Kinetics of Novikoff Cytoplasmic Messenger RNA Methylation†

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ABSTRACT: Methylation patterns of Novikoff cytoplasmic mRNA were determined as a function of labeling time with L-[methyl-^{3}H]methionine. The 5′-terminal m^{7}G could be released from whole mRNA by treatment with nucleotide pyrophosphatase. Subsequent alkaline phosphatase treatment of this mRNA, followed by KOH digestion, yielded N′mpNp and N′mpN″mpNp from cap 1 (m^{7}GpppN′mpN) and cap 2 (m^{7}GpppN′mpN″mpN), respectively. Our results indicate that the relative amounts of labeled cap structures do change with time and that the amount of internal N^6-methyladenosine decreases, relative to 5′-cap structures, as the cytoplasmic mRNAs age and the average size decreases. The formation of cap-2 structures by the addition of the second 2′-*O*-methyl group at position N″m appears to be a cytoplasmic event. Thus, after very short labeling times, greater than 80% of the labeled methyl groups in cap 2 are found in this position. These results, along with earlier data obtained on L-cell heterogeneous nuclear RNA methylation, are consistent with a model in which the nucleus is the cellular site of three mRNA methylation events producing 5′-terminal m^{7}G, the first 2′-*O*-methylnucleoside (N′m) found in cap-1 structures and internal N^6-methyladenosine. Subsequently, these nuclear methylations are followed by the cytoplasmic methylation at N″m. Analysis of the methylnucleoside composition of cap-1 structures, along with comparable "core" structures (m^{7}GpppN′m) generated from cap-2 by removal of N″m, indicates that at any single labeling time the methylnucleoside composition of a given cap-1 and the cap-2 "core" structure is remarkably similar. On the other hand, comparisons of the methylnucleoside composition of the cap structures at different labeling times indicate an increase in Cm in the first 2′-*O*-methylnucleoside (N′m) with time.

There is an increasing body of evidence to suggest that the majority of eukaryotic mRNA molecules from both cells (Rottman et al., 1974; Adams and Cory, 1975; Desrosiers et al., 1975; Furuichi et al., 1975a; Perry et al., 1975a; Wei et al., 1975) and viruses (Furuichi and Miura, 1975; Furuichi et al., 1975b; Furuichi et al., 1975c; Keith and Fraenkel-Conrat, 1975; Moyer et al., 1975; Wei and Moss, 1975) contain a unique methylated cap structure, m^{7}GpppN′mp(N″mp)Np on their 5′ termini. Furthermore, recent reports indicate that similar structures are also present on hnRNA[1] (Perry et al., 1975b; Salditt-Georgieff et al., 1976), a class of molecules that contains mRNA precursors. In addition to a 5′-terminal cap structure, both cytoplasmic mRNA and hnRNA also contain N^6-methyladenylic acid located internally between the cap and 3′ poly(A) segment. Although this mononucleotide is apparently absent in many viral RNAs (Furuichi and Miura, 1975; Moyer et al., 1975; Wei and Moss, 1975), globin mRNA (Perry and Scherrer, 1975), and yeast mRNA (Sripati et al., 1976; Dekloet and Andrean, 1976), it can account for nearly 50% of the labeled methylnucleotides in cellular mRNA (Desrosiers et al., 1974).

In a recent characterization of Novikoff cytoplasmic mRNA, we noted the presence of two types of cap structures, m^{7}GpppN′mpNp (cap 1) and m^{7}GpppN′mpN″mpNp (cap 2) (Desrosiers et al., 1975). Similar results have been reported for mouse myeloma cells (Adams and Cory, 1975), L-cells (Perry et al., 1975a), and HeLa cells (Wei et al., 1975; Furuichi et al., 1975a). In an earlier publication (Desrosiers et al., 1975), it was suggested that the two cap structures might, in fact, represent separate classes of mRNA with different degrees of cytoplasmic stability. Previous studies on cap structures in cytoplasmic mRNA have utilized cells labeled for a single time period. Therefore, it was reasoned that the relative abundance of cap-1 and -2 structures might depend upon the length of labeling time, possibly reflecting different kinetics of labeling or turnover. The presence of such time-dependent changes might, however, also reflect other factors, such as additional cytoplasmic methylation of partially methylated precursors.

To study the possibility that differential cytoplasmic mRNA stability affects the distribution of cap structures and at the same time to permit continual cytoplasmic methylation to occur, we employed continuous labeling of Novikoff cells with L-[methyl-^{3}H]methionine for periods up to 24 h. The methylation patterns of mRNA were then analyzed as a function of time.

Materials and Methods

Cell Culture and Labeling Conditions. Novikoff hepatoma cells (N1S1 strain) were grown in Swimm's S-77 medium (GIBCO) containing 10% calf serum, essentially as described (Desrosiers et al., 1974). For labeling with L-[methyl-^{3}H]-methionine (Amersham/Searle, 5 Ci/mmol), cells in mid-logarithmic growth phase were harvested aseptically and resuspended in fresh warm medium at a concentration of approximately 7.5 × 10^5 cells/ml. Labeling for the time course study was performed in the presence of 20 mM sodium for-

† From the Department of Biochemistry, Michigan State University, East Lansing, Michigan 48824. *Received June 8, 1976.* This work was supported by Public Health Service Research Grant CA 13175 from the National Cancer Institute. Michigan Agricultural Experiment Station Journal No. 7723. Portions of this work were presented earlier at a Symposium on mRNA, The Relation of Structure to Function, sponsored by the Biology Division of Oak Ridge National Laboratory (April, 1976).

[1] Abbreviations used are: hnRNA, heterogeneous nuclear RNA; m^{7}G, 7-methylguanosine; m^{6}A, *N*^{6}-methyladenosine; m^{6}Am, *N*^{6}-,2′-*O*-methyladenosine; HSLC, high-speed liquid chromatography; Nm, 2′-*O*-methylnucleoside; N′, nucleoside adjacent to pyrophosphates in 5′ cap; N″, penultimate nucleoside to pyrophosphate in 5′ cap; EDTA, (ethylenedinitrilo)tetraacetic acid; DEAE, diethylaminoethyl.

mate, 40 μM adenosine, and 40 μM guanosine to suppress nonmethylpurine ring labeling; normal methionine levels were present for all labeling times, except for 20 min, in which medium without methionine was used.

Labeling conditions for the distributional analysis experiments were altered to increase the specific activity of the methylated nucleotides in mRNA. Adenosine, guanosine, and formate were omitted and the 5- and 24-h samples were labeled in 5 mM methionine, one-half the normal concentration. Under these conditions, there was no discernible change either in cell doubling time or in cell appearance when examined by phase microscopy.

In most experiments, 15–20 μCi/ml of L-[methyl-^{3}H]-methionine was used. At each time point an aliquot of the cells was harvested aseptically and the radioactive medium returned to the growing culture. Final cell concentrations at the time of harvest never exceeded 1.3×10^6/ml.

Isolation and Characterization of Poly(A)-Containing Cytoplasmic mRNA. Total cytoplasmic RNA was isolated as previously described (Desrosiers et al., 1974). Poly(A)-containing mRNA was isolated by oligo(dT)-cellulose chromatography, including a heat step prior to a second passage over the column (Desrosiers et al., 1975). This step is necessary to eliminate traces of rRNA that otherwise interfere with methylation analysis.

Sedimentation analysis of poly(A)-containing mRNA was performed using 4.8-ml gradients of 5–20% sucrose in 99% Me_2SO, 10 mM LiCl, 1 mM EDTA. The mRNA was made 91% Me_2SO, 10 mM LiCl, 1 mM EDTA in total volume of 100 μl, and heated at 60 °C for 2 min prior to layering onto the gradient. Centrifugation was for 14.5 h at 25 °C and 45 000 rpm in a Beckman SW 50.1 rotor.

Nucleotide Pyrophosphatase Treatment of Whole mRNA. Poly(A)-containing cytoplasmic mRNA, essentially free of tRNA and rRNA contamination, was digested with nucleotide pyrophosphatase from *Crotalus atrox* (Sigma). A 200-μl reaction contained 0.25 unit of enzyme, 9 A_{260} units of RNA, 20 μmol of Tris-HCl, pH 7.8, and 0.2 μmol of magnesium acetate. After incubation at 37 °C for 35 min, the reaction was stopped by heating in a boiling water bath for 5 min. The high level of carrier RNA was added to suppress nonspecific diesterase activity, which contaminates this enzyme. The RNA was separated from the released pm^{7}G by chromatography on Bio-Gel P2 (100–200 mesh; 1.5 × 22 cm column) and treated with 0.25 unit of bacterial alkaline phosphatase (PL Biochemicals, electrophoretically pure) in 0.05 M Tris-HCl, pH 7.8, 0.001 M magnesium acetate for 45 min at 37 °C to remove the newly exposed 5′-terminal phosphates. The dephosphorylated RNA was then digested for 18 h at 37 °C with 0.4 N KOH to obtain N′mpNp from cap-1 and N′mpN″mpNp from cap-2 structures. These oligonucleotides were resolved from each other and m^{6}Ap by chromatography on Pellionex WAX in the presence of 7 M urea (Desrosiers et al., 1975).

Preparation of mRNA for Methylnucleoside Distributional Analysis. Internal methylnucleosides and intact 5′-terminal caps were produced by enzymatic digestion of poly(A)-containing cytoplasmic mRNA with RNase T_2 (Sigma) at 2 units/A_{260} unit of RNA in 0.9 M NaCl, 0.15 M sodium acetate, pH 4.5, 0.01 M EDTA, for 2 h at 37 °C. The reaction mixture was then adjusted to pH 8 with 1 M NaOH and made 0.017 M in magnesium acetate. Alkaline phosphatase that had been dialyzed against 0.05 M NH_4HCO_3 was added (0.25 unit/A_{260} unit of RNA) and the reaction continued for 30 min at 37 °C. The products of this reaction were resolved on DEAE-Sephadex (7 M urea). Intact cap structures to be used for subsequent analysis were desalted on Bio-Gel P-2 (100–200 mesh). Nucleosides were adsorbed to charcoal and eluted with 20% pyridine.

Cap-2 structures (m^{7}GpppN′mpN″mpN) were purified on Pellionex-WAX to remove remaining traces of urea, which were found to inhibit subsequent digestion with nuclease P_1. Cap-2 oligonucleotides contained in a volume of 500 μl were applied to Pellionex-WAX (⅛ in. × 30 cm) and urea was removed by eluting with 10 ml of 0.1 M ammonium acetate. The buffer was changed to 6 M ammonium acetate and the cap structure was eluted in 2-ml total volume. Ammonium acetate was removed by lyophilization. Cap-2 structures were digested with 160 μg/ml of nuclease P_1 (Yamasa Shoyl Co., Ltd.) in 0.01 M sodium acetate, pH 6.1. After 45 min at 37 °C, the sample was made 0.05 M Tris-HCl, pH 7.8, 0.001 M in magnesium acetate, and 0.3 unit of alkaline phosphatase/100 μl was added; incubation was continued for 30 min at 37 °C. The reaction mixture was diluted to 500 μl with water and reapplied to the Pellionex Wax column described above. N″m was eluted with 0.1 M ammonium acetate (2-ml total volume) and "core" oligonucleotide, m^{7}GpppN′m, eluted with 6 M ammonium acetate.

Both cap structures and "core" oligonucleotides generated from cap 2 were completely digested to nucleosides by incubation for 45 min at 37 °C with 0.25 unit of nucleotide pyrophosphatase and 0.4 unit of alkaline phosphatase in 100-μl reactions, containing 0.1 M Tris-HCl, pH 7.8, 0.1 mM magnesium acetate.

Acid Hydrolysis. Acid hydrolysis of whole mRNA and 5′-terminal oligonucleotides and mononucleotides can be used to cleave the N-glycosidic bond of purine-containing nucleotides, thereby releasing free purine bases. Generally, 1.5 A_{260} units of RNA was dissolved in 0.5 ml of concentrated formic acid, the tube was sealed, and the hydrolysis was carried out at 100 °C for 2 h, similar to the procedure of Munns et al. (1974). The released bases were resolved by high-speed liquid chromatography (HSLC) on Aminex A-5 (Desrosiers et al., 1975). [^{14}C]Adenosine was added as an internal standard to the ^{3}H-labeled RNA before hydrolysis to permit determination of ^{14}C/^{3}H ratios after digestion and thus provide a measure of the methanol lost from 2′-*O*-methyl groups in the presence of strong acid.

Results

Cap-1 and -2 structures differ from each other by containing one and two 2′-*O*-methylnucleosides, respectively. These structures can be resolved in the presence of 7 M urea on DEAE columns or on Pellionex WAX. Frequently, the resolution obtained on intact whole cap structures on Pellionex WAX is not satisfactory, even in the presence of 7 M urea, which is added to suppress base-composition effects. Removal of terminal m^{7}G with nucleotide pyrophosphatase, followed by treatment with bacterial alkaline phosphatase, leaves the mRNA with a 5′-terminal end of N′mpNp··· or N′mpN″-mpNp··· , corresponding to cap-1 and -2 structures, respectively. Subsequent alkaline hydrolysis of the remaining portion of mRNA produces N′mpNp and N′mpN″mpNp, which are easily and quickly separated by HSLC on Pellionex WAX.

Novikoff mRNA was labeled for varying times with L-[methyl-^{3}H]methionine and rigorously purified to eliminate rRNA, as described under Materials and Methods. Whole mRNA treated with nucleotide pyrophosphatase yielded a mononucleotide which was hydrolyzed with formic acid, and the hydrolysate was chromatographed on Aminex A-5. Greater

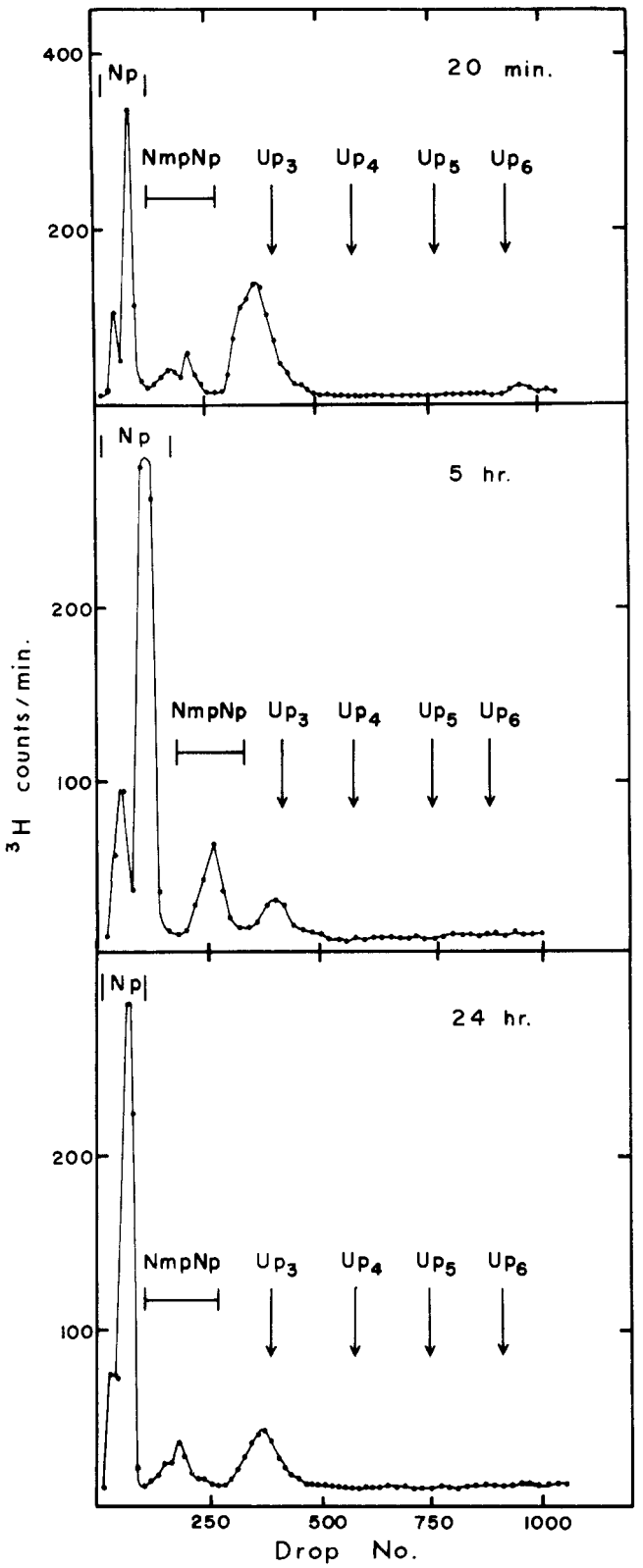

FIGURE 1: HSLC resolution on Pellionex WAX of KOH digestion products from mRNA which had previously been treated with nucleotide pyrophosphatase and alkaline phosphatase. A 1/8 in. × 40 cm column was developed at room temperature with a 100-ml gradient of 0–0.2 M $(NH_4)_2SO_4$ in 7 M urea, 0.005 M sodium phosphate, pH 7.7, at a flow rate of 25 ml/h. The position of the oligo(Up) standards added as uv markers and carrier rRNA digestion products are shown. Poly(A)-containing RNA was isolated at the times indicated.

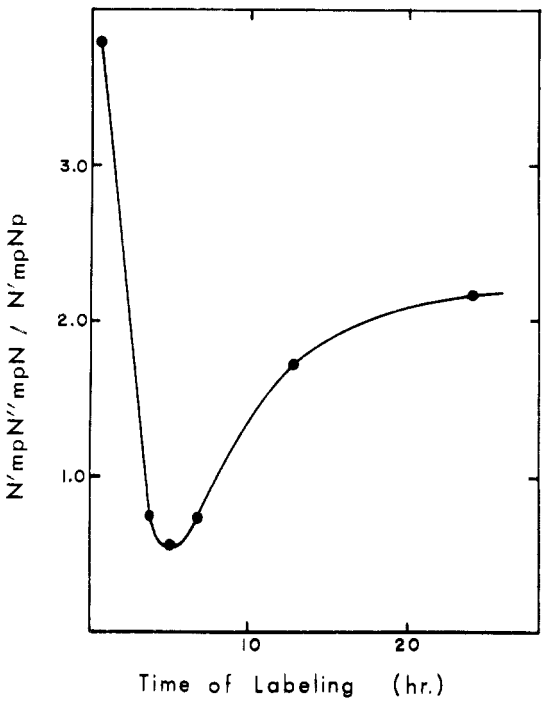

FIGURE 2: Change in ratio of N'mpN''mNp to N'mpNp in mRNA with time. The data was obtained from mRNA that had been labeled with L-[methyl-^{3}H]methionine for the times indicated and treated as described in the legend to Figure 1. Dinucleotide contamination from rRNA was determined by KOH hydrolysis of intact mRNA and chromatography on Pellionex WAX as in Figure 1. Appropriate corrections were made. Ratio of radioactivity in N'mpN''mpNp, eluting with the $(Up)_3$ marker, vs. N'mpNp was calculated.

than 85% of the released material eluted as 7-methylguanine (data not shown). The mRNA remaining after nucleotide pyrophosphatase treatment was further hydrolyzed with alkaline phosphatase and KOH. The internal base-methylated mononucleotide m^6Ap and the 5'-terminal oligonucleotides N'mpNp and N'mpN''mpNp were separated on Pellionex WAX (Figure 1).

One of the main objectives of these studies was to examine the relative distribution of cap-1 and -2 structures as a function of time of continuous labeling with L-[methyl-^{3}H]methionine. A pronounced change in labeling of these cap structures was observed with different labeling times (Figure 1). After a short exposure of only 20 minutes, most of the label in cytoplasmic mRNA was contained in cap 2, while further labeling yielded an increase in cap 1. Similar determinations were made at later time points and the ratios of radioactivity between caps 1 and 2 were observed to change (Figure 2).

To further identify and study the methylnucleoside distribution in internal and cap positions in poly(A)-containing mRNA, three periods of labeling were chosen: 20 min, 5 h, and 24 h. Although Pellionex WAX efficiently resolves N'mpNp and N'mpN''mpNp derived from caps 1 and 2, respectively, DEAE-Sephadex (7 M urea) is better suited for obtaining intact cap structures. Whole mRNA was digested with RNase T_2 and alkaline phosphatase to produce nucleosides from internal base methylations plus the 5'-terminal caps 1 and 2. These products were resolved on DEAE-Sephadex, developed in the presence of 7 M urea (Figure 3). It is again apparent that the distribution of ^{3}H radioactivity between nucleoside and caps 1 and 2 varies significantly with time of labeling.

The nucleosides recovered from urea buffer by charcoal adsorption were analyzed on Aminex A-5 under conditions that resolve base-modified nucleosides (Desrosiers et al., 1974).

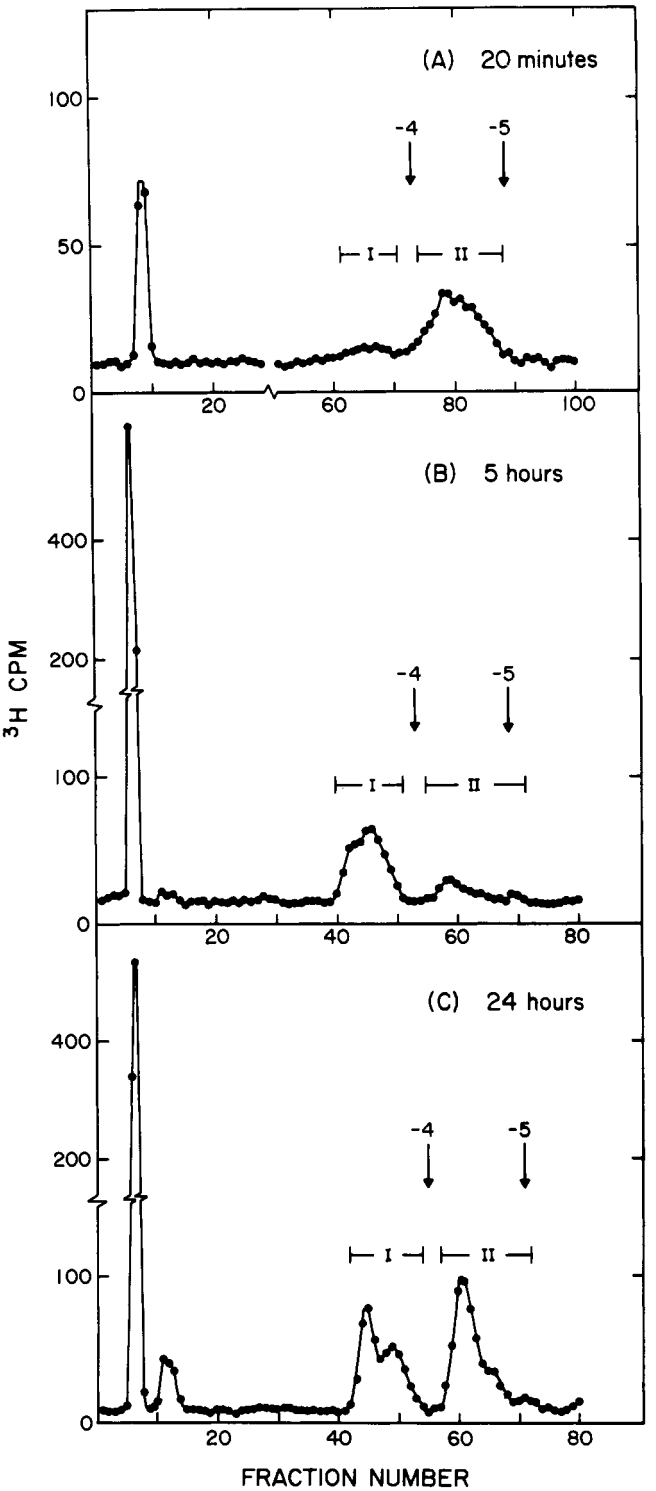

FIGURE 3: DEAE-Sephadex column separation of RNase T_2 and alkaline phosphatase digestion products from whole mRNA. Poly(A)-containing mRNA which had been labeled with L-[methyl-^{3}H]methionine for various time periods was digested with RNase T_2 and alkaline phosphatase, as described under Materials and Methods. Following digestion, the reaction mixture was diluted with 9 volumes of 7 M urea, 0.02 M Tris-HCl, pH 7.4, and applied to a 0.9 × 25 cm DEAE-Sephadex column. The mononucleosides were eluted with 0.1 M NaCl, 7 M urea, 0.02 M Tris-HCl, pH 7.4. To resolve oligonucleotides a 200-ml gradient of 0.1 M to 0.4 M NaCl in 0.02 M Tris-HCl, pH 7.4, 7 M urea was used at a flow rate of 12 ml/h; 2-ml fractions were collected. Standard oligonucleotides (pUm_3 and pUm_4) were included as markers to indicate approximate charge.

TABLE I: Amount of Internal m^6A per Average mRNA; Variation with Time.

Time	% of Label in mRNA as: m^7G[a]	m^6A[b]	Ratio m^6A/m^7G
20 min	5.5	28	5.1
5 h	17	58	3.4
13 h	19	53	2.8
24 h	27	35	1.3

[a] Determinations were made by acid hydrolysis of whole mRNA and analyzed on Aminex A-5 HSLC. [b] Determination of m^6A was the amount of mononucleotide from the DEAE-Sephadex (7 M urea) column of T_2 and alkaline phosphatase digest (cf. Figure 3) corrected for ring labeling when necessary.

6-Methyladenosine comprised greater than 95% of the base-methylated nucleosides at all times of labeling, but small amounts of radioactivity eluting with 5-methylcytidine were observed at later times (data not shown).

To correlate the number of internal methylations per messenger RNA molecule with labeling time, the amount of internal m^6A was compared to the amount of m^7G in whole poly(A)-containing mRNA, which had been labeled for the times indicated (Table I). The amount of m^7G in mRNA should reflect the absolute number of modified 5′ termini without being complicated by the number of ribose methylations in each message (i.e., cap 1 vs. cap 2). As indicated in Table I, the ratio of internal m^6A to terminal m^7G decreases with time, indicating that the average number of internal methylations in poly(A)-containing mRNA is reduced in longer labeling periods.

Since other reports have shown that larger hnRNA (Salditt-Georgieff et al., 1976) and mRNA (Perry and Kelley, 1976) have a higher average number of internal m^6A residues, it was of interest to examine the size of methyl-^{3}H labeled mRNA as a function of time. Poly(A)-containing mRNA from three labeling periods was analyzed by Me_2SO–sucrose gradient centrifugation (Figure 4). Messenger RNA labeled for shorter times was substantially larger in size than mRNA labeled for longer periods; i.e., with increased labeling time the percent of the molecules sedimenting at less than 18 S changes from 32 to 54%.

Since there is a time-dependent change in m^6A content, size of message, and cap 1 to cap 2 ratio, the methylnucleoside distribution within the caps was also studied. Cap-1 and -2 structures produced by RNase T_2 and alkaline phosphatase were resolved on DEAE-Sephadex (Figure 3) and desalted on Bio-Gel as described under Materials and Methods.

Cap-1 structures were digested with a mixture of nucleotide pyrophosphatase and alkaline phosphatase to produce 2′-*O*-methylnucleosides and m^7G. The separation of methylnucleosides found in cap-1 structures is presented in Figure 5. Only results from mRNA obtained at 5 and 24 h are included, since the amount of radioactivity in cap 1 at 20 min is too small to analyze (Figure 3). Two important aspects of this data should be mentioned: first, the major change in methylnucleoside composition as a function of time is the increase in Cm content, and, second, it appears that most of the Am is present as a doubly methylated derivative, N^6,2′-*O*-dimethyladenosine (m^6Am). Verification of the m^6A content in this dimethylated nucleoside was obtained by subjecting an aliquot of isolated cap 1 to acid hydrolysis and subsequently analyzing the free bases produced, as described earlier. After 5-h labeling, 50% of the label in m^6Am was converted to m^6A (cf. Figure 5,

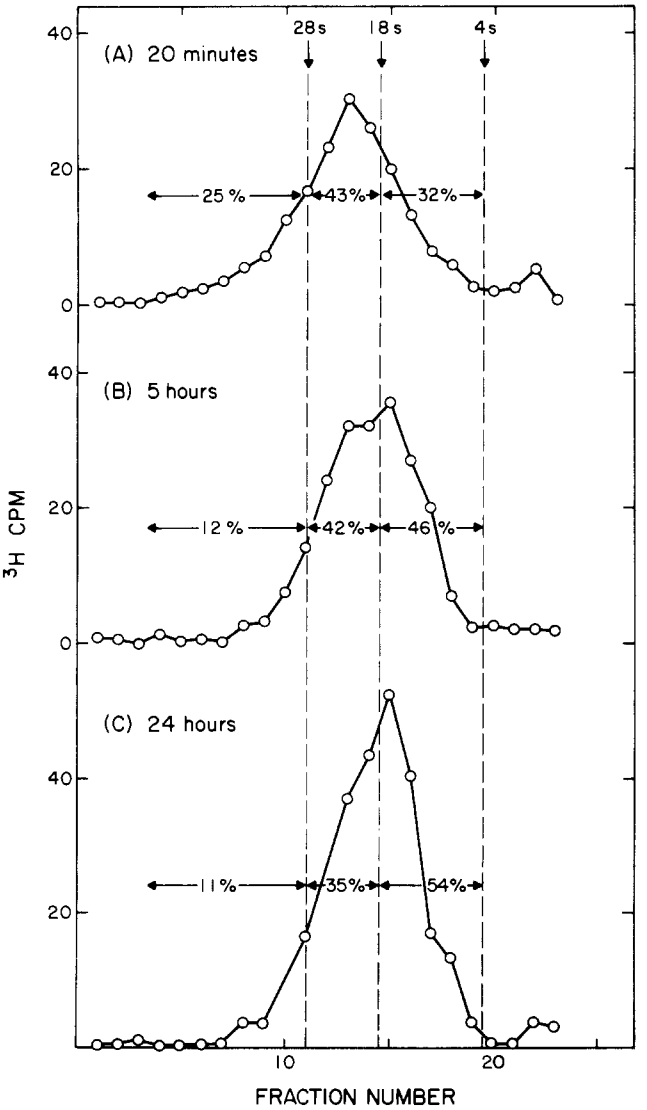

FIGURE 4: Me_2SO-sucrose gradient profiles of poly(A)-containing cytoplasmic RNA. Preparation of mRNA and gradient centrifugation was as described under Materials and Methods. The percent of the total radioactivity present in fractions smaller than 18 S, between 18 and 28 S, and greater than 28 S was calculated for each RNA sample. Messenger RNA was isolated after labeling with L-[methyl-^{3}H]methionine for (A) 20 min, (B) 5 h, and (C) 24 h, as described under Materials and Methods.

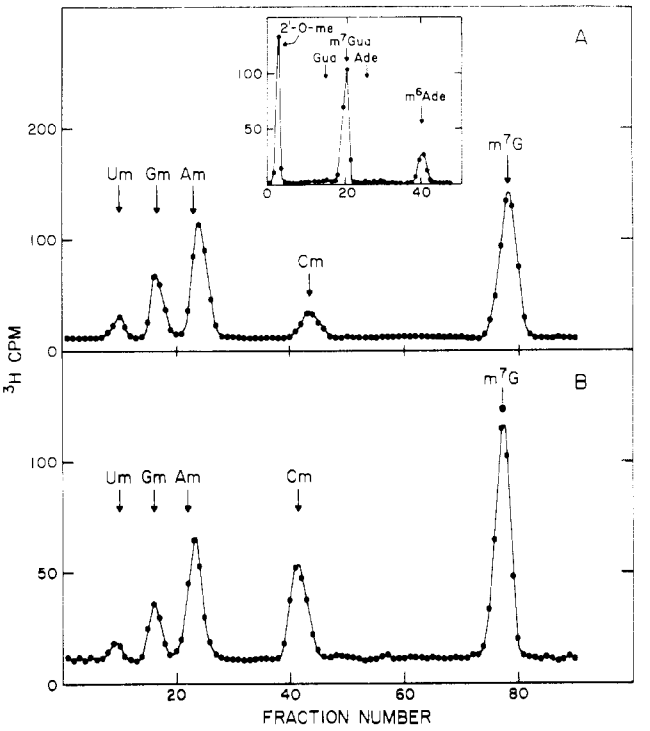

FIGURE 5: The distribution of methylnucleosides in cap-1 structures. Cap-1 structures produced by RNase T_2 and alkaline phosphatase treatment were eluted from a DEAE-Sephadex (7 M urea) column in a volume of 10–20 ml and desalted on a 1.9 × 42 cm Bio-Gel P2 column by elution with 0.02 M NH_4HCO_3. Material in the void volume was made 20% with ethanol and evaporated. Cap-1 structures were then digested with nucleotide pyrophosphatase and alkaline phosphatase as described under Materials and Methods. The reaction mixture was dried with N_2 and dissolved in 125-μl column buffer. HSLC on Aminex A-5 ($\frac{1}{8}$ in. × 90 cm) was in 0.4 M ammonium formate, pH 4.25, 40% ethylene glycol at 40 °C. Flow rate was ~7 ml/h (2500 psi) until Am was eluted; the rate was then increased to ~12 ml/h (4750 psi) for remainder of the run. Fraction size was 10 drops (~0.4 ml) until Cm was eluted; fraction size was then doubled. (A) Cap 1 from mRNA labeled for 5 h. Inset is the acid hydrolysis of the same 5 h cap-1 structure analyzed on Aminex A-5 in 0.4 M ammonium formate at pH 5.3. (B) Cap 1 from mRNA labeled for 24 h. 2'-*O*-Methylnucleosides and m^{7}guanosine were added as markers and detected at 260 nm.

inset). Also, the percent of label in m^{7}G equals the percent of label in 2'-*O*-methyl products. Similar results were obtained with acid hydrolysis of cap 1 derived from 24-h mRNA; i.e., 87% of the Am is found in the form of m^{6}Am.

The methylnucleoside distribution in the N″m position of cap 2 was determined by digesting cap-2 structures with nuclease P_1 which produces m^{7}GpppN′m + N″m. The released methylnucleoside N″m can be separated from the remainder of the cap structure by HSLC on Pellionex WAX in ammonium acetate and subsequently analyzed on Aminex A-5. Of the label in cap 2 after 20 min, 80% was released as N″m. The N″m position of 24-h labeled cap 2 appears to be particularly rich in Um, and contains a significant amount of Am (Figure 6). Acid hydrolysis of this N″m nucleoside produced no m^{6}A (data not shown).

The overall distribution of methylnucleosides at each specific site of Novikoff mRNA methylation after 20 min, 5 h, and 24 h of continuous labeling with L-[methyl-^{3}H]methionine is shown in Table II.

Discussion

In earlier studies (Desrosiers et al., 1975), we examined the distribution of methylnucleosides in Novikoff mRNA after continuously labeling the cells for 13 h with L-[methyl-^{3}H]-methionine. Analysis of mRNA methylnucleoside composition indicated that an average Novikoff mRNA contained 5.7 methyl groups, 3 of which were present as internal m^{6}A, 1 terminal m^{7}G, and 1.7 Nm's. The nonintegral number of Nm's could result from an unequal distribution of cap-1 and -2 structures, as well as m^{6}Am in the N′m position. An explanation for the necessity of two different types of cap structure in mRNA was not readily apparent. However the stabilizing effect that 2'-*O*-methyl groups have on RNA molecules in the presence of specific nucleases (Stuart and Rottman, 1973) led us to suggest that the ratio of these two cap structures might reflect multiple classes or fractions of mRNA with different cellular stabilities (Desrosiers et al., 1975). If this were true, one might expect the relative levels of these two structures to change as a function of labeling time.

TABLE II: Distribution of [^{3}H]Methylnucleosides in Various Positions of Cap-1 and -2 Structures as a Function of Labeling Time.[a]

		% of Total Label in:						
	Labeling Time	Um	Gm	Am	m^{6}Am	Cm	m^{7}G	% of Cap 2 as N″m
Cap 1 (m^{7}GpppN′mpN)								
	5 h	4.6	14.9	0	28.8	8.7	42.2	
	24 h	2.4	9.4	3.5	19.6	21.1	44.0	
Cap 2 "core" (m^{7}GpppN′m)								
	5 h	4.7	11.0	4.1	25.2	13.0	42.0	
	24 h	1.8	8.0	9.3	20.4	18.4	42.1	
Cap 2 (N″m)								
	20 min	44	11	28		15	2	80
	5 h	35	21	26		18	0	35
	24 h	36	18	23	0	23	0	26

[a] Whole poly(A)-containing mRNA was digested with RNase T_2 and alkaline phosphatase; cap 1 and 2 structures were separated on DEAE-Sephadex (7 M urea). Following digestion of cap structures with penicillium nuclease, the "core" oligonucleotide and N″m nucleoside were resolved on a Pellionex WAX column (ammonium acetate). The distribution of nucleosides in N″m was determined as in Figure 6. Core oligonucleotide and cap-1 structures were digested with nucleotide pyrophosphatase and analyzed as in Figure 5. The data is presented as a percentage of the total radioactivity present in the structural position indicated.

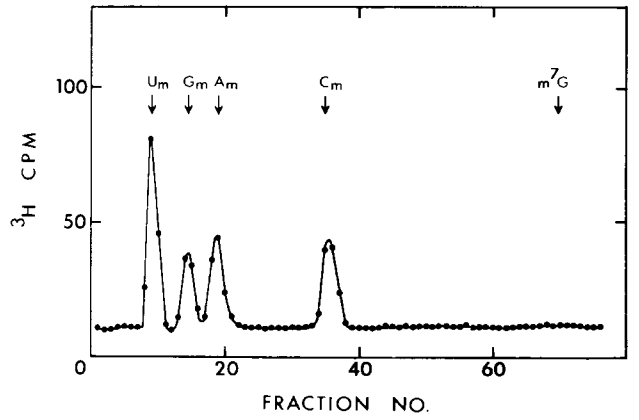

FIGURE 6: Methylnucleoside distribution analysis by Aminex A-5 HSLC of N″m nucleoside of mRNA labeled for 24 h. The N″m nucleoside obtained from nuclease P_1 digestion of cap 2 was eluted from Pellionex WAX (ammonium acetate), lyophilized, dissolved in 0.4 M ammonium formate pH 4.25 in 40% ethylene glycol, and chromatographed as in Figure 5.

Previous determinations of cap-1 to cap-2 ratios utilized mRNA that had been treated by periodate oxidation and β elimination to remove terminal m^{7}G prior to alkaline digestion. Since the elimination of m^{7}G by periodate oxidation of mRNA was not reproducible in our laboratory, we explored the possibility of using the enzyme nucleotide pyrophosphatase on intact mRNA molecules and found this method of m^{7}G removal to be superior. It should perhaps be noted that the susceptibility of the 5′-terminal m^{7}G-containing cap to nucleotide pyrophosphatase implies that it must be open and accessible to the enzyme and not buried within the folded structure of the mRNA molecules. Following removal of terminal phosphate and subsequent alkaline digestion, the N′mpNp and N′-mpN″mpNp obtained from cap-1 and -2 structures, respectively, were separated by HSLC on Pellionex WAX.

Poly(A)-containing mRNA was isolated from Novikoff cells that had been labeled with L-[methyl-^{3}H]methionine for different periods of time. Analysis of cap structures obtained from these mRNA samples, indeed, indicated that the relative labeling of caps 1 and 2 changed with time (Figure 2). At 20 min, there was much more L-[methyl-^{3}H] label in cap 2, reflecting enhanced labeling at the N″m position of cap structures that were earlier methylated in the nucleus at m^{7}G and N′m from cold methyl precursors. After 5 h, the ratios were reversed (Figures 1 and 3) and, after longer periods (24 h), the ratio approaches a "steady-state" level of cap 2 to cap 1 of approximately 2. These studies employed continuous labeling with L-[methyl-^{3}H]methionine to permit continued formation of newly methylated mRNA sequences, since the ratio of cap structures present at a particular time reflects both synthetic and degradative events.

The time-dependent changes observed in the relative labeling of each cap structure prompted us to further characterize the methylation occurring at each specific site within mRNA as a function of time. Such an approach necessitated the prior separation and isolation of 5′-terminal cap-1 and -2 structures, as well as the m^{6}A located within the mRNA. DEAE-Sephadex (7 M urea) columns provided excellent resolution of the material obtained from 20-min, 5-h, and 24-h labeled mRNA (Figure 3). Analysis of the material present in the mononucleotide fractions by subsequent HSLC on Aminex A-5 showed it to be mainly N^6-methyladenosine (data not shown). Since there have been several reports of 5-methylcytidine (m^{5}C) in viral and cellular mRNA (Dubin and Stoller, 1975; Salditt-Georgieff et al., 1976) the mononucleotide fraction was further analyzed. HSLC on Aminex A-5 provided separation of m^{6}A and m^{5}C and indicated a small peak of radioactive material eluting with m^{5}C at later labeling times (data not shown). After 20 min, very little m^{5}C was detected. The maximum levels at 5 and 24 h were 2 and 4% of the total internal methylnucleoside, respectively, indicating a low but possibly significant amount of this methylnucleoside that accumulates with time.

Another interesting time-dependent comparison involves the internal methylnucleoside m^{6}A and 5′-terminal m^{7}G. With longer labeling times, the amount of m^{6}A relative to m^{7}G decreases (Table I). This could be due to a selective time-dependent loss of mRNAs rich in m^{6}A or an increase in the number of 5′-terminal m^{7}G residues on mRNA. Determina-

tion of the average size of Novikoff mRNA sequences on denaturing sucrose gradients indicates a distinct reduction in mRNA size as a function of labeling time (Figure 4). At 20 min, most of the mRNA sediments in Me_2SO gradients at approximately 20 S. At 5 h, a bimodal distribution is obtained with components sedimenting at 20 and 15 S, while after 24 h most of the mRNA sediments at 15 S. This indicates a reduction in the average size of mRNA sequences with time, accompanied by a loss of m^6A, which, may be distributed at approximately equal intervals throughout the mRNA. Similar results have been obtained in duplicate experiments in which cells were grown in the presence or absence of adenosine, guanosine, and sodium formate, which were added to suppress purine ring labeling. Also, it should be noted that these results on mRNA are in essential agreement with recent studies on HeLa hnRNA (Salditt-Georgieff et al., 1976) and L-cell mRNA (Perry and Kelly, 1976). However, the HeLa and L-cell studies concentrated on the relative reduction in the number of internal m^6A residues with reduction in size of RNA at a fixed labeling time. Thus, in Novikoff cells, it can be stated that with increased labeling time the average size of methyl-labeled mRNA gets shorter and the content of internal m^6A is reduced.

The isolation of labeled caps 1 and 2 permits a systematic analysis of specific methylated positions within these oligonucleotides as a function of labeling time. Comparisons can be made between cap 1 from different labeling times, as well as between cap 1 and the analogous "core" structure, $m^7GpppN'm$, from cap 2. Earlier analyses were performed on either mixtures of caps 1 and 2 (Desrosiers et al., 1975) or on cap structures obtained at a single fixed time of labeling (Perry et al., 1975b). The methylnucleoside composition of cap-1, particularly at position N′m, appears to change with time. Although the *relative* labeling of Um, Gm, and Am is nearly equivalent at 5 and 24 h (Figure 5 and Table II), there is a significant increase in Cm with time. This increase probably reflects a time-dependent enrichment of a subclass of mRNA sequences with enhanced cytoplasmic stability. Also, as shown in Figure 5, virtually all of the Am present in the N′m position is found as the doubly methylated nucleoside, N^6,2′-*O*-methyladenosine. At 5 h the amount of radioactivity recovered as N^6,2′-*O*-methyladenine is consistent with the presence of only m^6Am and no Am, while at 24 h the distribution is 87% m^6Am, 13% Am. This result differs from comparable analyses on L-cell mRNA, which contained larger amounts of singly methylated Am in the N′m position (Perry and Kelley, 1976).

In an attempt to determine if the flux in cap 1 and 2 labeling was primarily due to labeling of a specific site within the cap structure or, alternatively, general labeling at all positions, the N″m nucleoside was selectively removed and separately analyzed. After 20 min, greater than 80% of the methyl label is in the N″m position (Table II). The distribution of methylnucleosides in the N″m position at 24 h labeling (Figure 6) indicates a high level of both Um and Am. None of the Am appears as the doubly methylated nucleoside m^6Am.

Removal of N″m from cap 2 results in the production of "core" structures that can be easily separated from N″m and recovered on Pellionex WAX in the presence of ammonium acetate. Following complete degradation with nucleotide pyrophosphatase and alkaline phosphatase, the methylnucleosides in the N′m position and m^7G can be analyzed on Aminex A-5. These data enable one to make an interesting comparison between the methylnucleoside composition of cap 1 and that of the analogous "core" structure derived from cap 2. The results, summarized in Table II, indicate that at a given labeling time the distribution between these two structures is remarkably similar. This correspondence in methylnucleoside distribution even extends to the relative increase in Cm observed at later labeling times. Data obtained with L-cell caps 1 and 2 "core" structures showed differences in composition at the N′m position (Perry and Kelley, 1976). Aside from different cell types used in both experiments, it should be noted that studies with L-cells were performed under pulse-chase labeling conditions, while for Novikoff cells continuous labeling was employed. The data obtained with Novikoff mRNA do not suggest a selective methylation of certain mRNA's containing a unique cap-1 N′m content to which a N″m is added to form cap-2 structures. The presence of altered methylnucleoside compositions in mRNA's with different cytoplasmic half-lives could produce the differences observed in pulse chase vs. continuous labeling.

Earlier data on hnRNA methylation patterns indicated the presence of 5′-terminal cap-1 structures and internal m^6A but no cap 2 (Perry et al., 1975b). These data, in combination with the present study, are consistent with a model in which m^7G, N′m and internal m^6A are products of nuclear methylation events. After exit of mRNA sequences containing these modifications into the cytoplasm, there is a cytoplasmic methylation event at the N″m position, forming cap 2. The selective enrichment, with time, of mRNA sequences containing a relatively high Cm composition suggests an enhanced stability of a subclass of mRNAs that happen to have this altered methylnucleoside composition.

The complexities of this system in which thousands of mRNA sequences are being simultaneously synthesized and degraded, are indeed enormous. Examination of methylnucleoside composition in mRNAs collectively grouped by cap-1 and -2 content is only a first step in following these mRNA modifications as a function of time. Of real interest will be those studies dealing with a specific homogeneous eukaryotic mRNA sequence.

Acknowledgment

The authors gratefully acknowledge the contributions of Dr. Ronald C. Desrosiers who participated in experiments leading to this work and provided helpful suggestions and discussion.

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Double-Stranded DNA in Methanol–Ethanol–Buffer Solvent System†

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ABSTRACT: DNA in a solvent system consisting of roughly equal volumes of methanol and ethanol and 5% buffer has a conservative circular dichroism (CD) spectrum of very low intensity above 220 nm and an increase of ϵ_{258} comparable to that of denatured DNA (about 40%). A direct comparison of this spectrum with the CD of single-stranded DNA reveals many differences, indicating DNA in this solvent system has a conformation different from that of denatured DNA. When the alcohols are removed, the B form conformation and normal ϵ_{258} are restored in native DNA, while single-stranded DNA remains denatured. A double-stranded structure of DNA in the methanol–ethanol–buffer solvent system is confirmed by the neutral cesium chloride density gradient centrifugation of DNA in which one chain is labeled with [^{14}C]thymidine and the other [^{3}H]5-bromodeoxyuridine. The doubly labeled DNA exposed to the alcohol solvent system has a centrifugal pattern identical with that of control DNA; the two radioactivities cosediment and form a superimposing band, distinctly different from that of single-stranded DNA; ^{3}H-labeled (thymidine) chains sediment further than ^{14}C-labeled chains (5-bromodeoxyuridine). Denatured DNA exhibits varying CD spectra depending on solvents. It is suggested that single-stranded DNA in different solvent systems assumes different modes of base stacking.

Within the restriction of the Watson–Crick pairing scheme, complementary nucleic acids display a remarkable diversity of secondary structure. A variety of distinct conformations has been characterized in DNA fibers by the x-ray diffraction technique (Arnott, 1970). In solution, changes in conformation can be brought about by changes in pH, solvent, temperature, relative humidity, or nature and concentration of counterions, or by complexing with other macromolecules (Bush and Brahms, 1973). Circular dichroism (CD[1]) has been employed extensively to monitor these transformations as it is a very sensitive technique to measure conformational changes in polynucleotides. Such studies may permit an assessment of the relative importance of different conformational forces in conferring the stability of DNA in solution.

Conformational changes of DNA in different solvents have attracted considerable interest. Mixed-type media are commonly used with water as one of the components. Low-molecular-weight alcohols are often used as the second component because of their low polarity and ability to mix well with water. For example, DNA in buffer assumed the B form but transforms to the C form in the 95% methanol–5% buffer and 65% ethanol–35% buffer solvent systems (Girod et al., 1973). In contrast to these organic solvent systems where DNA is found to retain a secondary structure, it was reported to be completely and irreversibly denatured and lose all secondary structure in a solvent system which contained roughly equal volumes of methanol and ethanol and a buffer concentration below 15% (Johnson and Girod, 1974). This conclusion was based on ultraviolet absorption and CD studies. No cogent interpretation was given to this unexpected finding.

However, a close examination of the reported CD spectra (Johnson and Girod, 1974) revealed that the intensity of two CD bands above 230 nm was too low compared with that of denatured DNA, even compared with that of spectra recorded at the denaturing temperature (Brahms and Mommaerts, 1964; Usatyi and Shlyakhtenko, 1973). Moreover, the validity of comparing absorption and CD spectra in buffer and those in alcohols has never been established. Because of these uncertainties, the structure of DNA in the methanol–ethanol–buffer solvent system has been reexamined using the same spectroscopic techniques. The results show that native and heat-denatured DNA have different CD spectra in the meth-

† From the Laboratory of Nuclear Medicine and Radiation Biology, University of California, Los Angeles, California 90024. *Received May 3, 1976.* This study was supported by Contract E(04-1) GEN-12 between the Energy Research and Development Administration and the University of California, by the Division of Cancer Treatment Contract N01-CM-43791, and by the National Science Foundation Grant MBS 74-13253 A02.

[1] Abbreviations used: CD, circular dichroism; SSC, standard saline-citrate buffer (150 mM NaCl, 15 mM Na citrate, pH 7.5); dThd, thymidine; BrdUrd, 5-bromodeoxyuridine; $\Delta\epsilon$, molar extinction coefficient for left circularly polarized light minus that for right circularly polarized light; EDTA, ethylenediaminetetraacetic acid.