INCORPORATION OF LABELED RIBONUCLEOSIDE-5'-MONOPHOSPHATES INTO RIBONUCLEIC ACID IN A CYTOPLASMIC FRACTION OF RAT-LIVER HOMOGENATES

EBERHARD HARBERS* AND CHARLES HEIDELBERGER

McArdle Memorial Laboratory, University of Wisconsin, Madison, Wisc. (U.S.A.)

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SUMMARY

The incorporation of [^{14}C] and [^{32}P]ribonucleoside-5'-phosphates into RNA has been studied in a fortified cytoplasmic fraction from rat-liver homogenates. It was found that AMP is attached as an end group adjacent to a cytidine residue. Both purine and pyrimidine ribonucleotides are incorporated adjacent to pyrimidine nucleotide residues. However, at least in the case of CMP, another nucleotide is attached afterwards, since the CMP is not terminal. The RNA was fractionated on ECTEOLA resin and the pattern of incorporation was found to be highly heterogeneous. The significance of these findings has been discussed.

INTRODUCTION

It was previously reported from this laboratory[1] that in a cytoplasmic fraction from rat-liver homogenate, similar to that described by SCHNEIDER AND POTTER[2], [^{32}P]-adenosine-5'-phosphate (AM^{32}P) is incorporated into the ribonucleic acid (RNA). Degradation by alkaline hydrolysis and snake-venom diesterase demonstrated that the radioactive phosphate was attached to the 5'-carbon of adenosine and the 3'-carbon of cytidine[1]. These studies have been continued, using [^{32}P]guanosine-5'-monophosphate (GM^{32}P), cytidine-5'-monophosphate (CM^{32}P), uridine-5'-monophosphate (UM^{32}P), and also with some [^{14}C]ribonucleotides.

These investigations were started with the idea that such expts. might give some information about the nucleotide sequence within the labeled RNA molecule. However, the results that we obtained with AMP[1], together with subsequent findings obtained by others with similar systems[3–6] indicated that the labeled nucleotide was not utilized for a net synthesis of RNA but was attached to the end of the RNA chain. The recent studies of HECHT *et al.*[6] have shown that this reaction is involved in the transfer of activated amino acids by the soluble RNA to microsomal ribonucleoprotein granules.

* Present address: Institut für Medizinische Physik und Biophysik, University of Göttingen, Germany.

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METHODS

The livers of female rats, weighing 120–150 g (Holtzman Co., Madison, Wis.) were homogenized in 0.25 *M* sucrose and centrifuged at 600 × *g*. The sediment was washed once with 0.25 *M* sucrose and the combined supernatant fractions were used as the "cytoplasmic fraction". In most expts. a 1 g equiv. of tissue was incubated in air at 30° for 45 min in a total volume of 10 ml of medium containing the following components: 0.01 *M* glutamate, 0.01 *M* pyruvate, 0.004 *M* fumarate, 0.01 *M* phosphate buffer (pH 7.2), 0.005 *M* $MgCl_2$, 0.03 *M* fructose, 0.225 *M* sucrose, 0.001 *M* ATP, and 1 μmole of the labeled ribonucleotide. The reaction was stopped with perchloric acid (final concn. 4%), and after extraction of the acid-soluble fraction and lipids the sodium ribonucleate was extracted with neutralized 10% NaCl at 100° for 1 h. The vol. of NaCl was 5 times that of the tissue residue, and the extraction was repeated with 3 volumes of 10% NaCl at 100° for 30 min. The combined extracts were treated with 3 vols. of ethanol to precipitate the sodium ribonucleate, which was centrifuged after 12 h in the cold, and dialyzed for 12 h against distilled water. The dialysate was then lyophilized.

For the degradation of the RNA, 1/3 was hydrolyzed with 0.1 *N* NaOH for 18 h at 37°, and the rest with snake-venom diesterase[1]. The ribonucleotides were separated either by gradient elution chromatography on Dowex-1-formate[7], or by stepwise elution from 1 × 5 cm hand columns of Dowex-1-formate as described by HECHT AND POTTER[8]. In the latter system, 10-ml samples were collected in the following order: 5 × 10 ml of water (nucleosides and free bases), 5 × 10 ml 0.1 *M* formic acid (CMP), 5 × 10 ml of 0.5 *M* formic acid (AMP), 5 × 10 ml of 0.1 *M* ammonium formate (inorganic phosphate), 7 × 10 ml of 0.2 *M* ammonium formate (UMP), water to remove ammonium ions, and 8 × 10 ml of 2 *M* formic acid (GMP). The elutions with 0.2 *M* ammonium formate and with 2 *M* formic acid were followed in the spectrophotometer to ensure complete elution. Because of some overlapping of inorganic phosphate and UMP, the latter fractions were adsorbed on charcoal, and the radioactivity of the inorganic phosphate was determined and subtracted from the total activity. The nucleotide samples were freeze-dried in vacuum desiccators over NaOH and $CaCl_2$, and were redissolved in water. The [^{32}P]radioactivity was determined in dipping counters[7]. In the [^{14}C]expts., the ammonium ions were removed from the UMP samples with Dowex 50 columns, and all samples were plated and counted in windowless flow counters. The individual nucleotides were identified on the basis of their positions on the chromatograms and the ratio of their u.v. absorption at 275 and 260 mμ.

In order to fractionate the RNA, 1-mg samples were put on columns[9] containing 0.5 g of ECTEOLA resin (kindly provided by Dr. AARON BENDICH of the Sloan-Kettering Institute). The resin was washed before use with 0.5 *M* NaOH until no u.v.-absorbing material was eluted, and then with 0.01 *M* phosphate buffer (pH 7.0) until the eluate was neutral. The different RNA fractions were eluted with NaCl in 0.01 *M* phosphate buffer at the following concns. of NaCl: 0.05, 0.10, 0.15, 0.20, 0.40, 0.60, 1.0 *M*, and then with 0.5 *M* NaOH.

In the partial hydrolysis of RNA with ribonuclease the samples (1 ml each in 0.1 *M* citrate buffer, pH 6.8) were placed in dialysis bags and incubated at 37° with shaking in tubes containing 4 ml of the same buffer[10]. The solutions were changed

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after 0.5, 1, 2, 4, 8, and 24-h incubation. All fractions were counted and read at 260 mμ. The snake venom diesterase incubations were performed in 3-ml solutions containing 5 mg of partially purified enzyme at 37° for 8 h. The incubation mixture was 0.1 *M* with respect to Tris buffer (Sigma, pH 9.2) and 0.01 *M* in $MgCl_2$. After incubation, the mixture was put directly on Dowex-1-formate columns for chromatography.

Incubations with prostatic phosphomonoesterase[12] were carried out at pH 5.2 (0.1 *M* acetate buffer) at 37° for 24 h. Half the incubation mixture was put on charcoal columns and washed with water to separate the inorganic phosphate, which was dried and counted; the other half was used for the determination of total radioactivity.

Synthesis of [^{32}P]ribonucleotides

In the initial expts. with AM^{32}P the labeled precursor was prepared by biosynthesis in rats[1]. The animals were partially hepatectomized, injected with labeled inorganic phosphate 12 h later, and killed after 4 h. The AM^{32}P was isolated from the acid-soluble fraction of liver by ion-exchange chromatography. In order to obtain the other labeled ribonucleotides, attempts were made by the method described by EGGLESTON[13] for AM^{32}P. It was found that with an extract from baker's yeast under the conditions described[13] highly radioactive [^{32}P]purine ribonucleotides were formed, but there was almost no formation of pyrimidine nucleotides. Therefore yeast was grown (we are indebted to Dr. HARLYN HALVORSON for growing the yeast and hydrogen bacteria) in a medium containing a high content of ^{32}P but little total phosphate. The radioactive nucleic acids were extracted with hot 10% NaCl as described above, dialyzed against distilled water, and hydrolyzed with snake-venom diesterase to get the ribonucleoside-5′-phosphates. There was no detectable contamination with deoxyribonucleoside phosphates, because the diesterase does not attack DNA before it is partially hydrolyzed with DNase. The individual nucleotides were isolated by gradient elution chromatography as above. The final yield was 2–5 μmoles of each nucleotide with a specific activity of $1–4 \cdot 10^6$ counts/min/μmole (dip counter, 15% efficiency) when 15 mC of ^{32}P was added to the yeast culture.

Because the incorporation of the ribonucleotides into RNA was very low under the homogenate conditions, it was desirable to obtain labeled precursors with specific activities as high as possible. Unfortunately the nucleotides were found to be very radiosensitive. This was particularly true of AM^{32}P, which after lyophilization was found to have been converted into inosine and hypoxanthine. The same trouble arose when [^{14}C]ribonucleotides were prepared by biosynthesis with *Hydrogenomonas faecalis* grown in an atmosphere of 50 mC of $^{14}CO_2$.

RESULTS

Because the incorporation of the ribonucleotides into RNA was very small (about 1% of the total radioactivity added to the system) attempts were made to find conditions under which the uptake of precursors might be increased. SCHNEIDER AND POTTER had observed[2] that storage of the liver homogenate in the cold for several hours before incubation caused an increased incorporation of [^{14}C]orotic acid into RNA. However, when we aged the homogenate similarly prior to incubation with AM^{32}P, GM^{32}P, and CM^{32}P there was actually a decrease in the incorporation

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into RNA. Thus the aging effect may be involved in the initial conversion of the orotic acid into nucleotides, rather than at the stage of polynucleotide formation. It was also observed that adding the other 3 non-labeled ribonucleotides had no effect on the incorporation of the labeled nucleotide. Therefore all further studies were carried out under the conditions described for the initial expt.[1].

In all cases, aliquots of the labeled RNA were degraded by alkaline hydrolysis and diesterase, thus locating the labeled phosphorus atom as being attached to the 5'-carbon of the original precursor and the 3'-carbon of the adjacent nucleotide residue in the polynucleotide chain. It can be seen from Table I that the ^{32}P was transferred from the precursor to other 5'-ribonucleotides to a greater extent with CMP, UMP, and GMP, than with AMP. Although these results are not as clear-cut as in the expt. with AM^{32}P, they show that under the conditions of this *in vitro* cytoplasmic homogenate system AMP and CMP are attached primarily to CMP: on the other hand, GMP and UMP are attached predominantly to UMP.

TABLE I

DISTRIBUTION OF [^{32}P]RADIOACTIVITY AFTER HYDROLYSIS OF RNA LABELED *in vitro* IN CYTOPLASMIC LIVER HOMOGENATE

Precursor	*Hydrolysis with snake venom diesterase*		*Hydrolysis with alkali*	
	Nucleotide	*% of total activity in RNA*	*Nucleotide*	*% of total activity in RNA*
AM^{32}P	5'-CMP	0.6	2',3'-CMP	87.4
AM^{32}P	5'-AMP	92	2',3'-AMP	5.4
AM^{32}P	5'-UMP	0.5	2',3'-UMP	1.2
AM^{32}P	5'-GMP	0.4	2',3'-GMP	1.2
CM^{32}P	5'-CMP	82	2',3'-CMP	41
CM^{32}P	5'-AMP	8	2',3'-AMP	17
CM^{32}P	5'-UMP	2	2',3'-UMP	22
CM^{32}P	5'-GMP	8	2',3'-GMP	20
UM^{32}P	5'-CMP	11	2',3'-CMP	18
UM^{32}P	5'-AMP	11	2',3'-AMP	18
UM^{32}P	5'-UMP	72	2',3'-UMP	50
UM^{32}P	5'-GMP	6	2',3'-GMP	14
GM^{32}P	5'-CMP	34	2',3'-CMP	19
GM^{32}P	5'-AMP	15	2',3'-AMP	11
GM^{32}P	5'-UMP	8	2',3'-UMP	61
GM^{32}P	5'-GMP	43	2',3'-GMP	9
Inorganic ^{32}P	5'-CMP	33	2',3'-CMP	27
Inorganic ^{32}P	5'-AMP	24	2',3'-AMP	21
Inorganic ^{32}P	5'-UMP	27	2',3'-UMP	25
Inorganic ^{32}P	5'-GMP	16	2',3'-GMP	27

When inorganic ^{32}P was used as the precursor, the distribution of label among the ribonucleotides was essentially random, particularly after alkaline hydrolysis. Thus it can be concluded that under these conditions labeled purine nucleotides are attached in RNA to pyrimidine nucleotides, and that labeled pyrimidine nucleotides are attached to a pyrimidine nucleoside of the same structure.

The results thus far indicated that in this *in vitro* system the nucleotides were

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TABLE II

RELEASE OF INORGANIC ^{32}P RADIOACTIVITY BY INCUBATION OF LABELED RNA WITH PROSTATIC PHOSPHOMONOESTERASE

Precursor	*% of inorganic ^{32}P liberated from total ^{32}P in the RNA*
$CM^{32}P$	4.5
$AM^{32}P$	12.7
$UM^{32}P$	12.8
$GM^{32}P$	10.7

TABLE III

In vitro INCORPORATION OF [^{14}C]AMP AND CMP INTO THE CYTOPLASMIC RNA OF RAT LIVER

The RNA was hydrolyzed with alkali, and the nucleotides and nucleosides isolated by ion-exchange chromatography.

Precursor	*Nucleic acid fraction*	*% of total radioactivity in RNA*
[^{14}C]AMP	Nucleosides	93
[^{14}C]AMP	2',3'-AMP	7
[^{14}C]CMP	Nucleosides	12
[^{14}C]CMP	2',3'-CMP	83
[^{14}C]CMP	2',3'-UMP	5

not incorporated as a result of net synthesis of RNA. In order to gain some information on the amount of label in the phosphate-terminal groups of the RNA chains, the labeled RNA was incubated with prostatic phosphomonoesterase and the release of inorganic phosphate was measured. As shown in Table II, there was relatively little inorganic phosphorus liberated by this treatment from the RNA obtained from all 4 precursors. These results suggest that the labeled ribonucleotides were not incorporated into RNA as end-groups with a free 5'-phosphate atom. In order to test this hypothesis [8-^{14}C]AMP (kindly provided by Dr. G. A. LEPAGE AND A. SARTORELLI) and [^{14}C]CMP were used as precursors in a similar expt. After alkaline hydrolysis, most of the radioactivity in the RNA sample incubated with [^{14}C]AMP was found in the nucleoside fraction (Table III), whereas in the RNA derived from incubation with [^{14}C]CMP the radioactivity was found mainly in the 2',3'-CMP fraction. Thus this expt. confirms the observations of CANELLAKIS[5] and HECHT *et al.*[6] that under the conditions of these *in vitro* systems AMP is attached to a terminal cytidine residue such that the 5'-phosphate of the AMP is attached to the hydroxyl group on the 3'-carbon of the cytidine. On the other hand [^{14}C]CMP is also attached primarily to cytidine in the RNA, but not in a terminal position since only 12 % of the radioactivity appeared in the nucleoside fraction after alkaline hydrolysis (Table III). From these observations and those of HECHT *et al.*[6] it appears that the incorporation of CMP to a terminal cytidine residue most probably precedes the terminal attachment of AMP to the RNA polynucleotide chain.

Previous studies from this laboratory had demonstrated the metabolic and intramolecular heterogeneity of RNA biosynthesis[10], and consequently attempts were

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made to fractionate the RNA, labeled in the cytoplasmic liver homogenate, with ECTEOLA resin. It can be seen in Table IV that the labeled ribonucleotides were incorporated into various RNA fractions in a very heterogeneous fashion. With all 4 precursors, the highest specific activities were found in the 0.05 *M* NaCl eluate. A similar but not so striking, heterogeneity has recently been reported by GOLDTHWAIT[14] following incubation of a similar system with AM^{32}P. The pattern was somewhat similar for the 2 pyrimidine nucleotides, but the 2 purine nucleotides exhibited dissimilar patterns. Thus there was little uniformity of the degree of incorporation of the various ribonucleotides into the various species of RNA molecules that were separated by the ECTEOLA resin.

The high specific activity of the 0.05 *M* NaCl eluate from the GM^{32}P incubation permitted alkaline degradation of this fraction. As can be seen from Table V, 90 % of the radioactivity was in the 2′,3′-UMP, a higher degree of specificity than that obtained with GM^{32}P in the expt. shown in Table I. Thus, in this species of RNA molecule, the GMP is adjacent to uridine in the polynucleotide chain.

TABLE IV

FRACTIONATION OF RNA SAMPLES LABELED *in vitro* WITH [^{32}P]RIBONUCLEOSIDE-5′-PHOSPHATE ON ECTEOLA RESIN

The fractions were eluted stepwise with increasing concns. of NaCl in 0.01 *M* phosphate buffer.

Precursor RNA fraction	*Specific activities in counts/min/E_{260}*							
	CM^{32}P		*AM^{32}P*		*UM^{32}P*		*GM^{32}P*	
	Specific activity	*% of total activity*	*Specific activity*	*% of total activity*	*Specific activity*	*% of total activity*	*Specific activity*	*% of total activity*
0.05 *M* NaCl	26.2	9.5	163	36	16.7	9.9	434	66
0.1 *M* NaCl	1.2	1.7	7.1	10.3	4.1	8.1	32	13.5
0.15 *M* NaCl	4.1	17.8	1.9	4.7	5.1	29.8	5.1	5.6
0.20 *M* NaCl	3.1	38.6	—	—	1.8	28.8	2.1	4.2
0.40 *M* NaCl	1.3	19.5	94	43	0.7	10.9	4.9	0.8
0.60 *M* NaCl	2.6	2.8	7.6	2.8	10.5	9.0	22.8	6.2
0.5 *M* NaOH	0.5	10.3	2.7	2.5	0.1	3.6	—	—

TABLE V

DISTRIBUTION OF RADIOACTIVITY IN ALKALINE HYDROLYSIS OF THE RNA FRACTION ELUTED FROM ECTEOLA

Elution with 0.05 *M* NaCl obtained following incubation with GM^{32}P.

Nucleic acid fraction	*% of total radioactivity in RNA*
2′,3′-CMP	1.9
2′,3′-AMP	0.9
2′,3′-UMP	90
2′,3′-GMP	7.2

DISCUSSION

It is well known from many tracer expts. that RNA is not a metabolically homogeneous compound within the cell. This is deduced from the fact that the rates of

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incorporation of labeled precursors into the RNA of cell fractions obtained by differential centrifugation are quite different[7,15]. Moreover, the RNA samples obtained by various extraction procedures[16,17] as well as by partial enzymic degradations[10] were of different specific activities. In the present study the RNA samples, obtained after incubation of fortified cytoplasmic fraction of rat-liver homogenate with the 4 [^{32}P]ribonucleoside-5′-monophosphates, were separated on ECTEOLA resin and the various fractions had widely differing specific activities. It was also found that one of these RNA samples obtained from an incubation with GM^{32}P showed a much more clear-cut position adjacent to uridine in the polynucleotide chain than did the total cytoplasmic RNA. Data of this sort point out clearly some of the pitfalls involved in the interpretation of incorporation expts. with total cellular RNA.

More recently the results of several investigations, including our own, have indicated that in homogenate systems adenosine phosphates can be incorporated into RNA as end groups without the synthesis of new RNA[1,5,6,18–20]. In those cases where it was determined, the labeled AMP was found to be attached only to cytidine[1,5,6]. Thus, in order for AMP to be incorporated, the terminal group in the polynucleotide chain must be cytidine with its 3′-position free. If the cytidine were not terminal, CTP had to be added in order for the incorporation of ATP to take place[6]. Although the ribonucleoside monophosphates were employed in the present research, it is clear from the expts. of CANELLAKIS[5] and HECHT *et al.*[6] that the triphosphates are the actual precursors. Thus in our system the kinases for the conversion of the mono- into the triphosphates must have been present. The fact that the alkaline hydrolysis of the RNA obtained from [^{14}C]CMP gave only labeled CMP, in contrast with the production of labeled nucleoside when [^{14}C]AMP was the precursor, demonstrates a repeating cytidine sequence. Furthermore, the expt. indicates that an unlabeled AMP residue most probably was added to the labeled CMP residue, otherwise the latter would have been terminal. The specific RNA that we have been discussing must have a sequence at the end of the polynucleotide chain as shown in Fig. 1.

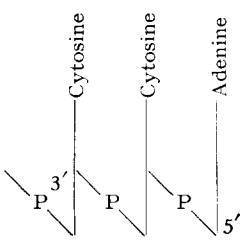

Fig. 1. Suggested structure for the terminal part of the specific RNA which incorporates AMP and CMP.

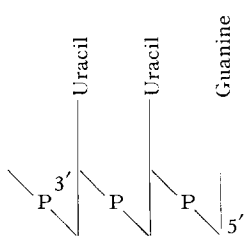

Fig. 2. Suggested structure for the terminal part of the specific RNA which incorporates GMP and UMP.

The information obtained from the incorporation of the 3 other ribonucleotides shows that they are also attached to pyrimidine residues; GMP and UMP were attached primarily to uridine, and CMP, mainly to cytidine. Thus the terminal sequence resulting from the incorporation of UMP and GMP may be represented as in Fig. 2.

The present findings seem to indicate that the part of the RNA molecule which is not terminal may be a more stable constituent of the cell than had thus far been

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fully recognized. This is in accord with recent observations of SIMINOVITCH AND GRAHAM[21] and of SCOTT AND TAFT[22]. Thus the incorporation of labeled precursors into RNA does not necessarily demonstrate the "dynamic state" of this compound. However, it does appear that the incorporation of nucleotides into nucleic acids without net synthesis is a more general phenomenon, which may even include DNA[23-25].

The functional importance of the incorporation of AMP as an end group attached to a terminal cytidine residue[1,5] was unknown until the studies of HECHT *et al.*[6] demonstrated that during the initial stages of protein synthesis the activated amino acid in the form of an acyl adenylate was attached to a soluble cytoplasmic RNA. For the attachment of the acyl adenylate a terminal cytidine was required, or CTP had to be added to the system[6]. Subsequently the activated amino acid is transferred to the microsomal ribonucleoprotein particles[6], and DECKEN AND HULTIN[26] have demonstrated a similar transfer of nucleotide-label from the soluble RNA to microsomal ribonucleoproteins. Thus it appears that this type of nucleotide attachment to terminal positions in the nucleic acids, without change in the core, may be a general expression of its functional activity as a means for the transfer of the necessary information. It is also conceivable that similar end group reactions might occur in the case of DNA.

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