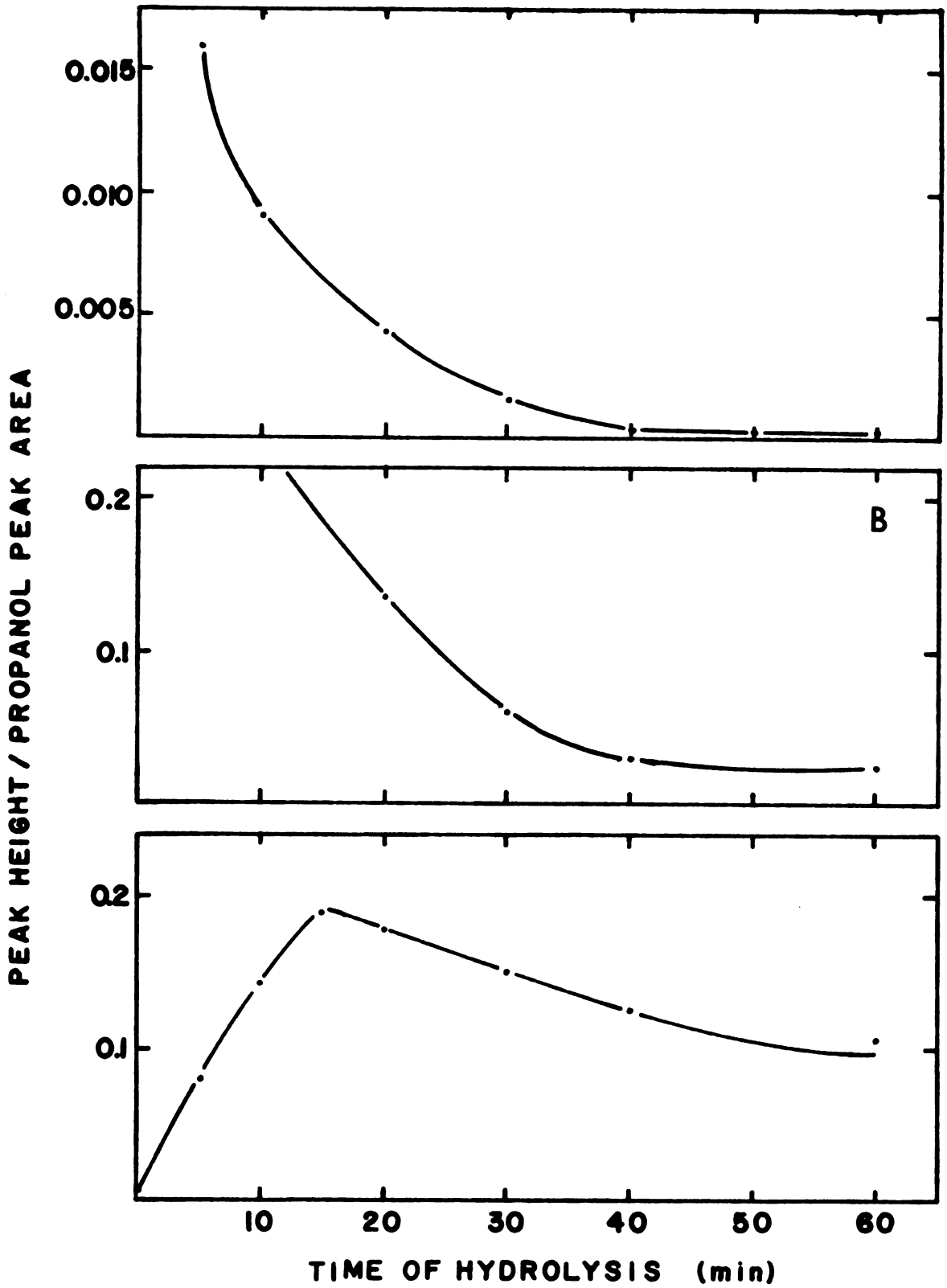
0.015
0.010
0.005
0.2
0.1
B
0.2
0.1
PEAK HEIGHT / PROPANOL PEAK AREA
10
20
30
40
50
60
TIME OF HYDROLYSIS (min)

Figure 5

Figure 6. Dependence of 2'-O-methyl determination on amount of sample hydrolyzed. Determinations were made in duplicate. A) E. coli rRNA, B) Novikoff cell rRNA.

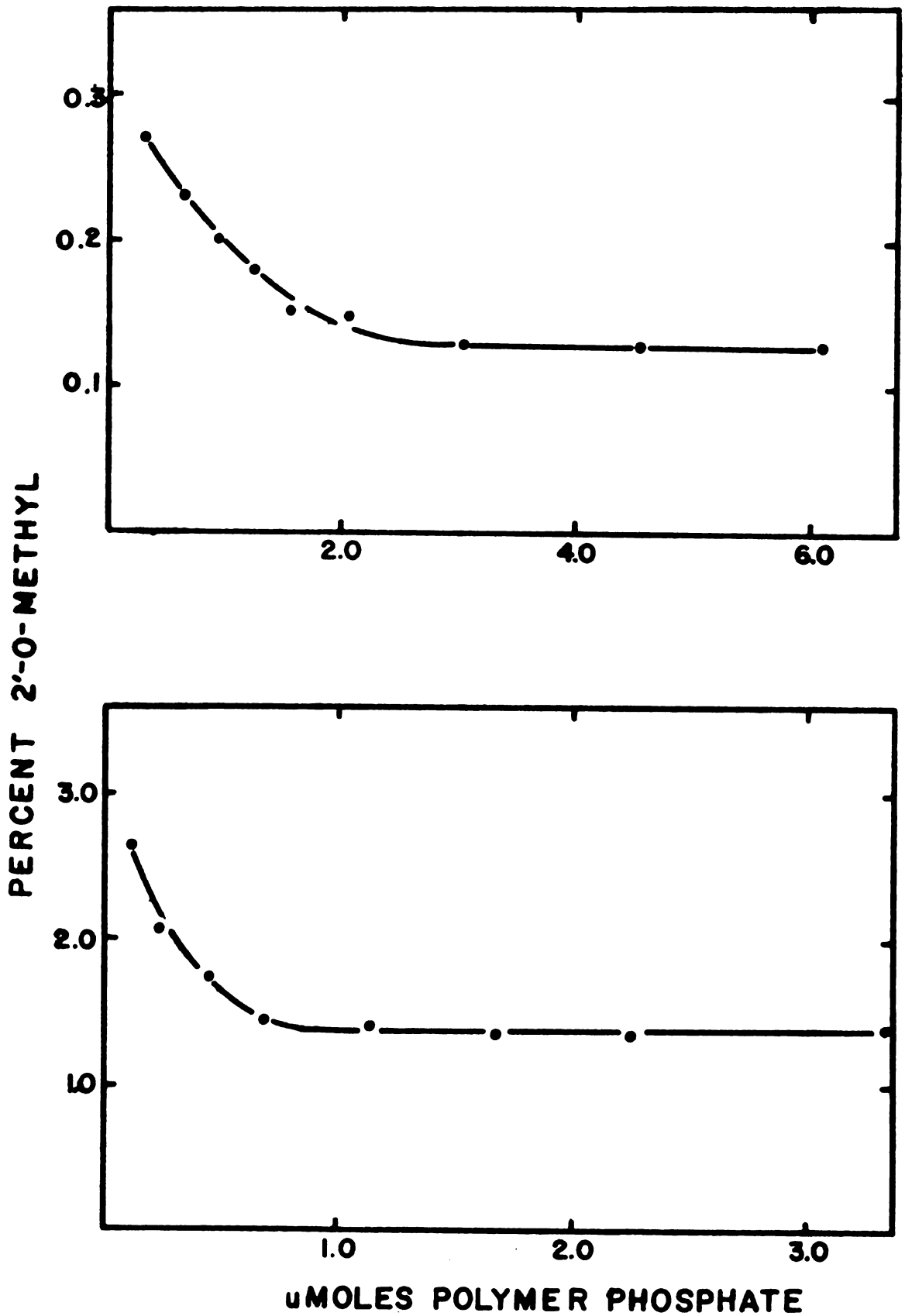
0.3
0.2
0.1
2.0
4.0
6.0
3.0
2.0
1.0
2.0
3.0
PERCENT 2'-O-METHYL
uMOLES POLYMER PHOSPHATE

Figure 6

easily lost during later manipulations. Poly (A) was assayed by itself to make certain that it contained no 2'-O-methyl components.

The accuracy of the CG assay was investigated using RNA with known 2'-O-methyl contents. The results of this study are shown in Table 3. As can be seen, the method is accurate over a range of 0-100% 2'-O-methylation.

The 2'-O-methyl levels for the various rat tissues is shown in Table 4. A nested analysis of variance was used to look for differences between the tissues (Table 5). An overall tissue difference was found at the 0.001 confidence level ($F=9.18$; critical value $F_{0.001,6,19}=6.18$). Using Scheffe's procedure each of the tissues was then compared to liver. Kidney rRNA was the only tissue with a significant difference from liver rRNA at the 0.05 confidence level. However, this tissue was also the only one in which degradation was a problem. Therefore Cochran's test was used to examine variability in the preparation of this tissue. It was found that kidney was the only tissue in which there was significant variability between preparations ($C = 0.66$; critical value $C_{0.05,6,2} = 0.62$) It is probable that kidney rRNA lost some of its non-methylated segments during isolation and thus showed an artifactual increase in 2'-O-methyl levels. Some of the kidney preparations showed less rRNA degradation and these gave lower 2'-O-methyl levels (Table 4). Therefore the variation in 2'-O-methyl levels between preparation of kidney rRNA was probably due to greater or lesser degrees of degradation.

Table 3. Accuracy of GC Assay.

	Percent 2'-O-methyl	
	Experimental	Expected
E. coli rRNA	0.13	0.1[a], 0.12[b], 0.17[c]
Novikoff rRNA	1.44	1.47[d]
Poly (Am,U)	8.43	8.6[e]
Poly (Am)	99.5	100
Poly (Um)	106.0	100
Poly (A)	N.D.	0

[a] Nichols and Lane (1966).

[b] Isaksson and Phillips (1968).

[c] Hayashi, et al. (1966).

[d] Egawa, et al. (1971).

[e] Determined by HSLC Method.

N.D. none detectable.

Table 4. 2'-O-Methyl levels for rat tissues.

	Percent 2'-O-Methyl	
	Average for each preparation	Average for each tissue
Rat liver rRNA		
Prep. 1	1.57 (5)[a]	
Prep. 2	1.58 (5)	
Prep. 3	1.47 (6)	1.51 ± 0.053[b]
Prep. 4	1.44 (9)	
Prep. 5	1.53 (6)	
Prep. 6	1.53 (14)	
Rat testes rRNA		
Prep. 1	1.44 (10)	
Prep. 2	1.50 (6)	1.47 ± 0.062
Prep. 3	1.47 (6)	
Prep. 4	1.47 (6)	
Rat brain rRNA		
Prep. 1	1.50 (6)	1.49 ± 0.086
Prep. 2	1.49 (6)	
Rat kidney rRNA		
Prep. 1	1.63 (9)	
Prep. 2	1.76 (8)	1.67 ± 0.062
Prep. 3	1.76 (6)	
Prep. 4	1.49 (5)	
Novikoff ascites rRNA		
Prep. 1	1.46 (8)	
Prep. 2	1.49 (9)	
Prep. 3	1.47 (10)	1.44 ± 0.049
Prep. 4	1.39 (7)	
Prep. 5	1.41 (17)	
Prep. 6	1.43 (8)	
Diethylnitrosomine treated rat liver rRNA		
Prep. 1	1.40 (8)	1.38 ± 0.084
Prep. 2	1.37 (13)	
L-ethionine treated rat liver rRNA		
Prep. 1	1.60 (2)	
Prep. 2	1.55 (5)	1.57 ± 0.081
Prep. 3	1.58 (3)	

[a]Number in parenthesis indicates the number of determinations.

[b]95% confidence interval calculated from nested analysis of variance.

Table 5. Nested Analysis of Variance; 2'-O-methyl levels.

	S.S.	d.f.	M.S.	E[M.S.]	f Ratio (df)
Tissues	1.43797	6	0.239662	0.025774	9.299 (19)
Preparations in each tissue	0.50189	20	0.025094	0.025094	
GC runs in each preparation	0.47167	65	0.007256	0.007256	
Samples in GC runs	0.36432	111	0.003282	0.003282	

Rats treated with ethionine could possibly incorporate 2'-O-ethyl groups in their rRNA. It has been shown that 2'-O-ethylation does occur in tRNA from animals given a single dose of H^3-L-ethionine. Perchloric acid hydrolysis of 2'-O-ethyl ribose releases ethanol and can be detected by the gas chromatography method. RNA from ethionine treated rats was lyophilized 3-4 times to remove residual ethanol remaining from ethanol precipitation of the rRNA. No ethanol was detected in rRNA from ethionine treated rats. tRNA from these rats was also studied. There was no decrease in methylation of this tRNA and no ethanol was produced. This could indicate that the treatment with ethionine was not sufficiently extensive to detect incorporation into the 2' position over the large background of non-altered tRNA.

Determination of nucleoside ratios

Enzymatic hydrolysis was performed as described in materials and methods. The action of Snake Venom phosphodiesterase was not always consistant. After repeated freezing and thawing, the enzyme became less potent and a contaminating deaminase seemed to become more active. When this occurred an Im peak was found in the 2'-O-methyl distribution analysis with a corresponding decrease in Am. Phosphodiesterase purchased from Sigma Biochemicals showed an even greater deaminase activity than that from Worthington.

To overcome decreased activity of the phosphodiesterase and increased deaminase activity several precautions were taken. Every reaction was monitored by paper electrophoresis after

twenty-four hours of incubation. If UV absorbing material was found in the dinucleoside, indicating incomplete hydrolysis, a new enzyme solution was used for subsequent degradations. To reduce deamination, the reaction was not stored after completion of hydrolysis but applied immediately to the DE-22 borate column. 2'-O-Methyl nucleosides were isolated from the normal nucleosides in RNA since they represent only one to two percent of the total mixture. This was accomplished by taking advantage of the ability of ribonucleosides to form a complex with the borate ion through which they could be retained on an anion exchange column. The 2'-O- methyl nucleosides could not form this complex with their blocked 2'-hydroxyl group and were not retained. Figure 7 shows a typical elution profile from the DE-22 column. After repeated use of the column the volume in which the 2'-O-methyl nucleosides eluted became larger.

Ribonucleoside ratios were determined by direct injection of small amounts of the reaction mixture, prior to borate chromatography, into the Chromtronix SS-2-500 HSLC column and elution with 0.4 M NH_4formate at pH 4.55. This had to be done soon after the reaction was complete as deamination could occur if the reaction was allowed to remain at room temperature. Freezing the reaction mixture caused precipitation of guanosine and phosphate salts. Alternatively, the non-2'-O-methyl nucleosides (ribonucleosides) could be collected from the DE-22 borate column with 0.7 M boric acid elution. An elution profile for the ribonucleotides compositional analysis is shown in Figure 8.

If the hydrolysis reaction was not complete, a peak eluting before uridine was seen. This was due to negatively charged nucleotides appearing in the solvent front.

The ratios of the ribonucleosides for the various rat tissue is shown in Table 6. All the tissues had very similar nucleoside ratios. This data was not subjected to statistical analysis.

The ratio of the 2'-O-methyl nucleosides was determined by HSLC on a 0.02 in. by 90 cm column eluted with 0.4 M NH_4 formate in 40% ethylene glycol, pH 4.15 (Figure 9). At this pH all the 2'-O-methyl components are well separated. The pH of this buffer must be checked each day, as the pH tends to increase as the buffer stands. At higher pH the Gm and Am peaks become less well resolved.

The ratio of the 2'-O-methyl nucleosides for the various tissues is shown in Table 7. Using a nested analysis of variance, no difference between tissues was found for any of the 2'-O-methyl nucleosides at the 0.01 confidence level (Table 8).

Figure 7. DE-22 borate chromatography of enzymatic hydrolysis. Rat liver rRNA was digested as described in Materials and Methods. A) Elution of 2'-O-methyl nucleosides with 0.15 M boric acid. B) Elution of ribonucleosides with 0.7 M boric acid. Order of elution of ribonucleosides is C, U, A, G.

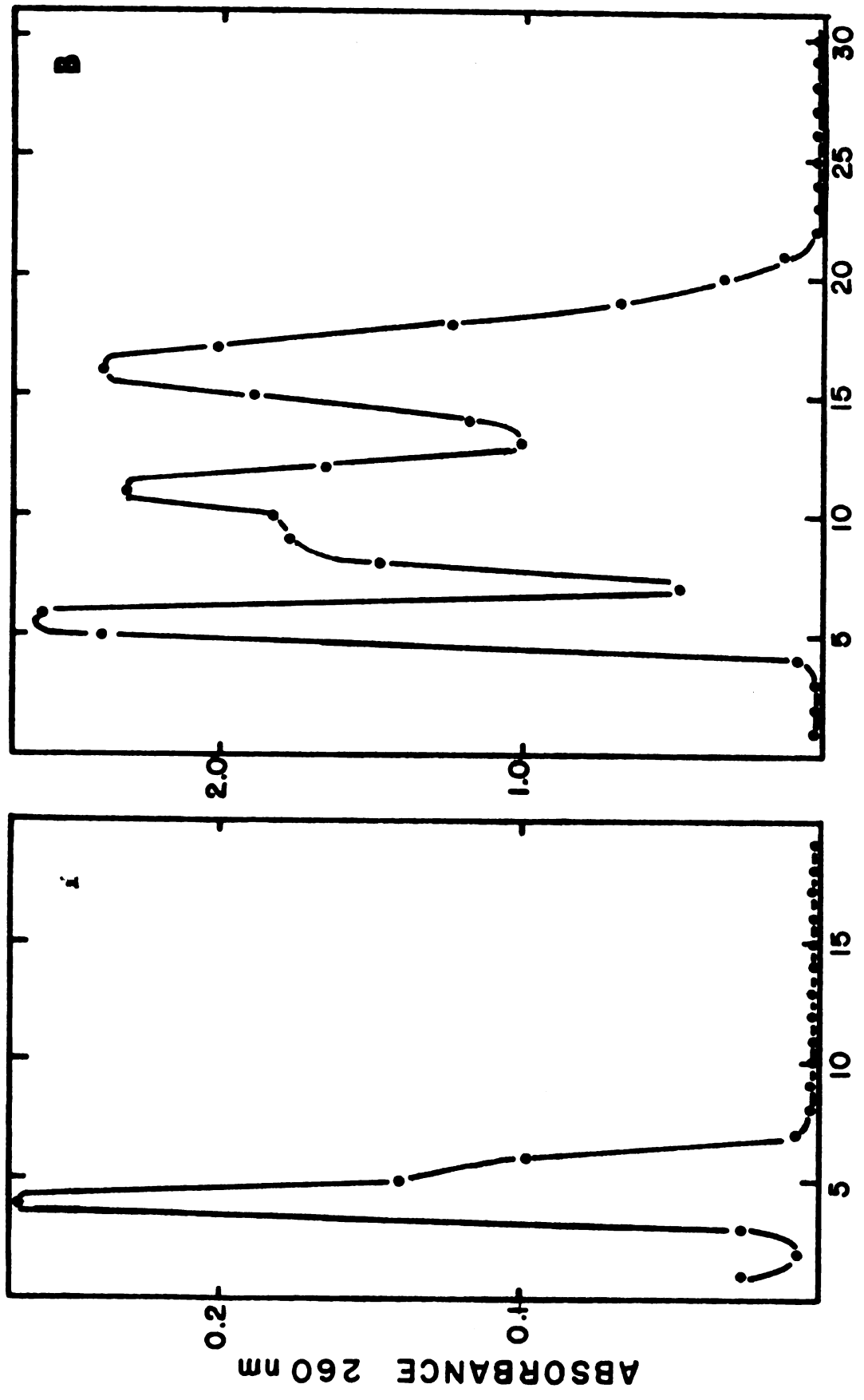
ABSORBANCE 260 nm
0.2
0.1
A
5
10
15
2.0
1.0
B
5
10
15
20
25
30
FRACTION NUMBER

Figure 7

Figure 8. HSLC elution of ribonucleosides from enzymatic hydrolysis. Column temperature was 26°C; column pressure was 2400 psi. Approximately three nmoles of nucleosides from rat liver rRNA were injected. Order of elution is U, G, A, and C.

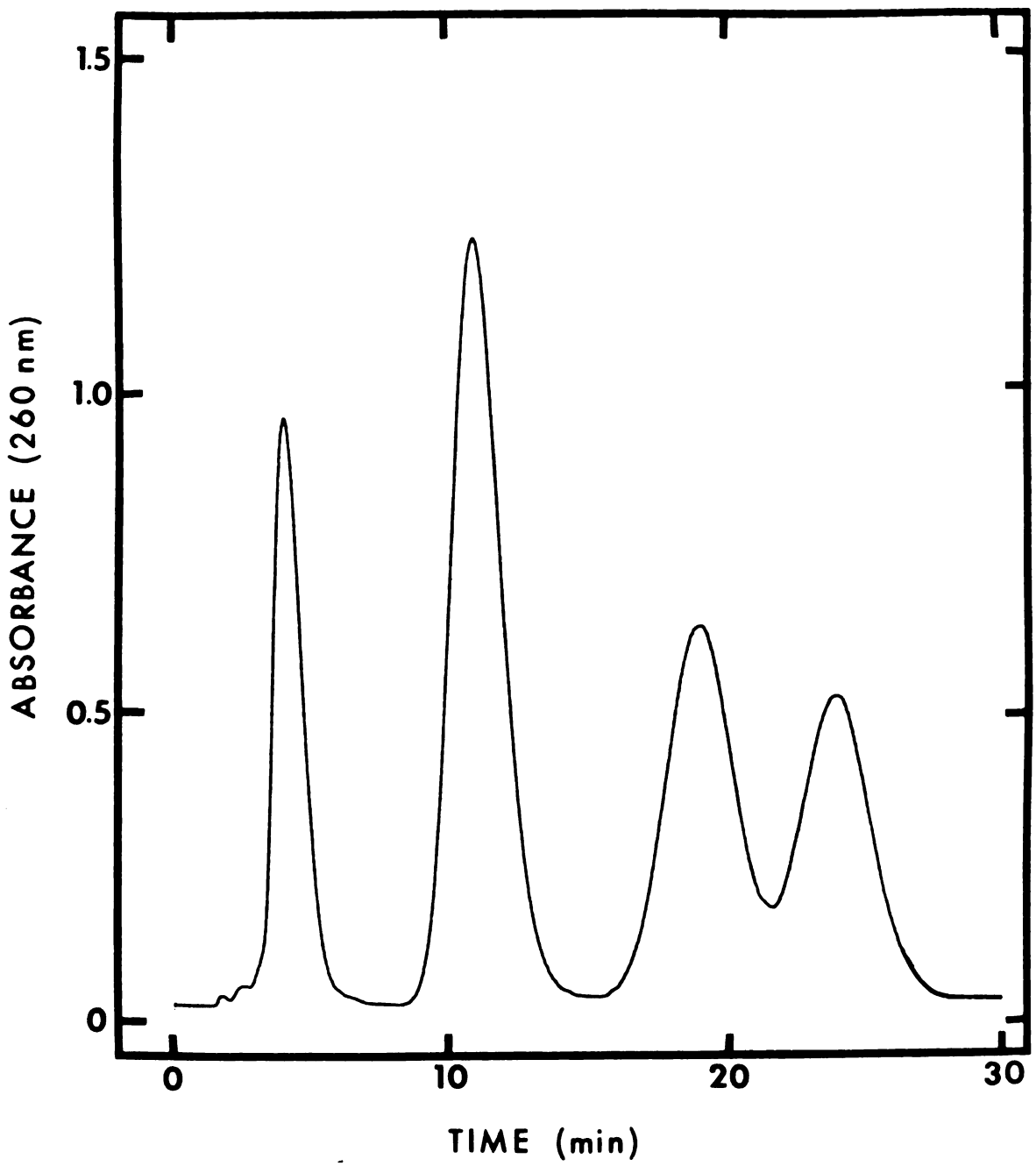

Figure 8

Table 6. Ribonucleoside ratios for rat tissue rRNA.

	Adenosine		Guanosine		Uridine		Cytidine	
	prep.	ave.	prep.	ave.	prep.	ave.	prep.	ave.
Rat liver rRNA								
Prep. 1	17.8		34.0		17.1		31.1	
Prep. 2	17.6	18.0	34.3	34.1	17.1	16.7	30.9	31.2
Prep. 3	18.6		33.8		16.0		31.6	
Rat brain rRNA								
Prep. 1	17.0	17.2	34.5	34.4	17.7	17.3	30.9	31.0
Prep. 2	17.5		34.4		16.9		31.2	
Rat testes rRNA								
Prep. 1	17.2		34.0		17.8		31.2	
Prep. 2	17.5	17.5	33.7	33.9	17.7	17.5	31.2	31.0
Prep. 3	17.7		34.0		17.1		31.8	
Rat kidney rRNA								
Prep. 1	17.5		34.3		17.2		31.8	
Prep. 2	17.4	17.8	34.0	34.3	17.2	17.2	31.2	31.0
Prep. 3	17.4		34.5		17.3		30.8	
Novikoff ascites rRNA								
Prep. 1	18.4		33.6		16.2		31.8	
Prep. 2	17.0	17.8	34.1	33.8	16.9	16.7	32.0	31.9
Prep. 3	17.9		33.8		16.3		32.0	
Diethylnitrosamine rRNA								
Prep. 1	17.6	18.0	34.4	33.8	15.9	15.8	31.0	32.2
Prep. 2	18.6		33.2		15.8		32.5	

Table 7. 2'-O-Methyl distribution for rat tissues.

	Am	Gm	Um	Cm
Rat liver rRNA (2)[a]	30.3 ± 0.47[b]	30.5 ± 0.72	18.4 ± 0.89	20.8 ± 1.45
Rat testes rRNA (3)	30.4 ± 0.51	29.8 ± 0.76	18.9 ± 0.92	20.8 ± 1.70
Rat brain rRNA (2)	29.8 ± 0.63	30.4 ± 0.94	18.4 ± 1.15	21.4 ± 1.88
Rat kidney rRNA (4)	30.1 ± 0.44	30.7 ± 0.67	19.0 ± 0.82	20.2 ± 1.39
Novikoff ascites rRNA (3)	31.2 ± 0.49	30.5 ± 0.75	16.5 ± 0.92	21.7 ± 1.57
Diethylnitrosamine rat liver rRNA (2)	30.7 ± 0.56	29.7 ± 0.87	17.7 ± 1.07	21.9 ± 1.76

[a]Number in parenthesis indicates number of tissue preparations.

[b]95% confidence interval calculated from nested analysis of variance.

Table 8. Nested Analysis of Variance; 2'-O-methyl distributions.

	S.S.	d.f.	M.S.	E[M.S.]	f ratio (df)
2'-O-Methyladenosine					
Tissues	11.79711	5	2.39423	0.53379	4.49 (5,11)
Preparations per tissue	5.45383	11	0.49580		
Runs per preparation	10.36749	44	0.23562		
2'-O-Methylguanosine					
Tissues	8.39806	5	1.67961	1.52400	1.10 (5,15)
Preparations per tissue	12.23783	11	1.11253		
Runs per preparation	11.86083	44	0.26956		
2'-O-Methyluridine					
Tissues	47.75237	5	9.5508	1.85795	5.14 (5,10)
Preparations per tissue	18.21796	11	1.65618		
Runs per preparation	12.05983	44	0.27409		
2'-O-Methylcytidine					
Tissues	24.16472	5	4.83294	1.57810	3.06 (5,10)
Preparations per tissue	15.75385	11	1.43217		
Runs per preparation	19.00700	44	0.43198		

Critical f ratio Am, $f_{0.01,5,11}=5.32$; Gm, $f_{0.01,5,15}=4.64$; Um, $f_{0.01,5,10}=5.64$; Cm, $f_{0.01,5,10}=5.64$;

Figure 9. HSLC elution of 2'-O-methylnucleosides from enzymatic hydrolysis. Column temperature was 39.5°C; column pressure was 2800 psi. Approximately three nmoles of nucleosides from rat liver rRNA was injected. Order of elution is Um, Gm, Am, Cm.

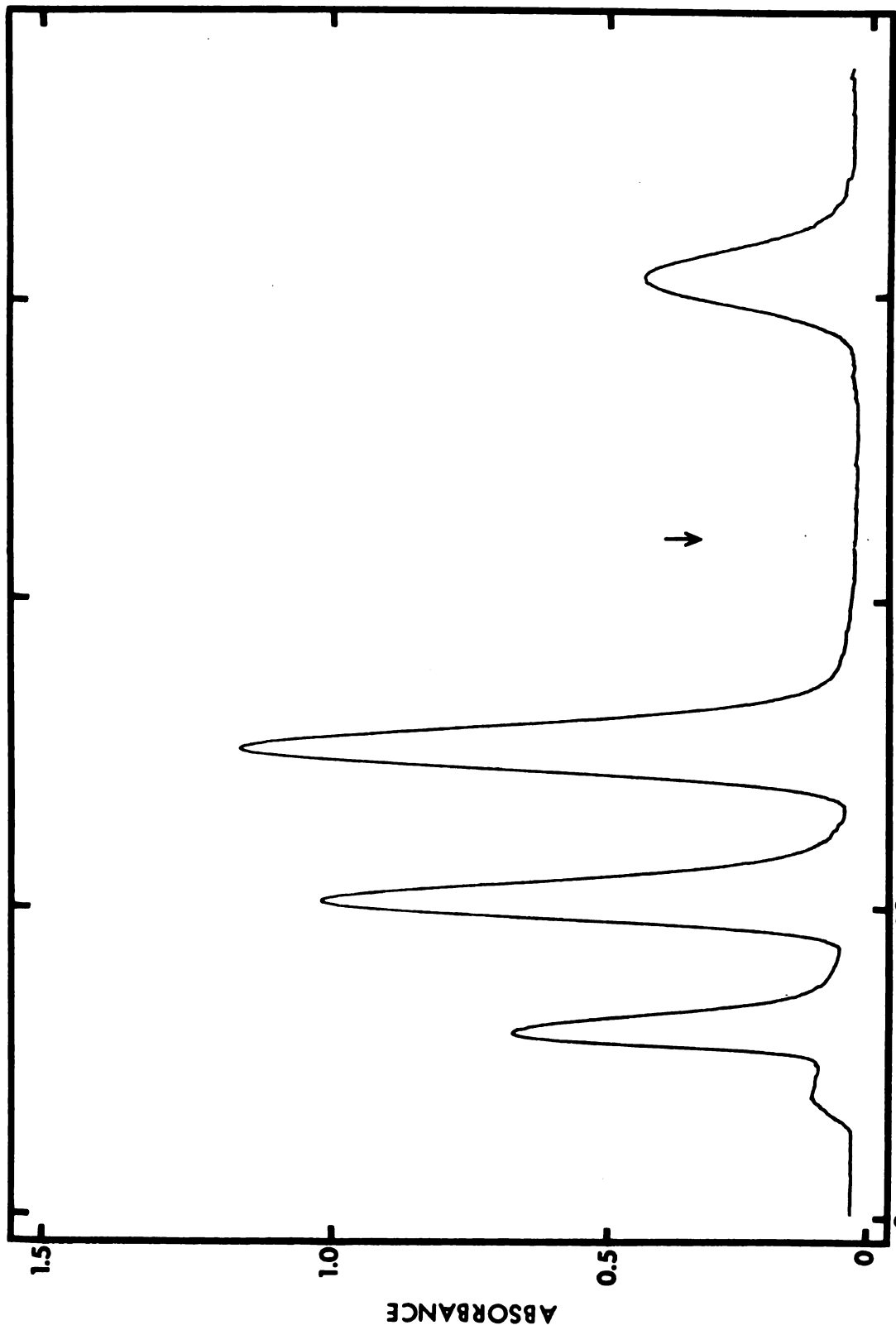

Figure 9

DISCUSSION

The use of the gas chromatography technique for determination of 2'-O-methyl levels in RNA has several advantages. Relatively small amounts of RNA can be analyzed without the necessity of radioactive labeling procedures. This means that tissues which are difficult to label or require high levels of precursor radioactivity, can be studied. Also, the problem of pool sizes and kinetics of labeling do not arise.

The G.C. procedure lends itself well to studies involving analysis of numerous RNA samples. However, certain aspects of the method require attention. It is very important that methanol and acetaldehyde are adequately resolved by the Porapak column. The methanol peak becomes difficult to quantitate if it trails into the acetaldehyde. This resolution is difficult to attain and the precautions described in Materials and Methods must be observed. When the procedure is carefully done, reasonably precise data can be obtained. If one assumes 6520 nucleotides in rRNA (28 S = 4500, Seeber and Busch, 1971; 18 S = 1900, Qualiarotti et al., 1970; 5 S = 120, Brown, 1974), the number of ribose methylated nucleotides in liver rRNA is 98.5 ± 3.5 at a 95% confidence interval. As is shown in Table 9 a difference of six to ten nucleotides per rRNA could be detected.

By combining the results from 2'-O-methyl level analysis with those from the distribution studies, the absolute amount of each 2'-O-methylated nucleoside can be determined (Table 9). A change of only two or three nucleosides could be detected at the 90% confidence limits.

The use of proper statistics is important when studying suspected quantitative differences. It is not adequate to look at one or two experiments and conclude that two similar samples are quantitatively the same or different. Confidence limits for the technique should be established since only in this way is it possible to determine how much variation could be detected and if differences are significant or merely due to imprecision in the method.

rRNA was prepared from purified ribosomes rather than from a high salt precipitation of cytoplasmic RNA, which would, admittedly, be simpler and quicker. However, RNA from purified ribosomes eliminated the possibility of contamination by DNA which would interfere with both GC analysis of 2'-O-methyl levels and the HSLC determination of 2'-O-methyl distribution.

It is known that endonucleolytic "nicks" in rRNA can occur in intact ribosomes. These "nicks" produce discrete RNA molecules with lengths smaller than eighteen or twenty-eight S. Presumably this cleavage occurs in intact ribosomes in portions of the RNA which are not protected by proteins or 2'-O-methyl groups. It was thought that if degradation did occur during purification, these shorter pieces would be retained in the RNA extracted from whole ribosomes. Apparently

Table 9. Number and distribution of 2'-O-methyl nucleosides per ribosome for rat tissues.

	Total 2'-O-methylated nucleosides per ribosome[a]	Number of each nucleoside per ribosome[b]			
		Am	Gm	Um	Cm
Rat liver rRNA	98.5 ± 3.5	29.8 ± 1.4	30.0 ± 1.7	18.1 ± 1.4	20.5 ± 2.1
Rat testes rRNA	95.8 ± 4.0	29.1 ± 1.7	28.6 ± 1.9	18.1 ± 1.6	19.9 ± 2.4
Rat brain rRNA	97.2 ± 5.6	29.0 ± 2.3	29.5 ± 2.6	17.9 ± 2.1	20.8 ± 3.0
Novikoff ascites rRNA	93.9 ± 3.2	29.3 ± 1.5	28.6 ± 1.7	15.5 ± 1.4	20.4 ± 2.2
Diethylnitrosamine rat liver rRNA	90.0 ± 5.5	27.6 ± 2.2	26.7 ± 2.4	15.9 ± 1.9	19.7 ± 2.8

[a]The total number of 2'-O-methyl nucleosides for each tissue was calculated using the 95% confidence interval for the 2'-O-methyl levels of each tissue. The total number of nucleosides was estimated at 6520 per ribosome (28 + 18 + 5 S rRNA).

[b]For the number of each nucleoside per ribosome, the percent of each nucleoside was multiplied by the average number of total 2'-O-methyl nucleosides present. The upper range was determined by multiplying the upper 95% confidence limit of the percent 2'-O-methyl nucleosides by the upper 95% confidence limit for each nucleoside. The lower range determined by using the lower confidence limits. The resulting confidence interval is 90%.

this is not true when extensive degradation occurs, as evidenced by the preparations from kidney.

In the case of kidney rRNA the 2'-O-methyl distribution was the same as for the other tissues. The 2'-O-methyl level, however, was significantly higher. This could result if there were selective loss of non-methylated portions of the rRNA. Methylation does protect RNA from the action of various nucleases (Dunlap, et al., 1971; Stuart and Rottman, 1973). Therefore preferential loss of unmethylated sequences could have occurred either by exonucleolytic attack or by endonucleolytic production of small pieces of RNA which would have been lost during ribosome purification or ethanol precipitation of rRNA.

To study possible differences between normal and cancerous tissue, three different carcinogenic states were chosen. Novikoff ascites hepatoma has been maintained for several years and has deviated considerably from its parent liver cells. It, therefore, represents a tissue which is dividing very rapidly and has no morphological resemblance to normal liver. Diethylnitrosamine is a potent carcinogen which is known to alkylate RNA bases. The mode of alkylation is presumably direct with no enzymatic action required to insert the alkyl group in the RNA. L-Ethionine also alkylates RNA but is thought to act through an S-adenosyl-ethionine intermediate. With S-adenosyl-ethionine substituting for S-adenosyl-methionine, ethylation can occur at sites that would normally be methylated. In this case the possibility of 2'-O-methylation could exist. These

three types of abnormal tissues have been shown to contain altered tRNA alkylation when compared to normal liver (Pegg, 1971; Nau, 1974; Swann and Magee, 1971). The question becomes; could these alterations extend to rRNA also?

In this study no differences in methylation of rRNA were found under any conditions. The studies with ethionine were not conclusive, however, since no ethylation of tRNA, from the treated rats, was found. Long term administration of ethionine to rats in their diet might still result in detectable 2'-O-ethylation of rRNA. This study would probably be better approached by use of radioactive ethionine. The 2'-O-alkylated products could then be isolated with the DE-22 borate column and the products characterized with the HSLC method.

It is significant that no variation in level or distribution of methylation was found either between normal tissues or between normal and carcinogenic material. Depending on the particular nucleoside, differences of methylation of two to five nucleosides would have been detected. This indicates that if variation in base sequences does exist it does not significantly alter the methylation pattern of the rRNA. The uniformity of methylation of rRNA, under conditions that affect tRNA alkylation, may indicate that proper methylation of RNA is vital in the processing of precursor RNA into mature rRNA, and that a complete complement of 2'-O-methyl groups are required for biological function. This constancy of structure suggests a major role of 2'-O-methylation.

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BIBLIOGRAPHY

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