Methylation of Newly Synthesized Ribonucleic Acid by Isolated Rat Liver Nuclei

CHARACTERIZATION OF THE RIBONUCLEIC ACID SYNTHESIZED BY NUCLEI FROM STARVED ANIMALS

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1. Nuclei from rat liver incubated with *S*-adenosyl[*methyl*- ^{14}C]methionine incorporated radioactivity into RNA and into lipid and protein. 2. All of the labelled RNA was extracted from the nuclei with trichloroacetic acid at 90°C. 3. The [^{14}C]methyl-group incorporation into the hot-trichloroacetic acid extract was 30% inhibited by the addition of actinomycin D (100 $\mu\text{g}/\text{mg}$ of DNA) or by the omission of CTP, GTP and UTP. 4. Assuming that the main substrate for this triphosphate-dependent methylation was newly synthesized precursor rRNA containing one methyl group/30 uridylyte residues, it was calculated that approx. 60% of the [^{14}C]UMP incorporated under similar conditions represented precursor rRNA synthesis. 5. In agreement with this, low concentrations of actinomycin D (approx. 1 $\mu\text{g}/\text{mg}$ of DNA) sufficient to abolish the triphosphate-dependent incorporation of [^{14}C]methyl group inhibited 68% of the [^{14}C]UMP incorporation. 6. The incorporation of [^{14}C]UMP by nuclei from starved animals decreased progressively with increasing periods of starvation, whereas the triphosphate-dependent [^{14}C]methyl-group incorporation was not further decreased after 1 day of starvation. 7. This suggests that precursor rRNA synthesis decreased within 1 day whereas other species of RNA were affected only after longer periods of starvation.

The RNA synthesized by isolated rat liver nuclei in the presence of Mg^{2+} appears to be predominantly ribosomal on the basis of its nucleotide composition and nearest-neighbour analysis (Widnell & Tata, 1966; Pogo, Littau, Allfrey & Mirsky, 1967; Chambon, Ramuz, Mandel & Doly, 1968). In agreement with this most of the RNA synthesized *in vitro* under these conditions is located in the nucleolus (Pogo *et al.* 1967), which is also the site where the 45S precursor of rRNA (precursor rRNA) is synthesized *in vivo* (see e.g. Perry, 1967), and where the Mg^{2+} -stimulated RNA polymerase responsible for the synthesis of precursor rRNA is located (Widnell & Tata, 1966; Roeder & Rutter, 1970).

Precursor rRNA is the predominant naturally methylated RNA synthesized by nuclei and the nascent chains are methylated during synthesis *in vivo* (Greenberg & Penman, 1966; Zimmerman & Holler, 1967; Muramatsu & Fujisawa, 1968). In HeLa cells the precursor rRNA contains 0.6 equivalent of methyl group/100 nucleotide residues (Wagner, Penman & Ingram, 1967), and 18% of these residues are uridylyte (Willems, Wagner,

Laing & Penman, 1968). Therefore the ratio of methyl groups to uridylyte residues in this precursor rRNA is 1:30.

Culp & Brown (1970) and Liau, Flatt & Hurlbert (1970) have demonstrated that isolated nucleoli incorporate [^{14}C]methyl groups into RNA from *S*-adenosyl-L-[*methyl*- ^{14}C]methionine. The present paper describes a similar incorporation of [^{14}C]methyl groups into RNA by intact nuclei and makes use of the ratio of methyl group/UMP incorporation to measure the proportion of precursor rRNA synthesized under these conditions.

MATERIALS AND METHODS

Animals. Male, Wistar-derived albino rats were maintained as previously described (Rickwood & Klemperer, 1970).

Materials. [4- ^{14}C]UTP in aqueous solution and *S*-adenosyl-L-[*methyl*- ^{14}C]methionine in 0.01 M- H_2SO_4 (pH 2.5-3.5) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.). For determination of the specific radioactivity of these labelled compounds, the

radioactivity of samples diluted in 5% (w/v) trichloroacetic acid was measured as described below. Unlabelled *S*-adenosyl-L-methionine, from Koch-Light Laboratories Ltd. (Colnbrook, Bucks., U.K.), was dissolved in 2M-H₂SO₄, pH 3, and stored at -20°C. RNA (mol. wt. 20 000–30 000, from *Torula*) was purchased from Calbiochem Ltd. (London W.1, U.K.). Pancreatic ribonuclease A was obtained from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

Preparation of nuclei. Nuclei were prepared as described previously (Rickwood & Klemperer, 1970).

Measurement of incorporation of radioactivity from [4-¹⁴C]UTP and *S*-adenosyl-L-[methyl-¹⁴C]methionine. The standard reaction mixture in both cases contained (in 0.5 ml): nuclei (0.3–0.4 mg of DNA), 100 μmol of sucrose, 50 μmol of tris-HCl buffer, pH 8.0, 2.5 μmol of MgCl₂, 0.02 μmol of *S*-adenosyl-L-methionine, 5 μmol of 2-mercaptoethanol and 0.1 μmol each of ATP, CTP, GTP and UTP. The appropriate labelled precursor in the reaction mixture was either [4-¹⁴C]UTP (2.4×10^6 c.p.m./μmol) or *S*-adenosyl-L-[methyl-¹⁴C]methionine (25×10^6 c.p.m./μmol). When the methyl-group incorporation was measured in the absence of CTP, GTP and UTP the amount of ATP was increased to 0.4 μmol.

The incubations were terminated by addition of 0.3 ml of 0.2M-sodium pyrophosphate, pH 7.4, 0.1 ml of RNA (*Torula*; 2 mg/ml), 0.1 ml of bovine plasma albumin (20 mg/ml) and 8 ml of 0.4M-HClO₄ at 0°C. The precipitate was collected by centrifugation at 1000g for 10 min and was washed twice with 8 ml of 0.4M-HClO₄ to remove acid-soluble material. Lipid in the pellet was extracted by two washings with 8 ml of ethanol at 0°C followed by two washings with 8 ml of ether-ethanol-chloroform (2:2:1, by vol.) at 37°C (Allfrey, Mirsky & Osawa, 1957). Potassium acetate (1%, w/v) was added to the ethanol used for washing at 0°C to minimize the extraction of protein and RNA into the lipid solvents (Hutchinson & Munro, 1961; Steele, Okamura & Busch, 1964).

The pellet was then suspended in 2.0 ml of 5% (w/v) trichloroacetic acid and heated at 90°C for 20 min. The suspension was cooled to 0°C and after the addition of 0.05 ml of bovine plasma albumin (20 mg/ml) was centrifuged at 1000g for 10 min. The supernatant ('hot-trichloroacetic acid extract') was taken and the radioactivity of a 1.0 ml sample was counted in 9 ml of Triton X-100 scintillation fluid [33% (v/v) Triton X-100 in toluene scintillator containing 4 g of 2,5-diphenyloxazole and 0.05 g of 1,4-bis-(5-phenyloxazol-2-yl)benzene/litre], for measurement of radioactivity in a Nuclear-Chicago 725 system scintillation counter (background 35 c.p.m.; counting efficiency approx. 68%). The hot-trichloroacetic acid extract contained more than 95% of the 0.4M-HClO₄-precipitable radioactivity incorporated from [¹⁴C]UTP.

Radioactivity recovered in the hot-trichloroacetic acid extracts from duplicate incubations agreed within 4%. When the lipid solvent extraction was omitted the radioactivity in the hot-trichloroacetic acid extract was approximately doubled, presumably owing to the presence of [¹⁴C]methyl-labelled lipid. On the other hand [¹⁴C]UMP incorporation into the hot-trichloroacetic acid extract was unaffected by lipid extraction. Therefore this procedure caused no loss of RNA.

To measure the radioactivity that remained insoluble

after extraction with hot trichloroacetic acid, the pellet was washed with 8 ml of 0.4M-HClO₄ and after centrifugation was resuspended in 1 ml of 5% (w/v) trichloroacetic acid, collected quantitatively by filtration on 2.5 cm-diam. glass-fibre disks (Whatman GF/C) and washed on the filter with 4 ml of 5% (w/v) trichloroacetic acid. After drying in an oven at 37°C overnight, the disks were placed in 10 ml of toluene scintillator and left for 48 h to allow the chemiluminescence to decay before measurement of radioactivity in the scintillation counter.

Determination of RNA methylase activity of nuclei. The capacity of nuclei to methylate RNA was measured in standard reaction mixtures in the presence of added *Torula* RNA (1 mg/0.5 ml) and the incorporation of radioactivity into the hot-trichloroacetic acid extract was measured after a 5 min incubation. This concentration of RNA permitted the maximum rate of [¹⁴C]methyl-group incorporation and the incorporation was unaffected by the presence or absence of CTP, GTP and UTP.

Phenol extraction of methylated RNA. The incubation was scaled up to 1 ml and was terminated by the addition of an equal volume of 0.1M-sodium acetate (pH 5.0) and 0.2 ml of RNA (10 mg/ml) at 0°C. The mixture was adjusted to pH 5.0, sodium dodecyl sulphate was added to a final concentration of 0.5% and an equal volume of a mixture containing phenol, *m*-cresol, water and 8-hydroxyquinoline (500:70:55:0.5, by wt.) (Kirby, 1965) was added. The RNA was then extracted at 60°C by the method of Scherrer & Darnell (1962), the mixture was centrifuged and the RNA was precipitated from the aqueous phase with 3 vol. of ethanol. After standing overnight at -20°C the RNA was collected by centrifugation and redissolved in 2 ml of 0.05M-tris-HCl buffer, pH 7.4. Samples (1 ml) were taken for the determination of acid-precipitable radioactivity before and after digestion with ribonuclease (100 μg of pancreatic ribonuclease/ml, incubated for 60 min at 37°C). Acid-precipitable radioactivity was measured after the addition of 0.1 ml of RNA (2 mg/ml), 0.1 ml of bovine plasma albumin (20 mg/ml) and 1 ml of 10% (w/v) trichloroacetic acid. The precipitate was collected quantitatively by filtration on a 2.5 cm-diam. glass-fibre disc and its radioactivity counted as described above.

RESULTS

Incorporation of label into nuclei. When nuclei were incubated with [¹⁴C]UTP or *S*-adenosyl-L-[methyl-¹⁴C]methionine in the presence of all four ribonucleoside triphosphates, radioactivity was incorporated into acid-precipitable material (Table 1). The incorporation was abolished if the nuclei had been previously heated at 90°C for 10 min. More than 95% of the radioactivity incorporated from [¹⁴C]UTP was soluble in hot trichloroacetic acid, but only 15% of the radioactivity incorporated from *S*-adenosyl-L-[methyl-¹⁴C]methionine could be so extracted. Thus the hot-trichloroacetic acid extract contained essentially all of the newly synthesized RNA, but only a small part of the [¹⁴C]methyl-group incorporation. Most of the [¹⁴C]methylation occurred into material

Table 1. *Incorporation of radioactivity from [¹⁴C]UTP and S-adenosyl-L-[methyl-¹⁴C]methionine*

The complete system contained (in 0.5 ml): nuclei (0.3–0.4 mg of DNA); 100 μ mol of sucrose; 50 μ mol of tris-HCl buffer, pH 8.0; 2.5 μ mol of MgCl₂; 5 μ mol of 2-mercaptoethanol; 0.02 μ mol of *S*-adenosyl-L-methionine; and 0.1 μ mol each of ATP, CTP, GTP and UTP. The labelled precursor was either [4-¹⁴C]UTP or *S*-adenosyl-L-[methyl-¹⁴C]methionine. After incubation at 37°C for 5 min the acid-precipitable material was collected and fractionated as described in the Materials and Methods section. Where indicated nuclei were heated for 10 min at 90°C before incubation. Treatment with ribonuclease (100 μ g/0.5 ml of reaction mixture) at the end of the incubation was for 20 min at 37°C after first heating the reaction mixture at 90°C for 10 min.

Fractionation procedure	Fraction	[¹⁴ C]UMP incorporated (pmol/mg of DNA)	[¹⁴ C]Methyl groups incorporated (pmol/mg of DNA) % of total	
—	Total acid-precipitable radioactivity	930	350	100
—	Total acid-precipitable radioactivity (heated nuclei)	10	1	—
Standard	Hot-trichloroacetic acid extract	920	54	15
Standard	Hot-trichloroacetic acid-insoluble residue	17	67	19
Standard	Hot-trichloroacetic acid extract (ribonuclease-treated nuclei)	46	25	7
Lipid extraction omitted	Hot-trichloroacetic acid extract	921	128	37

that was extractable with lipid solvents or insoluble in hot trichloroacetic acid. The latter fraction probably contained [¹⁴C]methylated protein formed by the action of the protein methylases that are known to occur in the nucleus (Allfrey, Faulkner & Mirsky, 1964; Sekeris, Sekeri & Gallwitz, 1967).

In other experiments RNA was isolated by phenol extraction from nuclei that had been incubated with *S*-adenosyl-L-[methyl-¹⁴C]methionine and all four nucleoside triphosphates, and it was found that of the radioactivity associated with the extracted RNA (equivalent to approx. 26 pmol of methyl groups/mg of DNA in the original nuclei) more than 98% became acid-soluble after incubation with pancreatic ribonuclease. Although the [¹⁴C]-methyl-labelled RNA was extracted in the hot-trichloroacetic acid fraction, Table 1 shows that only 55% (54–25 = 29 pmol in Table 1) of the [¹⁴C]methyl label in this fraction could be eliminated by digestion of the nuclei with ribonuclease before extraction. The ribonuclease-resistant portion was not characterized further but it may include proteins (e.g. histone) that were soluble in hot trichloroacetic acid.

Table 1 also shows that when the lipid solvent extraction was omitted there was a large increase in the [¹⁴C]methyl label in the hot-trichloroacetic acid extract, indicating that under these circumstances ¹⁴C-labelled lipid-soluble materials were present in the extract.

Characteristics of the incorporation into the hot-trichloroacetic acid extract. Fig. 1 shows that there was a rapid incorporation of [¹⁴C]UMP and [¹⁴C]methyl groups into the hot-trichloroacetic acid extract over the first 5 min, after which the rate diminished. The incorporation of [¹⁴C]UMP ceased

after 10 min, but the [¹⁴C]methylation continued for at least 20 min. When the nucleoside triphosphates other than ATP were omitted the incorporation of radioactivity from *S*-adenosyl-L-[methyl-¹⁴C]methionine was only partially inhibited, e.g. from 53 pmol to 36 pmol/mg of DNA after 5 min. Thus in the complete reaction mixture about 30% of the [¹⁴C]methyl-group incorporation into the hot-trichloroacetic acid extract was nucleoside triphosphate-dependent after incubation for 5 min.

Table 2 shows that the omission of CTP, GTP and UTP inhibited [¹⁴C]methyl-group incorporation into the hot-trichloroacetic acid extract to the same extent as the addition of a concentration of actinomycin D (100 μ g/mg of DNA) sufficient to inhibit more than 90% of the [¹⁴C]UMP incorporation. This nucleoside triphosphate-dependent methylation (17 pmol of [¹⁴C]methyl group/mg of DNA) therefore probably represented the methylation of RNA that had been synthesized during the incubation. The addition of *Torula* RNA (mol. wt. 20 000–30 000) increased the incorporation of methyl groups into the hot-trichloroacetic acid extract, presumably as a result of the methylation of the added RNA. On the other hand, in the presence of ribonuclease the incorporation was inhibited to a greater extent than by actinomycin D, suggesting that pre-existing RNA in the nuclei was methylated in addition to that synthesized during the incubation.

In other experiments further characteristics of the [¹⁴C]methyl-group incorporation into the whole hot-trichloroacetic acid extract were studied. The incorporation showed a broad alkaline pH optimum (relative rates: pH 7.4, 0.7; pH 8.0, 1.0; pH 8.5, 1.1),

Fig. 1. Incorporation of radioactivity from [4- ^{14}C]UTP and *S*-adenosyl-L-[methyl- ^{14}C]methionine into the hot-trichloroacetic acid extract. Standard reaction mixtures (Table 1) containing either [^{14}C]UTP or *S*-adenosyl-L-[methyl- ^{14}C]methionine were incubated at 37°C for the times indicated. The acid-precipitable material was collected and fractionated as described in the Materials and Methods section. The radioactivity in the hot-trichloroacetic acid extract was measured. ●, [^{14}C]UMP incorporation; ▲, [^{14}C]methyl-group incorporation in the presence of all four nucleoside triphosphates; Δ, [^{14}C]methyl-group incorporation in the absence of CTP, UTP and GTP.

and non-enzymic methylation in heat-inactivated nuclei was negligible over the pH range 7.4–8.5. The apparent concentration of *S*-adenosyl-L-methionine for maximum velocity was 30 μM and approx. 8 μM for half-maximum velocity. The conditions normally used to measure the activity of the Mn^{2+} -ammonium sulphate-stimulated RNA polymerase were inhibitory. Thus the addition of manganese chloride (3mM) and of ammonium sulphate (350mM) together inhibited the incorporation into the whole trichloroacetic acid extract by more than 90%.

Inhibition by actinomycin D of the incorporation of radioactivity into the hot-trichloroacetic acid extract. Fig. 2 shows that the incorporation of

[^{14}C]methyl group into the hot-trichloroacetic acid extract was more sensitive to actinomycin D than was the incorporation of [^{14}C]UMP. At 1.2 μg of actinomycin D/mg of DNA the nucleoside triphosphate-dependent incorporation of [^{14}C]methyl group was almost completely inhibited, whereas the [^{14}C]UMP incorporation was only 68% inhibited. It is known that low concentrations of actinomycin D can inhibit the synthesis of precursor rRNA without affecting the synthesis of other species (see, e.g., Perry, 1963). Therefore it is likely that the 68% inhibition of the [^{14}C]UMP incorporation, which abolished the nucleoside triphosphate-dependent methyl-group incorporation, represented the proportion of precursor rRNA,

Table 2. Incorporation of [^{14}C]methyl groups and [^{14}C]UMP into the hot-trichloroacetic acid extract

Nuclei (0.4 mg of DNA) were incubated as described in Table 1 except that the conditions were modified as shown by the omission of triphosphates or by the addition of actinomycin D (40 μg), *Torula* RNA (500 μg) or ribonuclease A (100 μg). The triphosphates omitted were CTP, GTP and UTP (for [^{14}C]methyl-group incorporation) and CTP and GTP (for [^{14}C]UMP incorporation).

Incubation conditions	Incorporation (pmol/5 min per mg of DNA)	
	[^{14}C]Methyl group	[^{14}C]UMP
Complete	53	930
Triphosphates omitted	36	71
Actinomycin D (40 μg) added	37	68
<i>Torula</i> RNA (500 μg) added	84	—
Ribonuclease A (100 μg) added	29	112

and the remaining [^{14}C]UMP incorporation (32%) represented the proportion of other non-methylated species of RNA that were synthesized. In approximate agreement with this is the proportion of precursor rRNA calculated on the assumption that the latter contained one methyl group/30 UMP residues, as in the case of HeLa cells (see the introduction). Thus in Fig. 1 the nucleoside triphosphate-dependent incorporation of [^{14}C]methyl groups was 17 pmol/mg of DNA, which is equivalent to 510 pmol of [^{14}C]UMP in the precursor rRNA or 57% of 900 pmol of [^{14}C]UMP actually incorporated.

In these experiments a 5 min period was adopted because the incorporation of UMP and of methyl group were both proportional to time over this period (see Fig. 1). Approximately similar results were obtained with longer incubations. Thus from the results in Fig. 1, 59% of the RNA synthesized after 10 min was precursor rRNA.

Fig. 2. Inhibition of the incorporation of radioactivity from [^{14}C]UTP and *S*-adenosyl-L-[methyl- ^{14}C]methionine into the hot-trichloroacetic acid extract by actinomycin D. Actinomycin D was added to the standard reaction mixtures (Table 1) containing either [^{14}C]UTP or *S*-adenosyl-L-[methyl- ^{14}C]methionine. After incubation for 5 min at 37°C the acid-precipitable material was collected and fractionated as described in the Materials and Methods section. ●, [^{14}C]UMP incorporation (100% = 940 pmol/5 min per mg of DNA); ▲, nucleoside triphosphate-dependent [^{14}C]methyl-group incorporation (100% = 17.1 pmol/5 min per mg of DNA).

Table 3. [^{14}C]UMP and nucleoside triphosphate-dependent [^{14}C]methyl-group incorporation by liver nuclei from fed and starved rats

Incubation mixtures similar to those described in Table 1 contained either [¹⁴ C]UTP and unlabelled <i>S</i> -adenosyl-L-methionine or <i>S</i> -adenosyl-L- <i>[methyl-¹⁴C]</i> methionine with or without nucleoside triphosphates as shown. After incubation for 5 min at 37°C the acid-precipitable material was collected and the hot-trichloroacetic extract was prepared as described in the Materials and Methods section. Results for two separate experiments are presented and all values are averages of results from duplicate incubations, which agreed within 4%. The total methylating activity of these nuclei measured in the presence of added <i>Torula</i> RNA (1 mg/0.5 ml of reaction mixture) was 83, 80 and 59 pmol of [¹⁴ C]methyl group/mg of DNA for nuclei from fed and 1 day- and 4 day-starved rats respectively.									
Starvation (days)	Expt. no.	Total [¹⁴ C]UMP incorporation			[¹⁴ C]Methyl-group incorporation (pmol/mg of DNA)			Calculated UMP incorporation into precursor rRNA	
		Plus all triphosphates (a)	Minus CTP, GTP and UTP (b)	Nucleoside triphosphate- dependent (a-b)	(pmol/mg of DNA) [(a-b) × 30]	Inhibition (%)	% of total UMP incorporation		
0	1	52.8	35.5	17.3	519	0	58		
	2	53.5	36.0	17.5	525		59		
1	1	39.6	30.3	9.3	279	48	46		
	2	39.6	30.8	8.8	264		44		
4	1	29.1	19.1	10.0	300	41	65		
	2	29.7	19.4	10.3	309		66		

Incubation mixtures similar to those described in Table 1 contained either [^{14}C]UTP and unlabelled *S*-adenosyl-L-methionine or *S*-adenosyl-L-[methyl- ^{14}C]methionine with or without nucleoside triphosphates as shown. After incubation for 5 min at 37°C the acid-precipitable material was collected and the hot-trichloroacetic extract was prepared as described in the Materials and Methods section. Results for two separate experiments are presented and all values are averages of results from duplicate incubations, which agreed within 4%. The total methylating activity of these nuclei measured in the presence of added *Torula* RNA (1 mg/0.5 ml of reaction mixture) was 83, 80 and 59 pmol of [^{14}C]methyl group/mg of DNA for nuclei from fed and 1 day- and 4 day-starved rats respectively.

Effect of starvation on nucleoside triphosphate-dependent methylation. Table 3 shows that both [^{14}C]UMP incorporation and nucleoside triphosphate-dependent [^{14}C]methyl-group incorporation were decreased in liver nuclei from starved rats. The calculated [^{14}C]UMP incorporation into precursor rRNA suggests that the synthesis of this species of RNA was decreased within 1 day of starvation.

In contrast to the nucleoside triphosphate-dependent methylation, which was halved after 1 day of starvation and then showed no further fall, the total methylase activity as measured in the presence of added RNA was unchanged after starvation for 1 day and was only decreased by 25% after 4 days (see the legend to Table 3). Thus the decrease in the nucleoside triphosphate methylation in starvation did not appear to be due to a decrease in the methylating ability of the nuclei, but probably resulted from a decreased synthesis of the substrate, namely the precursor rRNA.

Table 3 shows that the total [^{14}C]UMP incorporation fell progressively during starvation with the result that the proportion of precursor rRNA was decreased after 1 day but after 4 days was somewhat higher than in nuclei from fed animals. Thus after 1 day the decrease in synthesis affected mainly the precursor rRNA, but after 4 days the synthesis of the other species of RNA was decreased to about the same extent.

DISCUSSION

These results show that nuclei incorporated radioactivity from the methyl-group donor *S*-adenosyl-L-[methyl- ^{14}C]methionine into a variety of materials, which could be fractionated reproducibly by simple procedures. One of these products was ^{14}C -labelled RNA and all of this labelled RNA was extracted with hot trichloroacetic acid. The extract included other [^{14}C]methyl-labelled materials in addition to RNA, but 55% of the radioactivity was sensitive to ribonuclease and 30% of the incorporation was both inhibited by actinomycin D and dependent on the presence of all four ribonucleoside triphosphates. Thus part of the radioactivity incorporated into RNA from *S*-adenosyl-L-[methyl- ^{14}C]methionine appeared to represent the methylation of RNA that had been synthesized from the triphosphates during the incubation.

Liau *et al.* (1970) have described the methylation of RNA by isolated nucleoli, which are also known to synthesize precursor rRNA both *in vitro* (Hurlbert, Miller & Vaughan, 1969) and *in vivo* (see, e.g., Perry, 1967). The methylation reaction observed by Liau *et al.* (1970) resembled that described in the present paper, in that the reaction had an alkaline pH optimum, could

utilize added RNA as a substrate and was inhibited by ammonium sulphate.

Precursor rRNA is the main species of RNA that is methylated in the nucleus *in vivo* (see, e.g., Muramatsu & Fujisawa, 1968). The minor low-molecular-weight species of methylated nuclear RNA (Weinberg & Penman, 1968; Moriyama, Hodnett, Prestayko & Busch, 1969; Engers & Walters, 1970) and the partially methylated tRNA precursor in the nucleus (Bernhardt & Darnell, 1969; Burdon & Clason, 1969) constitute only a small fraction of the total methylated RNA in the nucleus. Moreover the RNA normally synthesized by isolated nuclei in the presence of Mg^{2+} is predominantly precursor rRNA (see the introduction). Thus it is likely that the nucleoside triphosphate-dependent [^{14}C]methyl-group incorporation observed under these conditions represented the methylation of newly synthesized precursor rRNA. This is borne out by the fact that in Fig. 2 the actinomycin D-sensitive fraction of the newly synthesized RNA (presumably precursor rRNA; see, e.g., Perry, 1967) included all of the nucleoside triphosphate-dependent methyl- ^{14}C -labelled RNA. Further, the actinomycin D-sensitive fraction (approx. two-thirds) was about the same as the proportion of precursor rRNA obtained assuming that the nucleoside triphosphate-dependent methylation was equivalent to one-thirtieth of the UMP incorporation into precursor rRNA, i.e. the same as the ratio of methyl groups: UMP residues in the precursor rRNA of HeLa cells (see the introduction).

The synthesis of rRNA is decreased in the livers of starved rats (Hirsch & Hiatt, 1966) and in tissue-culture cells deprived of amino acids (Maden, Vaughan, Warner & Darnell, 1969; Ellem, Fabrizio & Jackson, 1970). Calculation of the amount of newly synthesized precursor rRNA from the nucleoside triphosphate-dependent incorporation of [^{14}C]methyl-groups by nuclei showed that the synthesis of precursor rRNA was decreased after starvation for 1 day. Moreover, the observed decrease in UMP incorporation by nuclei after starvation for 1 day was predominantly due to the decreased synthesis of precursor rRNA, whereas after 4 days the synthesis of other types of RNA (including presumably DNA-like RNA) was also decreased. In agreement with this Henderson (1970) has found that RNA synthesis by isolated nuclei from starved rats was less susceptible to low concentrations of actinomycin D. These results also reflect the findings of Rickwood & Klempner (1970) that the Mg^{2+} -dependent RNA synthesis (mainly of precursor rRNA) was decreased after 1 day whereas the Mn^{2+} -dependent synthesis (of DNA-like RNA) decreased only after 4 days of starvation.

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REFERENCES

- Allfrey, V. G., Faulkner, R. & Mirsky, A. E. (1964). *Proc. natn. Acad. Sci. U.S.A.* **51**, 786.
- Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957). *J. gen. Physiol.* **40**, 451.
- Bernhardt, D. & Darnell, J. E., jun. (1969). *J. molec. Biol.* **42**, 43.
- Burdon, R. H. & Clason, A. E. (1969). *J. molec. Biol.* **39**, 113.
- Chambon, P., Ramuz, M., Mandel, P. & Doly, J. (1968). *Biochim. biophys. Acta*, **157**, 504.
- Culp, L. A. & Brown, G. M. (1970). *Archs Biochem. Biophys.* **137**, 222.
- Ellem, K. A. O., Fabrizio, A. M. & Jackson, L. G. (1970). *Biochim. biophys. Acta*, **209**, 425.
- Engers, M. D. & Walters, R. A. (1970). *Biochemistry, Easton*, **9**, 3551.
- Greenberg, H. & Penman, S. (1966). *J. molec. Biol.* **21**, 527.
- Henderson, A. R. (1970). *Biochem. J.* **120**, 205.
- Hirsch, C. A. & Hiatt, H. H. (1966). *J. biol. Chem.* **241**, 5936.
- Hurlbert, R. B., Miller, E. G. & Vaughan, C. L. (1969). In *Advances in Enzyme Regulation*, vol. 7, p. 219. Ed. by Weber, G. Oxford: Pergamon Press Ltd.
- Hutchinson, W. C. & Munro, H. N. (1961). *Analyst, Lond.*, **86**, 768.
- Kirby, K. S. (1965). *Biochem. J.* **96**, 266.
- Liau, M. C., Flatt, N. D. & Hurlbert, R. B. (1970). *Biochim. biophys. Acta*, **224**, 282.
- Maden, B. E. H., Vaughan, M. H., Warner, J. R. & Darnell, J. E. (1969). *J. molec. Biol.* **45**, 265.
- Moriyama, Y., Hodnett, J. L., Prestayko, A. W. & Busch, H. (1969). *J. molec. Biol.* **39**, 335.
- Muramatsu, M. & Fujisawa, T. (1968). *Biochim. biophys. Acta*, **157**, 476.
- Perry, R. P. (1963). *Expl Cell Res.* **29**, 400.
- Perry, R. P. (1967). In *Progress in Nucleic Acid Research*, vol. 6, p. 220. Ed. by Davidson, J. N. & Cohn, W. E. New York and London: Academic Press.
- Pogo, A. O., Littau, V. C., Allfrey, V. G. & Mirsky, A. E. (1967). *Proc. natn. Acad. Sci. U.S.A.* **57**, 743.
- Rickwood, D. & Klempner, H. G. (1970). *Biochem. J.* **120**, 381.
- Roeder, R. G. & Rutter, W. J. (1970). *Proc. natn. Acad. Sci. U.S.A.* **65**, 675.
- Scherrer, K. & Darnell, J. E. (1962). *Biochem. biophys. Res. Commun.* **7**, 486.
- Sekeris, C. E., Sekeri, K. E. & Gallwitz, D. (1967). *Hoppe-Seyler's Z. physiol. Chem.* **348**, 1660.
- Steele, W. J., Okamura, N. & Busch, H. (1964). *Biochim. biophys. Acta*, **87**, 490.
- Wagner, E. K., Penman, S. & Ingram, K. N. (1967). *J. molec. Biol.* **29**, 371.
- Weinberg, R. A. & Penman, S. (1968). *J. molec. Biol.* **38**, 289.
- Widnell, C. C. & Tata, J. R. (1966). *Biochim. biophys. Acta*, **123**, 478.
- Willems, M., Wagner, E. K., Laing, R. & Penman, S. (1968). *J. molec. Biol.* **32**, 211.
- Zimmerman, E. F. & Holler, B. W. (1967). *J. molec. Biol.* **23**, 149.