INCORPORATION OF LABEL FROM 5-FLUOROOROTATE INTO NON-RIBOSOMAL CYTOPLASMIC RNA IN RAT LIVER*

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1. Introduction

Studies from several laboratories, notably that of Heidelberger [1, 2], and others [3] have demonstrated that upon administration of radioactively labeled 5-fluoroorotic acid *in vivo*, the label occurs in free pyrimidine nucleotides in liver and is subsequently incorporated into pyrimidine nucleotide residues of RNA. However, few studies have been carried out in mammalian tissues on the actual types of RNA within the hepatic cell that are labeled by fluoropyrimidines. The studies described in this brief communication demonstrate that administration of 2-^{14}C-5-fluoroorotic acid leads to the labeling of RNA which has none of the characteristics of ribosomal RNA and a number of the characteristics of cytoplasmic "messenger" RNA as described for mammalian systems [4–6].

2. Methods

Male Holtzman rats weighing 150 to 160 g were utilized. The animals were maintained on a laboratory chow diet fed *ad libitum* in rooms alternately lighted 12 hr and kept dark for 12 hr. Prior to administration of the labeled compound, the animals were starved for 14 hr. At the end of the fasting period and 3 hr prior to sacrifice, 6-^{14}C-orotate (Calbiochem) or 2-^{14}C-5-fluoroorotate (Tracerlab) (5 μCi/μmole/rat) were administered intraperitoneally in 0.4 ml of water. The animals were sacrificed by decapitation, and the liver quickly removed and placed in ice-cold buffer. The liver was homogenized in 3 volumes of 0.25 M sucrose in 0.025 M tris-HCl, pH 7.6 with 0.005 M $MgCl_2$ and 0.05 M KCl, and the homogenate centrifuged at 12,000 *g* for 10 min to remove nuclei and mitochondria. The resulting supernatant solution was extracted with phenol by the modified method of Kirby [7] in order to isolate total cytoplasmic RNA. Transfer RNA and traces of phenol and sodium 4-aminosalicylate used for the extraction were removed by purification of RNA on a Sephadex G-200 column using 0.01 M tris-HCl.

Portions of isolated RNA were placed on linear sucrose density gradient (5–15%; w/v) and centrifuged for 17 hr at 24,000 rpm at 2°C in an SW-25.3 rotor in a Spinco L-2 ultracentrifuge. At the end of the centrifugation the tubes were removed, placed in tube holders and the bottom of the tube pierced with a pin. The contents of the tube were allowed to flow through the hole at the bottom by gravity. 0.5 ml fractions were collected after the flow was monitored by a Gilford Model 2000 Multiple Sample Absorbance Recorder. Radioactivity in the aliquots was determined utilizing a Packard scintillation counter.

Methylated albumin-kieselguhr columns were prepared according to the method of Mandell and Hershey [8]. RNA was applied to the column at 37°C and eluted using a 0.4 to 1.6 M linear gradient of buffered

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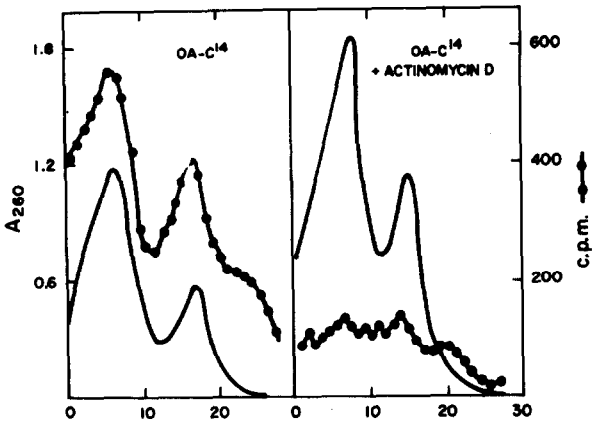

Fig. 1. Effect of actinomycin D on labeling of ribosomal RNA with 6-^{14}C-orotic acid. Holtzman male rats starved 14 hr were injected i.p. with 6-^{14}C-orotic acid (4 μCi/μmole per animal) 3 hr before killing. Actinomycin D was administered intraperitoneally 30 min before and 90 min after the label in a total dose level of 400 μg per kg.

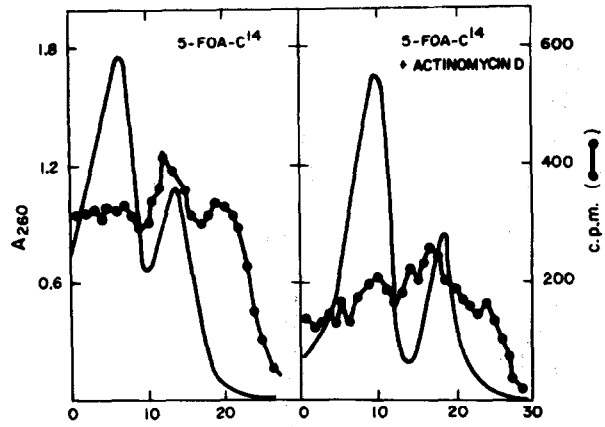

Fig. 2. Dissimilarity between the pattern of cytoplasmic ribosomal RNA absorbance and radioactivity after 2-^{14}C-5-fluororotic acid administration *in vivo*. Holtzman male rats starved 14 hr were injected i.p. with 2-^{14}C-5-fluororotic acid (5 μCi/μmole per animal) 3 hr before killing. Actinomycin D was administered as in fig. 1.

sodium chloride (pH 6.7). Fractions of 4.8 ml were collected at the rate of 10 fractions per hour. During the latter part of the chromatography the temperauture was raised [9] to 80°C.

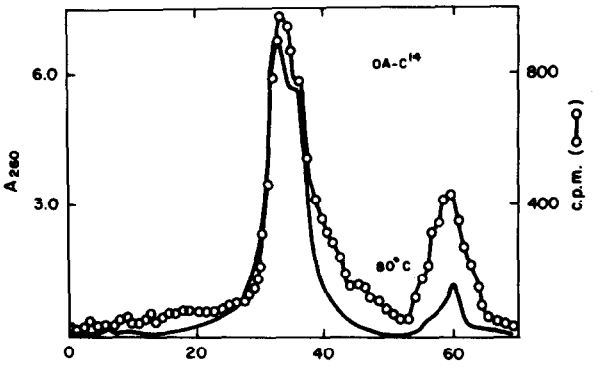

Fig. 3. Separation of cytoplasmic RNA labeled with 6-^{14}C-orotic acid on a methylated albumin-kieselguhr column. Separation of RNA was done at 37°C and 80°C using 0.4–1.6 M linear gradient of buffered NaCl (pH 6.7) as described in Methods.

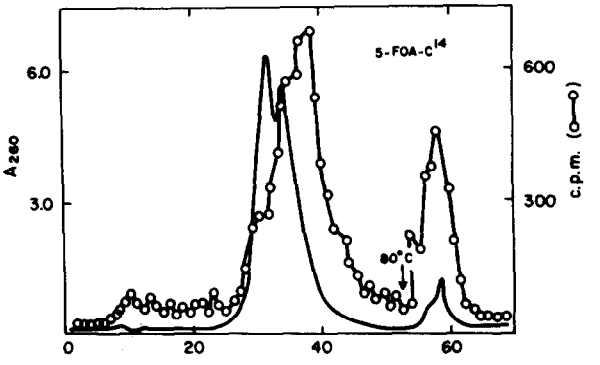

Fig. 4. Separation of RNA labeled *in vivo* after 2-^{14}C-5-fluororotic acid from ribosomal RNA on a methylated albumin-kieselguhr column. Separation was performed as in fig. 3.

3. Results

Fig. 1 shows the labeling patterns of RNA from livers of ^{14}C-orotate treated rats sedimented on 5–15% sucrose gradients. The RNA was obtained from animals administered the labeled compound as shown in the figures according to the methods described above. It can be seen that in untreated animals, orotic acid labels both the 18 and 28 S ribosomal peaks, as has been described previously [10]. Administration of actinomycin D (200 μg/kg; one dose 30 min before, and the second 90 min after injection of the isotope) resulted in a marked

decrease in total incorporation, but most important a complete loss of coincident labeling of the two ribosomal peaks. Previous studies in this and other laboratories [5, 11] have demonstrated that the RNA labeled in the presence of actinomycin D has a base composition quite similar to DNA and in this respect resembles a cytoplasmic messenger RNA fraction. In contrast to orotic acid, labeling with 5-fluoroorotic acid produces no coincident labeling of the 18 and 28 S ribosomal RNA peaks (fig. 2). In the animals not given actinomycin D, however, a peak is seen near the region of 18 S ribosomal RNA and a second peak in the region of approximately 10 S. When actinomycin D is administered, there is a small degree of inhibition of labeling, but no marked difference in the pattern which still exhibits a labeled peak in the region of 18 S as well as 10 S on the gradient. These data suggest that the RNA labeled by 5-fluoroorotic acid administration is not ribosomal RNA but of another species.

Fig. 3 shows the labeling patterns of the same RNA separated on a methylated albumin-kieselguhr column. The labeling pattern of ^{14}C-orotic acid follows the absorbance pattern rather closely, especially in the region of the major peak between fractions 30 and 40. The heavily labeled material eluted from the column after the temperature is raised to 80°C has been described by other authors and is felt to represent a more single-stranded form of RNA rich in adenylate-uridylate residues [12, 13]. In contrast, when 2-^{14}C-5-fluoroorotate is used to label *in vivo*, the major peak of labeling is shifted so that it does not coincide with the absorbance peak (fig. 4). There is still the heavily labeled peak coming off at the higher temperature.

4. Discussion

The data described here demonstrate that the labeling of cytoplasmic RNA in rat liver after the administration of 2-^{14}C-5-fluoroorotate is not identical to that occurring when 6-^{14}C-orotate is given as the label. The dissimilarities suggest that essentially 5-fluoroorotic acid administration does not label ribosomal RNA in the cytoplasm of the liver. Although the evidence is not at present complete, these data indicate that 2-^{14}C-5-fluoroorotate administration results in a rather selective labeling of a messenger RNA-like material in the cytoplasm. Preliminary data indicate that the RNA labeled by this analog in the nucleus remains in the nucleus for an extended period of time and the radioactivity in free cytoplasmic particles is relatively low. This might suggest that the newly formed nucleotides from 5-fluoroorotic acid may be incorporated into ribosomal precursor RNA and the RNA fraction formed but that this precursor is not treated by the nuclear apparatus in the same way as normal ribosomal RNA. It is possible that this analog-labeled RNA is not cleaved to smaller fractions and thus very small amounts appear not bound to cytoplasmic polysomes. Experiments are underway in this laboratory to clarify this particular point and to define further the type of cytoplasmic RNA labeled after 2-^{14}C-5-fluoroorotate administration.

References

[1] N.K.Chaudhuri, B.J.Montag and C.Heidelberger, Cancer Res. 18 (1958) 318.
[2] N.J.Wagner and C.Heidelberger, Biochim. Biophys. Acta 61 (1962) 373.
[3] J.L.Dahl, J.L.Way and R.E.Parks, Jr., J. Biol. Chem. 234 (1959) 2998.
[4] E.C.Henshaw, M.Revel and H.H.Hiatt, J. Mol. Biol. 14 (1965) 241.
[5] C.Lamar, Jr., M.Prival and H.C.Pitot, Cancer Res. 26 (1966) 1909.
[6] J.E.Darnell, Jr., Bacteriol. Rev. 32 (1968) 262.
[7] K.S.Kirby, Biochem. J. 96 (1965) 266.
[8] J.D.Mandell and A.D.Hershey, Anal. Biochem. 1 (1960) 66.
[9] K.A.O.Ellem, J. Mol. Biol. 20 (1966) 283.
[10] M.Muramatsu, J.L.Hodnett, W.J.Steele and H.Busch, Biochim. Biophys. Acta 123 (1966) 116.
[11] G.P.Georgiev, O.P.Samariua, M.I.Lerman and M.N.Smirnov, Nature 200 (1963) 1291.
[12] K.A.O.Ellem and J.W.Sheridan, Biochim. Biophys. Res. Commun. 16 (1964) 505.
[13] J.F.Houssais and G.Attardi, Proc. Natl. Acad. Sci. U.S. 56 (1966) 616.