METHYLATION OF NUCLEIC ACIDS IN HELA CELLS*

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Since the discovery of the presence of methylated bases in nucleic acids (Wyatt, 1951; Dunn and Smith, 1958; Littlefield and Dunn, 1958; Smith and Dunn, 1959), work in this area has centered around determining the extent of occurrence of these compounds in various types of nucleic acids and their identities. DNA from plants and animals contains 5-methylcytosine, but no methylated adenine or guanine (Wyatt, 1951; Dunn and Smith, 1958), whereas bacterial DNA apparently contains both 5-methylcytosine and 6-methylaminopurine (Gold, Hurwitz and Anders, 1963; Gold and Hurwitz, 1964). Transfer RNA (S-RNA) has been shown to contain a number of methylated bases (Dunn, 1959; Dunn, Smith, and Spahr, 1960; Bergquist and Matthews, 1962; Dunn, 1963; Hurwitz, Gold and Anders, 1964). Purified ribosomal RNA from E. coli is known to contain methylated adenine and methylated guanine (Starr, and Fefferman, 1964).

Most of the work described above has been done with bacterial RNA preparations. The work to be reported in this publication is the first attempt to ascertain to what degree the various types of nucleic acids are methylated in cultured animal (HeLa) cells and the nature of the methylated components of these nucleic acids.

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HeLa cells (S-3 clonal strain) were grown on Eagle's phosphate medium plus 5% dialyzed horse serum. The medium was prepared free of methionine and then supplemented with 2.8 μg of L-methionine-methyl-C^{14} (1.7×10^6 c.p.m. per μmole) per ml. After the cells had grown for approximately 2 generations (cell count increased from 5×10^4 ml to 2×10^5 ml), the cells were harvested by centrifugation, washed 3 times with 10 to 20 volumes of a solution containing 0.13 M NaCl, 0.005 M KCl, and 0.0075 M $MgCl_2$, and finally suspended in 3 volumes of 0.0025 M $MgCl_2$. The cells were ruptured by homogenization of this suspension for 1.5 minutes in a Potter-Elvehjem homogenizer at 0° C. One-third of a volume of a solution containing 1.0 M sucrose, 0.1 M KCl, 0.2 M Tris buffer (pH 7.4), and 0.016 M $MgCl_2$ was added and the nuclear fraction was then sedimented by centrifugation at 2,000 r.p.m. (no. 250 rotor in International Centrifuge) for 10 minutes. The supernatant solution (the cytoplasmic fraction) was then processed as described by Attardi and Smith (1962) to yield ribosomes and the soluble portion of the cytoplasmic fraction. Protein-free ribosomal RNA was prepared from the ribosomes by a phenol-extraction method modified from that described by Attardi, et. al. (1963). Details of this modified procedure will be published later. Transfer RNA (S-RNA) was purified from the soluble cytoplasmic fraction by the same phenol-extraction procedure. DNA was purified from the nuclear fraction by the method of Marmur (1961).

Ribosomal RNA was separated into its 28S and 18S components by sucrose gradient centrifugation according to the directions of Attardi, et. al. (1963). S-RNA was also purified by sucrose gradient centrifugation. Fractions (approximately 0.6 ml each) were collected from the sucrose gradient tubes and the optical density (to locate RNA) of each fraction and the radioactivity of the TCA-precipitable material of an aliquot of each fraction were determined. The radioactivity data provided a measure of the degree of methylation of RNA. The data of Figure 1 show that 28S and 18S ribosomal RNA were not equally methylated. 18S RNA contained

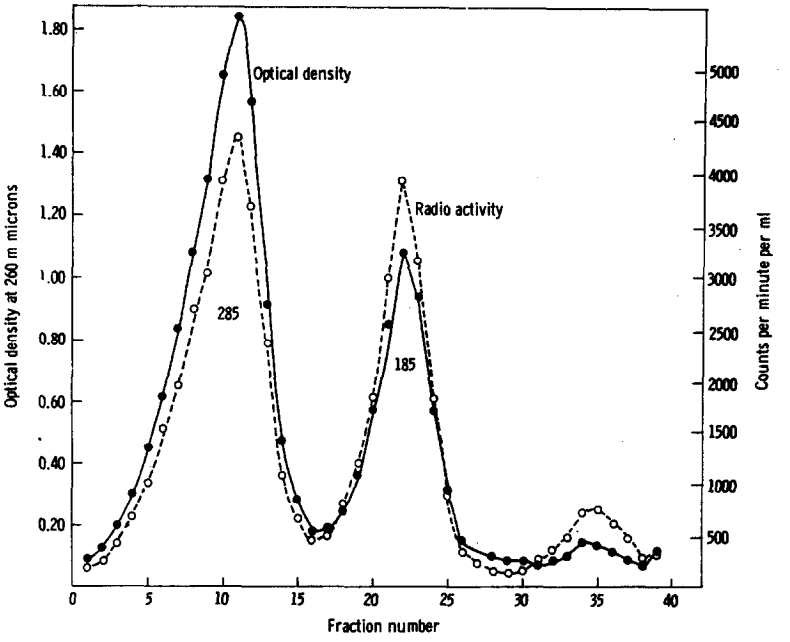

Fig. 1. Sedimentation pattern in a sucrose gradient of HeLa ribosomal RNA labeled with C^{14} from methionine-methyl-C^{14}. See Attardi et. al. (1963) for details of the centrifugation procedure and the determination of radioactivities and optical densities of the fractions collected from the sucrose gradient tubes.

approximately 1 $^1/_2$ times as many methyl groups per optical density unit as did 28S RNA. Similar analytical determinations indicated that S-RNA contained approximately 4 times as many methyl groups as the 18S ribosomal RNA.

The fractions from the sucrose gradients that contained the 18S, the 28S, and the transfer RNA were pooled separately and each of the pooled fractions was subjected to hydrolysis at 100° for 60 minutes in 70% perchloric acid. This procedure is known to result in the liberation of all bases (Marshak and Vogel, 1951). The treatment causes the ribose portion of the RNA to polymerize into a black insoluble material. This insoluble material was separated from the soluble material by centrifugation and washed until the wash water was free from radioactivity. The washings were combined with the

original supernatant solution and the solution was neutralized by the careful addition of 10 N KOH. The resulting insoluble $KClO_4$ was sedimented by centrifugation and washed free from radioactivity by repeated resuspension in water. The washings and the supernatant solution from the first centrifugation were combined. This solution would be expected to contain the free bases liberated from the RNA by hydrolysis with perchloric acid. Control experiments showed that the hydrolysis conditions and the washing procedure resulted in the recovery of 93 per cent of free pyrimidines from cytidine-5'-phosphate and uridine-5'-phosphate. Radioactivity determinations made on the RNA before and after hydrolysis indicated that 20-24% of the radioactivity of both 28S and 18S RNA and 64% of that of the S-RNA was present in the soluble material. The remaining radioactivity presumably was present in the black, insoluble material formed by the polymerization of ribose during hydrolysis. Thus, these data indicate that 20-24% of the methyl groups found in the ribosomal RNA consist of methylated bases, whereas 64% of the methyl groups of the S-RNA are present in the form of methylated bases. We have additional unpublished data which indicates that the major portion (76-80%) of the methyl groups of the ribosomal RNA and the minor portion (36%) in S-RNA, are present as methyl groups on the ribose portion of the nucleotide residues. The site of methylation of ribose remains uncertain, although it seems likely that these methyl groups would be present as 2-0 methyl ribose, since Hall (1963) has shown that such 0-methyl groups occur in RNA preparations.

The procedure of Cohn (1949) was used to separate, on a Dowex 50 column, the bases produced by hydrolysis with perchloric acid. Three radioactive peaks were obtained from the 18S RNA hydrolysate and only two from the 28S RNA hydrolysate. Two of the three peaks from 18S RNA were located in the general region of elution of adenine from the column; the third peak eluted with guanine. The two peaks from the 28S RNA hydrolysate were in the same positions as two of the three from the hy-

drolysate of the 18S RNA. Paper chromatographic experiments suggested that one of the three radioactive peaks obtained from the hydrolysate of the 18S RNA was due to the presence of 6-dimethylaminopurine. This compound was missing from the hydrolysate of the 28S RNA. The chromatographic properties of the two remaining unidentified methylated bases indicate that they are not identical with any of the following compounds: thymine, 5-methylcytosine, 1-methylguanine, 2-methylguanine, 7-methylguanine, 2-methyladenine, 6-methylaminopurine, and 7-methyladenine. Further work is in progress to identify these compounds.

Calculations made from the specific radioactivities of the methionine added to the growth medium and the purified nucleic acids show that S-RNA, 18S ribosomal RNA, 28S ribosomal RNA, and DNA contain one methyl group per 12, 48, 71, and 115 nucleotide residues, respectively. Chromatographic experiments similar to those described above for RNA showed that no radioactivity was found in the adenine and guanine fractions of the perchloric acid-hydrolysate of DNA. The single radioactive component present in the hydrolysate behaved chromatographically as 5-methylcytosine.

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