Biochemical Pharmacology, Vol. 21, pp. 2497–2507. Pergamon Press, 1972. Printed in Great Britain.

DUAL EFFECT OF 5-AZACYTIDINE ON THE SYNTHESIS OF LIVER RIBONUCLEIC ACIDS

LACK OF THE RELATIONSHIP BETWEEN METABOLIC TRANSFORMATION OF OROTIC ACID *IN VITRO* AND ITS INCORPORATION *IN VIVO*

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(*Received* 14 *January* 1972; *accepted* 17 *March* 1972)

Abstract—Administration of 5-azacytidine to rats results in inhibition of orotidylic acid decarboxylase in cell-free extracts of liver. Maximal effects were observed 2–8 hr after the administration of the analogue, and enzyme activity returned to control levels 30–36 hr later. The utilization of orotic acid for the synthesis of liver ribonucleic acids was inhibited only at 5–6 hr after drug. At subsequent times its incorporation was markedly enhanced, with maximal increases (400–450 per cent) being noted 18–24 hr after 5-azacytidine. Chromatographic separation of liver RNA isolated 2 and 24 hr after 5-azacytidine on a methylated albumin kieselguhr column did not indicate preferential inhibition or stimulation of the synthesis of individual types of RNA.

5-Azacytidine is subjected to anabolic transformations which are associated with its incorporation into nucleic acids.[1] In eukaryotic cells, besides being incorporated into RNA, the analogue affects pyrimidine nucleotide synthesis *de novo*.[2] Pronounced inhibition of the proliferation of various cells following 5-azacytidine has been observed,[3–5] and it has been also found that the drug inhibits the growth of mouse leukemic cells.[6–8] Although the mechanism of the inhibitory effects of 5-azacytidine in various systems has been studied since 1963 (see for example, Suhadolnik[9]), relatively little attention has been devoted to an investigation of the metabolic changes occurring at later stages after its administration *in vivo*. The finding of an increased uridine kinase activity and of uridine incorporation into liver RNA 24 hr after the administration of 5-azacytidine[10] led us in the present study to a more detailed investigation of its effect on metabolic transformation and incorporation of orotic acid in rat liver.

MATERIALS AND METHODS

Reagents. Adenosine 5′-triphosphate, uridine 5′-monophosphate, orotidine 5′-phosphate and 5-phosphorylribose 1-pyrophosphate, were obtained from Calbiochem, Los Angeles. 5-Azacytidine and 6-azauridine 5′-monophosphate were synthetized in this Institute. Orotic acid-6-^{14}C (48 μc/μmole), uridine-2-^{14}C 5′-monophosphate (44 μc/μmole) and 6-azauridine-4,5-^{14}C (80 μc/μmole) were provided by the Institute for Research, Production and Uses of Radioisotopes in Prague. Orotidine-6-^{14}C 5′-phosphate was prepared enzymatically as described.[2]

Animal and cell-free liver extracts. For these experiments, groups of female Wistar rats (170–180 g) were used. In general, experiments were started between 8–9 a.m. Substances were dissolved immediately before i.p. administration; controls received the same volume of physiological saline. The animals were killed by decapitation, bled, and excised livers were homogenized with cooling in a glass homogenizer with a tight-fitting Teflon pestle with 3 vol. of 0·025 M Tris–HCl buffer (pH 7·5) containing $2{\cdot}5 \times 10^{-3}$ M KCl and 5×10^{-3} M Mg^{2+} ions. Homogenates were centrifuged (10,000 rev/min, 20 min, 2°) and defatted supernatant fractions were used as the source of enzyme activity.

Assay of enzyme activities. Orotate phosphoribosyltransferase was determined[11] during a 2-min incubation period at 37° in a reaction mixture (0·5 ml) containing 4×10^{-2} M Tris–HCl buffer (pH 7·4), 1×10^{-4} M orotic-6-^{14}C acid, 4×10^{-4} M 5-phosphorylribose 1-pyrophosphate with equimolar Mg^{2+} ions, and 0·1 ml of liver postmitochondrial supernatant fractions corresponding to 25 mg liver tissue.

Orotidine 5′-phosphate decarboxylase was assayed under similar conditions[11] (5-min period of incubation[12]) following the addition of 2×10^{-5} M orotidine-6-^{14}C 5′-phosphate.

Uridine kinase was determined during a 10-min incubation period[10] with 1×10^{-4} M 6-azauridine-4,5-^{14}C as substrate and 1×10^{-3} M adenosine 5′-triphosphate with equimolar Mg^{2+} ions.

Uridinemonophosphate kinase was assayed under similar conditions[10] (10-min incubation period) in a reaction mixture containing 1×10^{-4} M uridine-2-^{14}C 5′-monophosphate with 4×10^{-3} M adenosine 5′-triphosphate and 2×10^{-3} M Mg^{2+} ions. Aliquots of incubation mixtures withdrawn during the linear course of respective enzymic reactions were separated chromatographically on Whatman paper No. 1 in a solvent system composed of isobutyric acid–ammonium hydroxide–water (66:1·5:33). For the determination of orotate phosphoribosyltransferase, further separation of orotidine 5′-phosphate and uridine 5′-monophosphate was carried out in a solvent system composed of propyl alcohol–ammonium hydroxide–water (7:1:2). Corresponding chromatographic spots were located according to standards and by radioactivity scanning.[2] Enzyme activities are expressed as millimicromoles of newly formed products of the respective reactions. The radioactivity of individual spots was measured with a Packard liquid scintillation spectrometer in 10 ml of scintillation fluid (4 g 2,5-diphenyloxazole, 0·15 g *p*-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene in 1 l. of toluene).

Utilization of orotic acid for the synthesis of RNA in rat liver. Orotic-6-^{14}C acid was administered intraperitoneally to groups of three to five rats at the dose level of 1 or 2 μc/0·2 μmole/animal 2 hr prior to killing. The excised liver was homogenized with 2 vol. of 0·15 M KCl, and repeated extraction of the acid-soluble pool was carried out with ice-cold 0·2 M $HClO_4$. The isolation of spectroscopically pure uridine 2′(3′)-phosphate after alkaline hydrolysis of RNA (1 M KOH, 18 hr, 20°) has been carried out as has been described previously.[2] The utilization of orotic-6-^{14}C acid is expressed as the specific radioactivity of isolated nucleotide in dis./min per micromole.

Isolation of total liver RNA and its separation on methylated albumin kieselguhr column. Rats were given i.p. orotic-6-^{14}C acid (4·8 μc/0·1 μmole/animal) at different time intervals following 5-azacytidine administration and 2 hr prior to killing. Excised liver was homogenized with 15 vol. of ice-cold 0·1 M Tris–HCl buffer (pH

9·0) with 1×10^{-3} M $MgCl_2$ and 0·5% sodium dodecylsulfate. An equal volume of phenol with 10% 0·1 M potassium acetate (pH 6·0) and 1×10^{-3} M disodium ethylenediaminotetraacetate preheated to 60° was added to the homogenate. Further extraction was carried out as described.[12] Following purification, an aliquot of ribonucleic acids (about 120 absorbancy units at 260 nm) was dissolved in 30 ml of 5×10^{-2} M sodium phosphate buffer (pH 6·7) with 4×10^{-2} M NaCl, and applied to a column of kieselguhr (2×5 cm) coated with methylalbumin. The separation was carried out at 37° by the method of Mandell and Hershey.[13] Ribonucleic acids were eluted with a continuous gradient of 0·4–1·3 M buffered saline at a flow rate of 28 ml/hr; 3-ml fractions were collected. For the complete elution, 1 M NH_4OH was employed.

RESULTS

Inhibition of orotidine 5'-phosphate decarboxylase in the liver of 5-azacytidine-treated rats. The amount of orotidine 5'phosphate in the reaction mixture containing orotic acid, 5-phosphorylribose 1-pyrophosphate and rat liver extract isolated after a 2-hr period of treatment with 5-azacytidine *in vivo* was 4–5 times greater than in the control. However, total anabolic conversion of orotic acid was 40–50 per cent decreased (Table 1). Using liver extract 24 hr following the administration of the analogue, the

TABLE 1. EFFECT OF 5-AZACYTIDINE ADMINISTERED TO RATS ON THE METABOLIC TRANSFORMATION OF OROTIC ACID IN CELL-FREE LIVER EXTRACTS*

5-Azacytidine treatment (hr)	OMP† (nmoles)	UMP (nmoles)	Uridine + uracil (nmoles)	Total transformation (nmoles)
Standard incubation				
0	5·01	24·10	17·00	46·1
2	17·37	2·58	2·18	22·1
24	4·61	19·48	14·11	38·2
6-Azauridine 5'-phosphate				
0	21·42	0·96	1·05	23·4
2	19·48	0·86	0·87	21·1
24	21·82	0·87	0·91	23·6

* 5-Azacytidine was administered i.p. at a dose level of 10 μmoles/100 g to groups of four female rats (175 g) 2 and 24 hr prior to killing. Metabolic transformations of orotic acid in cell-free liver extracts (0·1 ml, corresponding to 25 mg of liver tissue) were assayed at 37° in 0·5 ml of reaction mixture during a 2-min incubation period in 4×10^{-2} M Tris–HCl buffer (pH 7·4) (standard incubation) and in the presence of 1×10^{-3} M 6-azauridine 5'-phosphate.

† Abbreviations used: OMP, orotidine 5'-phosphate; UMP, uridine 5'-monophosphate.

metabolic transformation of orotic acid was almost unaffected. This phenomenon indicated that the 2-hr pretreatment of rats with 5-azacytidine *in vivo* led to an exclusive inhibition of orotidine 5'-phosphate decarboxylase as has been already shown in mouse leukemic cells.[2] In order to verify this assumption, 6-azauridine 5'-monophosphate, a known inhibitor of orotidine 5'-phosphate decarboxylase,[14] was added

to similar reaction mixtures containing cell-free liver extracts from animals killed at various time intervals after 5-azacytidine. Under these conditions, the level of orotidine 5′-phosphate was the same regardless of the extract used (Table 1). This finding indicated that administration of 5-azacytidine causes interference with orotidine 5′-phosphate decarboxylase without affecting orotate phosphoribosyltransferase activity.

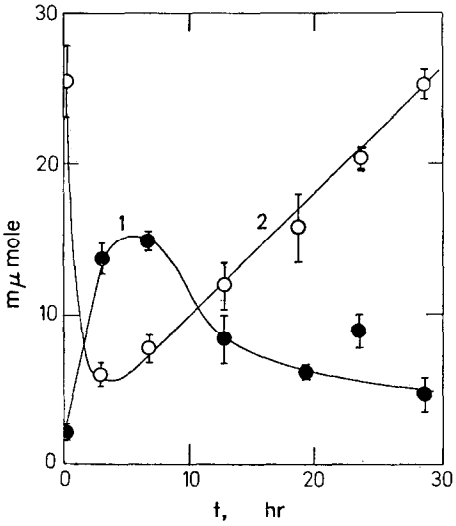

FIG. 1. Time course of the metabolic transformation of orotic acid in cell-free rat liver extracts after 5-azacytidine administration *in vivo*. The drug was administered i.p. (10 μmoles/100 g) to groups of three to five female rats (170–180 g). At different time intervals after the administration of drug (t, hr), the animals were killed and metabolic transformations of 1×10^{-4} M orotic-6-^{14}C acid in cell-free liver extracts were followed during a 2-min incubation period as described. (1) Newly formed orotidine 5′-phosphate; (2) uridine 5′-monophosphate, uridine and uracil expressed as millimicromoles.

The measurement of orotidine 5-phosphate levels at different stages after 5-azacytidine treatment revealed that maximal accumulation of the nucleotide occurred within 8 hr of treatment (Fig. 1). At longer time intervals, its level in the reaction mixture decreased. The changes in the activity of liver orotidine 5-′phosphate decarboxylase at different times following 5-azacytidine treatment are given in Fig. 2. The immediate decrease of enzyme activity was maximal between 1–8 hr after administration of the drug and was in accord with the accumulation of orotidine 5′-phosphate (Fig. 1). Although at later times after the drug enzyme activity gradually increases, it remains significantly depressed as late as 30 hr after 5-azacytidine.

In contrast to orotidine 5′-phosphate decarboxylase, uridine kinase reacts differently (Table 2). The administration of 5-azacytidine resulted in an enhanced activity of liver uridine kinase[10] associated with an increased incorporation of uridine into liver RNA. Our data show that the increase in uridine kinase activity occurred at a time when liver orotidine 5′-phosphate decarboxylase was inhibited.

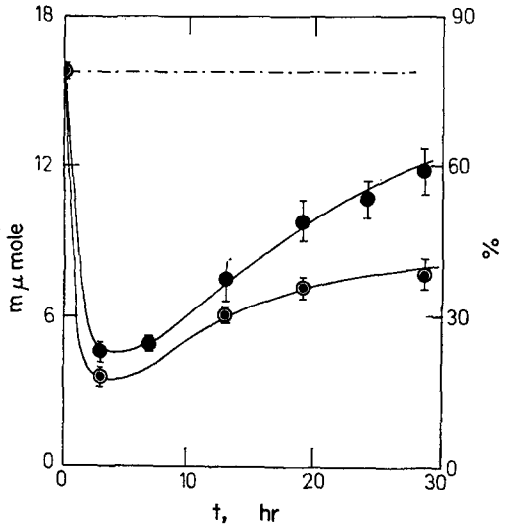

FIG. 2. Inhibition of orotidine 5′-phosphate decarboxylase in cell-free rat liver extracts after the administration of 5-azacytidine *in vivo*. The drug was administered i.p. to groups of five to six female rats at dosage levels of 10 (●) or 20 (◉)/μmoles/100 g. At different time intervals after injection (t, hr) the animals were killed and the enzyme activity (nmoles) was determined. (%) Decarboxylated substrate (2×10^{-5} M).

Utilization of orotic acid for the synthesis of RNA in rat liver during different phases of 5-azacytidine treatment. In agreement with the observed inhibition of orotidine 5′-phosphate decarboxylase, 2-hr treatment with 5-azacytidine resulted in a decreased utilization of orotic acid for the synthesis of liver ribonucleic acids.[2] Contrary to expectation, the inhibition disappeared rapidly and at later times the utilization of orotic acid was enhanced (Fig. 3), reaching a maximum at 18–24 hr after the injection of the analogue. Between 36–50 hr after drug the incorporation was again within normal limits.

TABLE 2. ACTIVITY OF SOME ENZYMES INVOLVED IN THE SYNTHESIS OF RIBONUCLEIC ACID IN RAT LIVER 24 HR AFTER *in vivo* TREATMENT WITH 5-AZACYTIDINE*

Enzymes	Substrate (mM)	Control (nmoles ± S. E.)	5-Azacytidine-treated (nmoles ± S. E.)	5-Azacytidine-treated (%)
Orotate phosphoribosyltransferase	OA† (0·1)	25·02 ± 3·12	23·30 ± 3·75	93·2
Orotidine 5′-phosphate decarboxylase	OMP (0·02)	13·64 ± 1·76	7·88 ± 1·02	57·8
Uridine kinase	6-AzUR (0·02)	0·81 ± 0·07	2·79 ± 01·7	346·2
Uridinemonophosphate kinase	UMP (0·1)	43·70 ± 5·22	43·05 ± 3·21	98·3

* 5-Azacytidine was administered i.p. at a dose level of 10 μmole/100 g to groups of four to seven female rats (175 g). The activities of enzymes in cell-free liver extracts were determined as described in Methods (orotate phosphoribosyltransferase was assayed in the presence of 1×10^{-3} M 6-azauridine 5′-phosphate). Activity of enzymes in control liver without 5-azacytidine = 100 per cent.

† Abbreviations used: OA, Orotic acid; OMP, orotidine 5′-phosphate; 6-AzUR, 6-azauridine; UMP, uridine 5′-monophosphate.

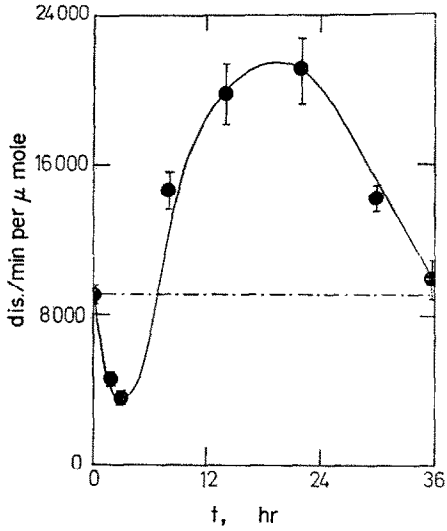

FIG. 3. Orotic acid utilization for liver RNA synthesis in 5-azacytidine-treated rats. Orotic-6-^{14}C acid (2 μc/0·2 μmole/animal) was administered i.p. at different time intervals after the application of 5-azacytidine (t, hr) 2 hr prior to killing to groups of four to seven female rats (175–180 g). 5-Azacytidine was given intraperitoneally at a dose level of 10 μmoles/100 g. The incorporation of orotic acid is expressed as disintegrations per min per micromole of uridine 2′(3′)-phosphate isolated from total liver RNA.

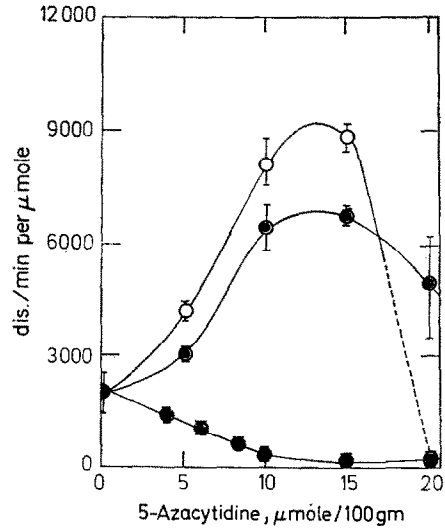

FIG. 4. Dual effect of 5-azacytidine treatment on orotic acid utilization for RNA synthesis in rat liver. 5-Azacytidine was administered i.p. to groups of three to five female rats at different dose levels (micromoles per 100 g) 2, (●); 8, (⊙) and 21, (○) hr prior to killing. Orotic-6-^{14}C acid (1 μc/μmole/animal) was administered i.p. 2 hr prior to killing and the incorporation of the label is expressed as the specific radioactivity of uridine 2′(3′)-phosphate (dis./min per micromole) isolated from total liver RNA's.

The dependence of inhibitory and/or stimulatory effects on the dose levels of 5-azacytidine at different stages of the treatment of animals is given in Fig. 4. The findings show that 2 hr after the administration of increasing doses of drug the inhibition rose until it reached 92–96 per cent. In contrast, at 8 hr and especially at 21 hr after 5-azacytidine the utilization of orotic acid was strongly enhanced. At later stages of treatment high doses of 5-azacytidine produce death of experimental animals due to toxicity.

Although liver orotidine 5′-phosphate decarboxylase after 5-azacytidine was decreased for 30–36 hr, the incorporation of orotic acid into liver RNA was increased as early as 5–6 hr after drug under identical conditions (Figs. 2 and 3). The time course

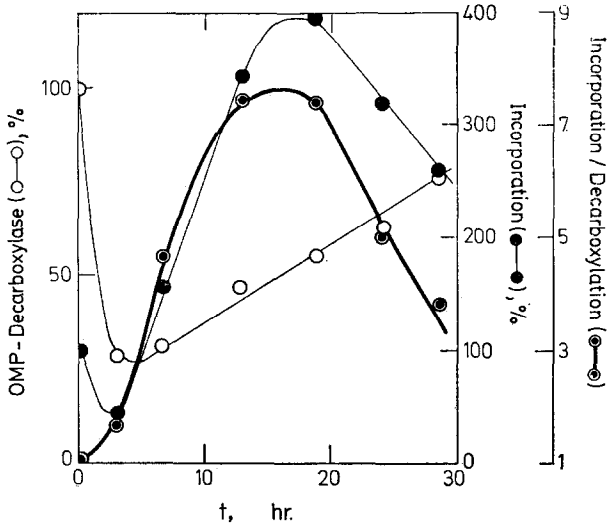

FIG. 5. Increase of orotic acid incorporation into RNA in rat livers with decreased orotidine 5′-phosphate decarboxylase activity. 5-Azacytidine was administered i.p. (10 μmoles/100 g) to groups of four to seven female rats. At different time intervals after its administration (t, hr), some of the animals were killed and the activity of decarboxylase in liver cell-free extracts was determined (○); control activity = 100 per cent. The remaining animals were given orotic-6-^{14}C acid (1 μc/μmole/animal) 2 hr prior to killing, and the specific radioactivity of isolated uridine 2′(3′)-phosphate (●) was determined; incorporation in controls = 100 per cent. Heavy line (◉) denotes the ratio of enhanced incorporation of orotic acid to the decreased orotidine 5-′phosphate decarboxylase after 5-azacytidine.

of the enzyme activity and simultaneously enhanced incorporation of orotic acid are shown in Fig. 5. A maximal 7- to 8-fold increase of orotic acid incorporation as compared to the control level of orotidine 5′-phosphate decarboxylase has been observed 16–18 hr after 5-azacytidine.

Labeling of different fractions of liver ribonucleic acids after 5-azacytidine administration. Since the synthesis of individual RNA types in the liver is controlled by regulatory mechanism which may be affected by 5-azacytidine to a different degree, it was of interest to investigate their labelling by orotic-6-^{14}C acid under the conditions of stimulation and/or inhibition following 5-azacytidine administration. For this reason the separation of total liver RNA on a methylated albumin kieselguhr column was carried out.

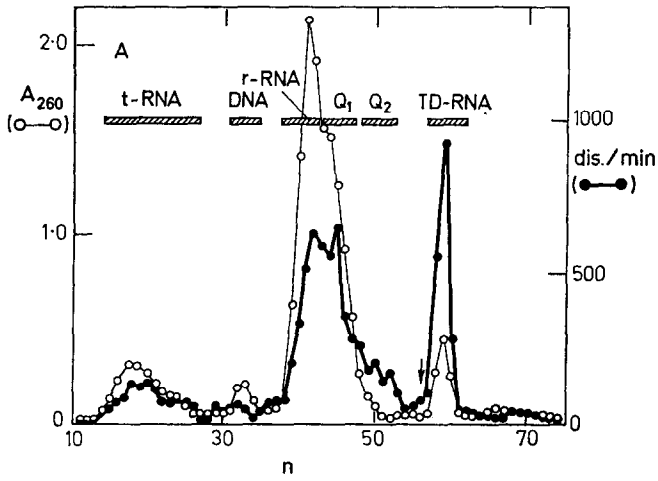

FIG. 6(A)

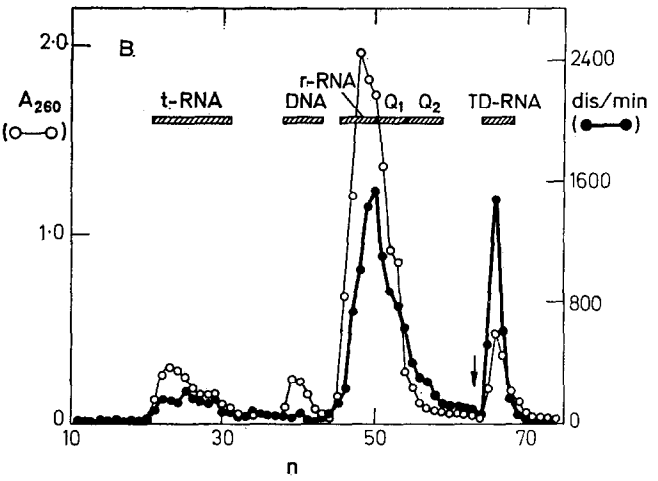

FIG. 6(B)

FIG. 6. Radioactivity distribution of total rat liver nucleic acids labeled with orotic-6-^{14}C acid after administration *in vivo* of 5-azacytidine. The separation was carried out on a methylated albumin kieselguhr column as described in Methods. The application of 1 M NH_4OH to the column is indicated by the arrow. (A) Control elution pattern of total liver RNA; orotic-6-^{14}C acid (4·8 μc/0·1 μmole/rat) was administered 2 hr prior to killing. (B) Orotic-6-^{14}C acid was injected 24 hr following 5-azacytidine (10 μmoles/100 g) 2 hr before the killing. A_{260}, Optical density of fractions (n) at 260 nm; dis./min, radioactivity of individual fractions.

A typical elution profile of liver RNA labeled with orotic-6-^{14}C acid 24 hr following 5-azacytidine administration is shown in Fig. 6B. As compared with the control (Fig. 6A), the pattern does not reveal significant differences in the radioactivity of individual RNA fractions although the total labeling is almost twice as great. Table 3 indicates the specific radioactivities of separated RNA species classified according to Yoshikawa-Fukada *et al.*[15] and Ellem.[16] The greatest inhibition of the labeling after

TABLE 3. RADIOACTIVITY OF INDIVIDUAL FRACTIONS OF RIBONUCLEIC ACIDS FROM RAT LIVER LABELED WITH OROTIC-6-^{14}C ACID AFTER 5-AZACYTIDINE TREATMENT*

Animals	Fraction, dis./min/A_{260} (%)				
	t-RNA	*r*-RNA	Q_1	Q_2	TD-RNA
Control	245	147	223	832	840
5-Azacytidine (2 hr)	146 (59·6)	25 (16·8)	43 (19·4)	85 (10·2)	149 (17·7)
5-Azacytidine (24 hr)	347 (142)	335 (228)	376 (169)	933 (112)	1155 (137)

* 5-Azacytidine was administered at a dose level of 10 μmoles/100 g to groups of two female rats (175 g) 2 and 24 hr prior to application of orotic-6-^{14}C acid (4·8 μc/0·1 μmole/animal). Two hr after the label, animals were killed; their liver RNA was isolated and separated on a methylated albumin kieselguhr column. Control = 100 per cent.

2-hr treatment with 5-azacytidine was observed in fraction Q_2 which is considered to be messenger RNA and is the most rapidly labeled RNA species in the cell.[16] In contrast, at 24 hr following the administration of 5-azacytidine, the greatest radioactivity was present in ribosomal RNA. The radioactivity of fraction Q_2 at that time was lowest, with the most marked deviations throughout individual experiments. Similar effects of the analogue have been noted on the labeling of the Q_1 fraction (Table 3) which has been identified[15,16] as a high molecular weight precursor of ribosomal RNA. The TD-RNA fraction, eluted from the column with ammonium hydroxide, is believed to contain RNA molecules with messenger activity,[16,17] in analogy to fraction Q_2; its labeling again does not differ from other fractions. The lower inhibition of transfer RNA at 2 hr following 5-azacytidine is apparently due to a different turnover of its terminal sequence.

The data suggest that using orotic-6-^{14}C acid 5-azacytidine does not influence, under given conditions, the preferential labeling of different RNA fractions. The same conclusion has been reached using 5-fluoroorotic-2-^{14}C acid* which is selectively utilized for the labeling of cytoplasmic messenger ribonucleic acid in the liver because of inhibition of ribosomal ribonucleic acid maturation.[18]

DISCUSSION

Orotic acid is utilized for the synthesis of liver nucleic acids;[19,20] studies *in vitro* have revealed indispensable enzymatic reactions taking part in its metabolic transformations.[21] The pathway of pyrimidine nucleotide synthesis *de novo* in liver was intensively studied also in relation to the effect of various analogues possessing anticancer action.[15,22,23] A number of substances that interfere with orotate phosphoribosyltransferase and/or orotidine 5′-phosphate decarboxylase have been discovered in recent years, and their biological activity has been attributed to their effects on pyrimidine nucleotide formation.

An increased utilization of orotic acid for the synthesis of liver RNA has been observed after prolonged application of different carcinogens; thioacetamide[24] or of substituted 4-dimethylaminoazobenzenes.[24,25] The incorporation of orotic acid into various RNA fractions was affected in different ways; following the application

* A. Čihák, unpublished observations.

of 4′-fluoro-4-dimethylaminoazobenzene, an increased uptake was observed[25] in the fraction of non-nucleolar nuclear RNA. This carcinogen also produced marked qualitative changes in messenger RNA synthesis in rat liver.[26] In another study,[27] 3-methylcholanthrene led to an increased uptake of orotic acid into nuclear RNA; more recently it has been demonstrated that 3-methylcholanthrene causes increased labeling of 45 S cytoplasmic particles in the liver.[28]

The enhanced incorporation of orotic-6-^{14}C acid into liver RNA following 5-azacytidine (Figs. 3 and 4) is apparently based on a different mechanism. It should be stressed that the increased utilization of orotic acid occurs under conditions of depressed orotidine 5′-phosphate decarboxylase (Figs. 3 and 5) as a result of its inhibition by newly formed 5-azacytidine 5′-monophosphate.[2] This phenomenon would suggest that a surplus of liver orotidine 5′-phosphate decarboxylase exists since even when this enzyme is inhibited by 70 per cent, the incorporation of orotic acid is 2–3 times higher in comparison to untreated controls (Fig. 5). An approximate calculation of the amount of uninhibited orotidine 5′-phosphate decarboxylase in the liver, after treatment with 5-azacytidine, suggests that under these conditions the remaining activity of the enzyme is sufficient to account for the observed increase in the incorporation of orotic acid into liver RNA. The increased uptake of orotic acid following its transformation to uridine 5′-monophosphate cannot be accounted for by the increased uridinemonophosphate kinase activity (Table 2). The greater specific radioactivity of total RNA could have been due to ncreased breakdown of its components into which 5-azacytidine has been incorporated;[3] however, this possibility was experimentally excluded.

Following 5-azacytidine, preferential labeling of individual RNA fractions has not been observed even though during a 2-hr pulse of orotic-6-^{14}C acid no appreciable labeling of cytoplasmic ribosomal RNA would be expected. In this connection it should also be noted that a 6-hr pulse of orotic-6-^{14}C acid or 5-fluoroorotic-2-^{14}C acid did not result in radioactivity profiles of respective RNA species that differed significantly in 5-azacytidine-treated and control animals. It cannot be excluded, however, that the application of a more precise method would lead to the detection of different radioactivity distributions, especially in the fraction of nuclear RNA's.

The enhanced uptake of orotic acid (and of uridine[10]) into total liver RNA is apparently related to the effect of 5-azacytidine on the functioning of degradative enzymes in the liver.[29–31] It was observed that 5-azacytidine administration resulted in a considerable enhancement of liver tyrosine aminotransferase while the rate of enzyme synthesis was not affected.[29,32] At present, measurements of the activity and degradation of DNA-dependent RNA polymerase during later stages of treatment with 5-azacytidine are under investigation.

Acknowledgements—We extend our appreciation to Dr. J. Veselý for helpful discussions and to Miss Z. Škachová for technical assistance.

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