DEGRADATION OF RAT LIVER POLYRIBOSOMES FOLLOWING THE ADMINISTRATION OF 5-AZACYTIDINE STABILITY OF ATTACHED NON-RIBOSOMAL RIBONUCLEIC ACIDS

A. Čihák[a]*, L.M. Narurkar[b] and H.C. Pitot[c]**

[a] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague 6,*
[b] *Cellular Differentiation Processes, Bhabha Atomic Research Centre, Trombay, Bombay 85, India*
[c] *McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin, USA*

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5-Azacytidine brings about the degradation of higher polyribosomal aggregates in rat liver accompanied by a cumulation of the fraction containing disomes and monosomes. The process starts immediately after the administration of the analogue. A maximum degree of degradation is obtained between 4 and 8 h while 18—24 h after 5-azacytidine is given the level of monosomes is decreased, often below that of the control. Furthermore the administration of the drug leads to a decreased binding of leucine-[^{3}H] charged transfer ribonucleic acids on the polyribosomes regardless of their size. The distribution of non-ribosomal ribonucleic acids attached to polyribosomes, labelled selectively with 5-fluoroorotic-[2-^{14}C] acid, is not significantly changed after 5-azacytidine evoked degradation of polyribosomes

5-Azacytidine has received increasing attention on account of its remarkable growth-inhibitory properties in various biological systems[1-5]. Studies of its inhibitory mechanism have shown that the compound is phosphorylated and incorporated into different types of ribonucleic acids; moreover 5-azacytidine 5'-phosphate formed *in vivo* blocks the *de novo* synthesis of pyrimidines by decreasing the activity of liver orotidylic acid decarboxylase[6]. In addition a marked decrease of the activity of various dietary and hormone induced amino acid metabolizing enzymes in rat liver after the administration of 5-azacytidine has been observed[1,7,8].

During the study of liver regeneration in 5-azacytidine-treated rats degradation of liver polyribosomes has been observed[3,9,10]. The present paper describes the phenomenon of this degradation in more detail.

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EXPERIMENTAL

Material. Ribonuclease-free density grade sucrose was purchased from Mann Research Laboratories, sodium deoxycholate was a product of Matheson, Coleman and Bell. 5-Azacytidine (obtained from Dr A. Piskala, Institute of Organic Chemistry and Biochemistry, Prague), 5-fluoroorotic-[2-^{14}C] acid (Tracerlab) and L-leucine-[4,5-^{3}H] (Schwarz Bioresearch) were used. The compounds were injected intraperitonealy in a maximal volume of 0·4 ml at different time intervals before killing. 5-Azacytidine was dissolved immediately before its administration in cold 0·9% NaCl solution to avoid any degradation. Groups of Holtzman male rats (180—220 g) maintained on laboratory chow diet (minimum 20% of crude proteins) and starved 14 h prior to use were utilized throughout the experiments.

Labelling experiments. 5-Fluoroorotic-[2-^{14}C] acid, used for the labelling of non-ribosomal ribonucleic acids, was injected i.p. in a dose level of 8 μCi/2 μmol per animal usually 3 h before killing. For the pulse-labelling of liver polyribosomes L-leucine-[4,5-^{3}H] was injected *i.p.* in the amount of 200 μCi/50 m μmol per animal 7 min before killing. The animals were sacrificed by cervical dislocation and the liver quickly removed and cooled.

Isolation of rat liver polyribosomes. Livers were weighed and homogenized[10,11] in 2 volumes of ice-cold 0·44M-sucrose in TKM-buffer, pH 7·6 (0·05M Tris-HCl with 0·07M-KCl and 0·002M-$MgCl_2$). Homogenization (20 strokes) was performed in a Potter-Elvehjem glass homogenizer

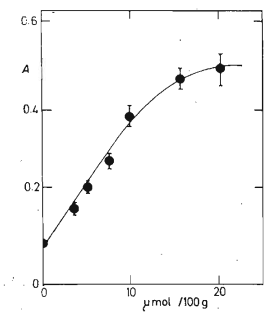

FIG. 1

Degradation of Rat Liver Polyribosomes after 5-Azacytidine

5-Azacytidine (μmol/100 g) was given *i.p.* to male rats (2—3 in each group, 160—180 g) previously starved 12—14 h, 3 h before killing. *A*; Ratio of the cumulative fraction of mono and disomes to the total fraction of polyribosomes ($n \geqq 3$).

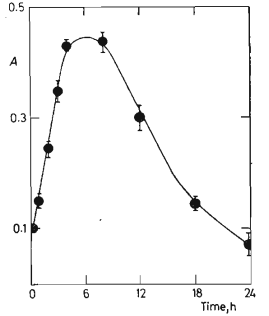

FIG. 2

Time Course of *in vivo* Degradation of Rat Liver Polyribosomes after 5-Azacytidine

5-Azacytidine was given *i.p.* to male rats (2—4 in each group, 180—200 g) previously starved for 12 h at a dose level of 25 mg per kg at different time intervals (h) before killing. *A*; Ratio of the cumulative fraction of mono and disomes to the total fraction of polyribosomes ($n \geqq 3$).

with a loose-fitted Teflon pestle at 4°C. The liver homogenate was centrifuged at 13000 r.p.m. for 10 min (4°C) in a Sorvall centrifuge. Postmitochondrial supernatant was carefully decanted and adjusted to 1·25% with respect to sodium deoxycholate. 30 min later, 4 ml portions of detergent-treated supernatant were layered over a discontinuous two-step gradient of 3 ml of 2·0M sucrose in TKM buffer and 4 ml of 1·3M sucrose in the same buffer. Centrifugation was carried out in a Spinco type 50 Ti rotor at 50000 r.p.m. for 135 min (4°C) using a Beckman model L 2 65-B ultracentrifuge. Supernatants were decanted and the tubes above the polyribosomal pellet were wiped, rinsed with 3 ml of ice-cold buffer, and wiped free of any remaining solution.

Density gradient analysis of polyribosomal components. Polyribosomes dissolved in TKM-buffer were layered over a pre-cooled 10—40% (w/v) sucrose density gradient in the same buffer. Centrifugation (12 ml, 0·5 mg of polyribosomes) was performed in an SW 41 rotor at 41 000 r.p.m. for 80 min (4°C) using a Beckman model L 2 ultracentrifuge. Polyribosome profiles were obtained by gravity flow through a 1 cm^3 flow cell in a Gilford model 2400 spectrophotometer with automatic recording. Individual polyribosomal peaks were collected by hand in ice and the radioactivity was measured in a Packard Tricarb Liquid Scintillation spectrometer in Scintisol liquid scintillation counting medium (Isolab).

RESULTS

Polyribosome Degradation after the Administration of 5-Azacytidine

Altered liver regeneration following 5-azacytidine is accompanied by an increased level of mono and disomes in the fraction of liver polyribosomes[11,12]. Similar degradation of polyribosomes was noted in normal liver after the administration of 5-azacytidine in studies on amino acid-metabolizing enzymes under conditions of dietary induction. The dependence of the degradation of liver polyribosomes on the dose of 5-azacytidine is apparent from Fig. 1.

We were interested to find out which fraction of liver polyribosomes was affected most profoundly by 5-azacytidine. For this reason individual liver polyribosomes of control and 5-azacytidine-treated rats were separated in a sucrose density gradient and their efflux from the gradient monitored by an automatic recorder. The results, given in Table I indicate a preferential degradation of higher polyribosomal aggregates; the per cent degradation decreases with the decreasing size of the particle. We examined further the time profile of the polyribosome degradation after a single dose of 5-azacytidine. The results given in Fig. 2 show an initial rapid increase in the level of disomes with the maximum at 4—8 h after the administration of the analogue[3].

Pulse-labelling of Rat Liver Polyribosomes after 5-Azacytidine Administration

The restorative process following the treatment with 5-azacytidine depends on the utilization of the drug by the liver as well as on its excretion. 24 h after the administration of medium doses of 5-azacytidine (6—10 μmol per 100 g) the level of disomes and monosomes drops to a value often lower than in control untreated liver and the pulse-labelling of liver polyribosomes with leucine-[^{3}H] is slightly increased (Table II).

In an effort to establish the character of labelling of liver polyribosomes after a short time-pulse of leucine-[^{3}H], polyribosomes after their dissociation into ribosomal subunits were fractionated in a sucrose density gradient. As is shown in Fig. 3, during the 7 min of labelling, leucine-[^{3}H] is not incorporated into proteins of ribosomal subunits and practically all the radioactivity is associated with the transfer RNAs which had been released during EDTA-dissociation[11]. The data obtained (Table II) thus can be accounted for totally as charged transfer RNAs bound to polyribosomes.

The binding of charged transfer RNAs to individual fractions of polyribosomes during a short-term labelling with leucine-[^{3}H] in control and 5-azacytidine-treated rats is shown in Table III. The comparison of specific radioactivities of individual

TABLE I

Degradation of Polyribosomal Aggregates in Rat Liver after the Administration of 5-Azacytidine

Groups of 4 male rats (200 g) were injected *i.p.* with 5-azacytidine (10 μmol/100 g) and saline, respectively 4 h before killing. Total liver polyribosomes were separated on 10—40% sucrose density gradient.

Polyribosomes	Control	5-Azacytidine		Polyribosomes	Control	5-Azacytidine	
n	A_{260}	A_{260}	%	*n*	A_{260}	A_{260}	%
>6	2·360	1·390	(58·8)	3	0·583	0·576	(99·0)
6	0·432	0·336	(77·8)	*total*	4·515	3·341	
5	0·555	0·508	(91·4)	2 + 1	0·745	1·899	(254·8)
4	0·585	0·531	(90·8)	*total*	5·260	5·240	

TABLE II

Pulse-labelling of Rat Liver Polyribosomes with Leucine-[^{3}H] after 5-Azacytidine

Four male rats (220 g) were injected *i.p.* with 5-azacytidine (10 μmol/100 g) and pulse-labelled 7 min before killing with L-leucine-[4,5-^{3}H] (200 μCi/50 m μmol per animal).

5-Azacytidine treatment h	Profile disomes/polyribosomes	Leucine-[^{3}H] labelling, d.p.m. per A_{260}, %			
		polyribosomes ($n \geqq 3$)		disomes ($n \leqq 2$)	
Control	0·142	630·3	(100)	398·4	(100)
3	0·431	359·8	(57)	70·1	(18)
24	0·077	704·8	(112)	517·6	(130)

oligosomes shows an overall decrease in their radioactivity. In view of the effect of 5-azacytidine on the turnover[13] of the terminal-CpCpA sequence of transfer RNA a decreased formation of the complex of aminoacyl t-RNAs with ribosomes can be expected. Any dilution effect during the pulse-labelling with leucine-[^{3}H] after 5-azacytidine administration was experimentally excluded.

Effect of 5-Azacytidine on the Fraction of Non-ribosomal Ribonucleic Acids Attached to Polyribosomes

The relatively selective labelling with 5-fluoroorotic-[2-^{14}C] acid of the fraction of non-ribosomal ribonucleic acids attached to polyribosomes[14,15] afforded a way to study the fate of these ribonucleic acids. 3 h after the administration of 5-fluoroorotic-[2-^{14}C] acid, practically no cytoplasmic ribosomal RNAs are labelled and the radio-

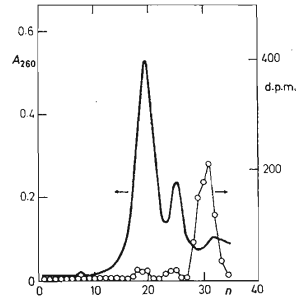

FIG. 3

Radioactivity of Ribosomal Subunits Released from Liver Polyribosomes Pulse-labelled with Leucine-[^{3}H]

Rats were pulse-labelled (○) for 7 min with L-leucine-[4,5-^{3}H] (200 μCi/50 m μmol per animal); isolated polyribosomes were dissociated and separated on 15—30% exponential sucrose gradients (5 ml) in 0·1M triethanolamine-HCl (pH 7·6) with 0·05M-KCl, 0·001M-$MgCl_2$ and 0·01M-EDTA. Fractions of 0·15 ml were collected (n) and the absorbancy at 260 nm was measured (A_{260}).

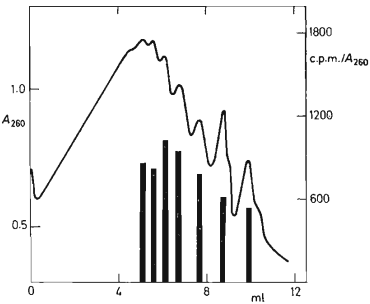

FIG. 4

Labelling of Non-ribosomal RNA Attached to Rat Liver Polyribosomes with 5-Fluoroorotic-[2-^{14}C]Acid.

5-Fluoroorotic-[2-^{14}C] acid (8 μCi/2 μmol per animal) was administered *i.p.* to four male rats (200 g) 3 h before killing. Polyribosomes were separated on 10—40% sucrose density gradient (ml) and measured at 260 nm (A_{260}). The radioactivity of individual fractions is expressed in c.p.m./A_{260} and represented by the bars.

activity in the cytoplasm is found in the fraction of non-ribosomal ribonucleic acids[15]. The distribution of these radioactive RNAs attached to liver polyribosomes after the separation on sucrose density gradient is shown in Fig. 4.

We attempted to examine the changes in the polysomal association of non-ribosomal ribonucleic acids after 5-azacytidine-induced degradation of polyribosomes pre-labelled with 5-fluoroorotic-[2-^{14}C] acid. The data presented in Table IV show a higher specific radioactivity of various fractions isolated from the polyribosomal pellet in the 5-azacytidine-treated animals than in controls. It would seem that an explanation for this finding is that while the polysome structure is degraded following 5-azacytidine administration, the non-ribosomal, probably mRNA component of the polyribosomes is not affected. Thus, on a specific radioactivity basis, the larger oligomers would be expected to have higher specific radioactivity in the partially degraded polyribosomes as seen in Table IV. The ribosomal subunits released from polyribosomes during EDTA-dissociation are practically unlabelled under these conditions and, as had been described[14,16], the radioactivity was distributed over the entire gradient. Thus, the degradation of polyribosomes resulting from 5-azacytidine administration is not paralleled by any significant change in the radioactivity distribution of non-ribosomal ribonucleic acids of cytoplasmic fractions.

DISCUSSION

The degradation of liver polyribosomes following 5-azacytidine[3,9,12] is not specific since a similar cumulation of disomes has been observed after the administration of a number of other substances[17–19]. A question arises whether the monosomes and disomes which accumulate are biologically active *in vivo*. According to Reader

TABLE III

Pulse-labelling of Individual Polyribosomal Fractions in Control and 5-Azacytidine-treated Rat Livers with Leucine-[^{3}H]

Groups of male rats (190—210 g) were injected *i.p.* with saline or 5-azacytidine (10 μmol/100 g). 3 h later and 7 min before killing L-leucine-[4,5-^{3}H] (200 μCi/50 m μmol per animal) was injected.

Polyribosomes	Control liver d.p.m. per fraction	5-Azacytidine-treated liver	
		d.p.m. per fraction	%
>6	879·9	461·7	(52·5)
6	253·9	96·3	(37·9)
5	375·8	123·3	(32·5)
4	343·8	113·1	(32·9)
3	213·5	56·3	(26·4)
2	274·7	122·4	(44·5)

TABLE IV

Effect of 5-Azacytidine on Non-ribosomal RNAs Attached to Rat Liver Polyribosomes

Groups of 4 male rats (180 g) were injected *i.p.* with 5-fluoroorotic-[2-^{14}C]-acid (8 μCi/2 μmol per animal), to label non-ribosomal RNAs, 3 h before 5-azacytidine (10 μmol/100 g) was given. Animals were killed 4 h thereafter.

Polyribosomes *n*	Control liver d.p.m. per fraction	5-Azacytidine-treated liver	
		d.p.m. per fraction	%
>6	70·0	150·1	(214)
6	78·2	241·3	(305)
5	104·3	335·3	(322)
4	111·5	341·0	(310)
3	104·9	330·8	(315)
2	103·2	319·1	(314)

and Stanners[20] free disomes are artifacts of isolation formed by aggregation of free monosomes, probably because of the absence of messenger RNA, transfer RNA and peptide chains that would hinder their nonspecific association. The experiments reported herein (Table II) on pulse-labelling of liver polyribosomes with leucine-[^{3}H] seem to indicate, however, that monosomes possess a partial capacity to generate proteins. This contention is supported by the finding that 70 S ribosomes actually take part during protein synthesis[21,22]. Investigation pertaining to the biological activity of such monosomes in an *in vitro* system are at present in progress.

At 18 h following 5-azacytidine administration when its effect is already levelling off, the distribution of liver polyribosomes is close to that of the control (Fig. 2). However, at 24 h the amount of monosomes is already significantly lower while the number of heavier polyribosomal aggregates is rising in comparison to controls (Table II). Simultaneously a slightly increased radioactivity of individual polyribosomal fractions following pulse-labelling with leucine-[^{3}H] has been observed. This would indicate an increased protein synthetic capacity of restored polyribosomes.

Selective labelling with 5-fluoroorotic-[2-^{12}C] acid of the fraction of ribonucleic acids[14,15] which exist in the cell as a relatively low density RNA-protein complex[22–24], and having properties of cytoplasmic messenger RNA, allows one to study the fate of this RNA fraction. The possibility of the contamination by nuclear ribonucleoprotein of polyribosomes during their isolation[25] has been previously excluded[15]. No significant decrease of the labelling of non-ribosomal RNAs attached to different polyribosomal fractions was observed following 5-azacytidine administration (Table IV). It seems therefore that during 5-azacytidine-evoked degradation of polyribosomes the 5-fluoroorotic-[2-^{14}C] acid-labelled ribonucleic acids do not undergo significant degradation.

After the completion of this work two reports have appeared describing similar

degradation of polyribosomes without simultaneous decay of mRNA. Sedimentation studies of rapidly labelled RNA prepared from rat liver polyribosomes after the incubation with sodium deoxycholate showed that the degradation of polyribosomes was not due to fragmentation of the mRNA. The results indicate[26] that deoxycholate causes a release of monosomes from the intact messenger RNA. Another study was made of the fate of messenger RNA which is released from L cell polyribosomes when the cells are exposed to a temperature shock resulting in about 80 per cent conversion of polyribosomes to monosomes and 98 S particles. At the elevated temperature previously tra nslated mRNA is not degraded and is retained in the cytoplasma as free ribonucleoprotein and as messenger ribonucleoprotein-monosome complexes[27]. These structures are capable of recombining with ribosomes and forming functional polyribosomes when the temperature is returned to normal. The experiments described in our paper seem to indicate that in case of 5-azacytidine-evoked degradation of hepatic polyribosomes a somewhat similar situation occurs. To verify this idea will require further confirmation.

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I. Wichterle, J. Linek: **Antoine Vapor Pressure Constants of Pure Compounds.** Published by Academia, Prague 1971. 101 pp., price 9 Kčs.

The monograph said contains the collection of constants of the Antoine equation expressing the dependence of saturated vapor pressures on temperature for many practically important organic and inorganic substances, both in the liquid and solid state. The authors used the Antoine equation for the given purpose which is suitable for the application to digital computers and which has, in addition, the advantage of general applicability and considerable flexibility.

The input data were taken over from the known Stull paper (1947) and the constants of the Antoine equation were evaluated using a digital computer. The book is divided into four parts, as follows:

1. Vapor Pressures of Organic Compounds up to 1 atm (1199 compounds);
2. Vapor Pressures of Inorganic Compounds up to 1 atm (295 compounds);
3. Vapor Pressures of Organic Compounds above 1 atm (97 compounds);
4. Vapor Pressures of Inorganic Compounds above 1 atm (39 compounds).

Besides the constants of the Antoine equation, on the one hand the standard deviation and on the other hand the temperature range of validity are indicated for each substance cited.

This monograph has a great practical importance, not only for those ones who are interested in phase equilibria. It is a pity it was not possible to make a work which would comprise the period from 1947 up to these days; however, it is to be realized that something like this would require many-years' proportionate work of a whole group of authors.

Only two comments can be, perhaps, pointed out on the book: firstly, it is not clear why in the first part (Vapor Pressures of Organic Compounds up to 1 atm), the deviation is inscribed with "AVG." (average) whereas in three other parts with "STD." (standard). Secondly, why the formulas of substances in Tables are set up so illegibly, as for instance: C8 H24 CL2 O3 SI4 (which is 1,7-dichlorooctamethyltetrasiloxane) though it is not the matter of copies of the tables printed by a computer printer.

The formal typographic arrangement of this book is not beyond the ordinary standard of monographs of that type and price; a remarkable thing, perhaps, is (or even not any more) that the binding is held together only by the van der Waals forces for after having been turned over several times, the book falls to two parts.

Č. Černý